Stopped-flow Kinetic Analysis of eIF4E and Phosphorylated eIF4E Binding to Cap Analogs and Capped Oligoribonucleotides

EVIDENCE FOR A ONE-STEP BINDING MECHANISM*

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Recruitment of eukaryotic mRNA to the 48 S initiation complex is rate-limiting for protein synthesis under normal conditions. Binding of the 5'-terminal cap structure of mRNA to eIF4E is a critical event during this process. Mammalian eIF4E is phosphorylated at Ser-209 by Mnk1 and Mnk2 kinases. We investigated the interaction of both eIF4E and phosphorylated eIF4E (eIF4E(P)) with cap analogs and capped oligoribonucleotides by stopped-flow kinetics. For m7GpppG, the rate constant of association, \( k_{on} \), was dependent on ionic strength, decreasing progressively up to 350 mM KCl, but the rate constant of dissociation, \( k_{off} \), was independent of ionic strength. Phosphorylation of eIF4E decreased \( k_{off} \) by 2.1–2.3-fold at 50–100 mM KCl but had progressively less effect at higher ionic strengths, being negligible at 350 mM. Contrary to published evidence, eIF4E phosphorylation had no effect on \( k_{on} \). Several observations supported a simple one-step binding mechanism, in contrast to published reports of a two-step mechanism. The kinetic function that best fit the data changed from single- to double-exponential as the eIF4E concentration was increased. However, measuring \( k_{off} \) for dissociation of a pre-formed eIF4E-m7GpppG complex suggested that the double-exponential kinetics were caused by dissociation of eIF4E dimers, not a two-step mechanism. Addition of a 12-nucleotide chain to the cap structure increased affinity at high ionic strength for both eIF4E (24-fold) and eIF4E(P) (7-fold), primarily due to a decrease in \( k_{off} \). This suggests that additional stabilizing interactions between capped oligoribonucleotides and eIF4E, which do not occur with cap analogs alone, act to slow dissociation.

The efficiency of mRNA translational initiation is strongly enhanced by the 5'-terminal cap, m7GpppN (1). The cap specifically binds to eIF4E, which is the first canonical initiation factors to interact with mRNA during its recruitment to the ribosome. eIF4E in turn binds to eIF4G, a protein that also interacts with the RNA helicase eIF4A to promote unwinding of mRNA secondary structure, with the multisubunit factor eIF3 to recruit the 43 S initiation complex, and with the cytoplasmic poly(A)-binding protein to enhance initiation of poly(A)-containing mRNAs. Initiation codon recognition is followed by dissociation of eIF4G and joining of the 60 S ribosomal subunit to form the elongation-competent 80 S initiation complex.

eIF4E has been extensively investigated in organisms that range from yeast to mammals (2–7). Besides translation, eIF4E also functions in nucleocytoplasmic transport of mRNA, sequestration of mRNA in a nontranslatable state, and stabilization of mRNA against decay in the cytosol (8–10). The three-dimensional structures of human, mouse, and Saccharomyces cerevisiae eIF4E have been solved (11–13). The complex of full-length human eIF4E with m7GpppA is bell-shaped, with the cap analog situated in a deep slot in the concave surface of the protein. The cap-binding pocket consists of separate recognition components for the m7G base, the triphosphate moiety, and the second nucleoside residue. The alkylated base is stacked between Trp-56 and Trp-102. (Amino acid numbers refer to the human sequence (14).) Glu-103 and Trp-102 form H-bonds with the N-1, N-2, and O-6 protons of m7G. Trp-56 also interacts directly with the ribose moiety, and Arg-157 and Lys-162 interact directly with the α- and β-phosphate oxygen atoms. The second nucleoside moiety of m7GpppA is fixed by the flexible C-terminal loop. To date, there have been no structures reported for eIF4E in complex with capped mRNA or even short oligoribonucleotides.

Cap analogs bind to eIF4E in a tight complex, a step that has been studied primarily by equilibrium techniques (15–33). Intrinsic Trp fluorescence quenching of N-terminal truncated mouse eIF4E (residues 28–217) by titration with cap analogs indicates that the free energy of m7G stacking and hydrogen bonding is separate from the free energy of triphosphate chain interactions (26). Electrostatically steered eIF4E-cap analog association is accompanied by hydration of the complex and a shift in ion equilibria. The kinetics of cap analog binding to wheat eIF(iso)4F (34) and mouse eIF4E (28–217) (35, 36) have also been studied with rapid mixing techniques. The authors of these studies have interpreted their data as indicating a two-step binding reaction. Another group measured the equilibrium dissociation constant, \( K_d \), and the rate constant of dissociation, \( k_{off} \), for human eIF4E by surface plasmon resonance (SPR) (37). Surprisingly, the experimentally determined \( k_{off} \) values and the calculated \( K_d \) values \((K_d = k_{off}/k_{on})\) differed from those reported for the stopped-flow studies (35, 36) by 2–3 orders of magnitude.

Mammalian eIF4E is phosphorylated at Ser-209 (38, 39). Although several eIF4E kinases have been reported, the strongest evidence points to Mnk1 and Mnk2 as the physiological kinases (40, 41). Mnk is activated via the extracellular signal-regulated kinase (ERK) and p38 pathways in response to mitogens, cytokines, or cellular stress (40–42).
Several types of observations link increased eIF4E phosphorylation with increased rates of protein synthesis, including stimulation of cultured cells with mitogens (43–45), use of the Mnk inhibitor CGP57380 (46, 47), sequence variants of eIF4E that cannot bind eIF4G (48), shut-off of cellular protein synthesis by adeno-virus infection (49), and induction of apoptosis (50). The opposite conclusion, that eIF4E phosphorylation does not affect the rate of protein synthesis, or even decreases it, has been drawn from studies with Mnk overexpression (51), cell-free translation (52), recovery of cultured cells from hypertonic stress (53, 54), and Indinavir, a human immunodeficiency virus protease inhibitor (55). Whole animal studies have also generated conflicting results. Transgenic Drosophila that express a non-phosphorylatable form of eIF4E are small, have morphological defects, and are less viable (56). The Drosophila homolog of Mnk, Lk6, is dispensable under a high protein diet, but its loss causes growth reduction when amino acids in the diet are restricted (57, 58). Yet when Mnk1 and Mnk2 are knocked out in mice, no phenotype is observed (59). Thus, at the levels of protein synthesis, cell growth, and intact animal physiology, the roles of Mnk and eIF4E phosphorylation are controversial.

The current study was motivated by several gaps in our understanding of eIF4E-cap interactions as well as discrepancies in the published literature. First, protein synthesis is a dynamic process and, during each round of initiation, the mRNA cap is presumably both bound and released by eIF4E. A full understanding of the biochemical consequences of eIF4E phosphorylation therefore requires a knowledge of $k_{on}$ and $k_{off}$ values for both phosphorylated and unphosphorylated eIF4E (eIF4E and eIF4E(P)). Second, it is not known whether the discrepancies in kinetic values noted above (35–37) from the methodologies employed (SPR stopped-flow), the forms of eIF4E studied (full-length versus N-terminal truncated), or the methods of producing eIF4E(P) (enzymatic phosphorylation versus intermediately ligation). Third, the $k_{on}$ for eIF4E(P)-cap analog association has not yet been measured directly. Fourth, kinetic parameters measured by the stopped-flow technique have not been reported for the binding of capped oligoribonucleotides to eIF4E. Fifth, all previous studies of eIF4E interactions with capped oligonucleotides (20, 22, 37, 60) have utilized a mixture of normally capped and reverse-capped oligoribonucleotides, the latter of which do not bind eIF4E. Sixth and finally, the proposed two-step mechanism for cap binding to eIF4E (26, 34, 35) is at variance with observations for a viral cap-binding protein (61, 62).

We report here stopped-flow kinetic results for full-length human eIF4E and eIF4E(P) binding to cap analogs and capped oligoribonucleotides, the latter being capped entirely in the correct orientation. A full understanding of the biochemical consequences of eIF4E phosphorylation therefore requires a knowledge of $k_{on}$ and $k_{off}$ values for both phosphorylated and unphosphorylated eIF4E (eIF4E and eIF4E(P)). Second, it is not known whether the discrepancies in kinetic values noted above (35–37) from the methodologies employed (SPR stopped-flow), the forms of eIF4E studied (full-length versus N-terminal truncated), or the methods of producing eIF4E(P) (enzymatic phosphorylation versus intermediately ligation). Third, the $k_{on}$ for eIF4E(P)-cap analog association has not yet been measured directly. Fourth, kinetic parameters measured by the stopped-flow technique have not been reported for the binding of capped oligoribonucleotides to eIF4E. Fifth, all previous studies of eIF4E interactions with capped oligonucleotides (20, 22, 37, 60) have utilized a mixture of normally capped and reverse-capped oligoribonucleotides, the latter of which do not bind eIF4E. Sixth and finally, the proposed two-step mechanism for cap binding to eIF4E (26, 34, 35) is at variance with observations for a viral cap-binding protein (61, 62).

We report here stopped-flow kinetic results for full-length human eIF4E and eIF4E(P) binding to cap analogs and capped oligoribonucleotides, the latter being capped entirely in the correct orientation. Our findings differ from those of previous reports with regard to the magnitude of kinetic constants, the effects of eIF4E phosphorylation on $k_{on}$ and $k_{off}$ and the manner of cap binding. With regard to the latter, our data support a one-step rather than two-step binding model. Furthermore, the stopped-flow results provide a kinetic basis for enhanced binding of eIF4E to capped oligoribonucleotides compared with cap analogs.

**EXPERIMENTAL PROCEDURES**

**Materials**—All common reagents were of analytical grade and were purchased from Sigma unless otherwise stated. m7GTP, m7GTP-Sepharose 4B, and glutathione-Sepharose 4B were purchased from Amersham Biosciences. Protease inhibitor mixture was from Roche Diagnostics. The syntheses of m7GpppG and m7,3′,5′-GpppG were performed as described previously (63). The concentrations of dinucleotide cap analog solutions were determined by absorbance ($\varepsilon_{295} = 22.6 \times 10^3$ M$^{-1}$ cm$^{-1}$ at pH 7.0).

**eIF4E Expression and Purification**—Full-length wild-type human eIF4E (14) was expressed in Escherichia coli BL21 (COE) pLys(S) from pET11d-eIF4E (64). Protein expression was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside overnight at 15 °C. We found that these conditions (lowering the isopropyl-β-D-thiogalactopyranoside concentration and temperature) increased the yield of soluble eIF4E compared with the published procedure (64) and produced 7–10 mg of cell suspension. Cells (3 g) were lysed with a French press in 25 ml of buffer B (50 mM HEPES/KOH, pH 7.6, 0.3 mM KCl, 10 mM EDTA, 1 mM DTT, 1% streptomycin sulfate, and protease inhibitor mixture). After centrifugation at 32,000 × g for 40 min at 4 °C, the soluble fraction was diluted 1:1 with 100 μM GTP and loaded onto a 3-ml m7G-Sepharose column. This and other chromatographic steps were monitored spectrophotometrically at 280 nm. The column was washed with 40 column volumes of buffer B (50 mM HEPES/KOH, pH 7.6, 0.2 mM KCl, 1 mM EDTA, and 1 mM DTT). eIF4E was eluted with buffer B except that the KCl concentration was 0.5 M KCl. Use of KCl for elution rather than the more traditional m7GTP (65) prevents formation of tight eIF4E complexes with the cap analog (66), which can affect the measurement of kinetic parameters for the binding reaction. eIF4E was at least 95% pure as judged by SDS-PAGE (see “Results”). For eIF4E purification from E. coli and Mnk purification from HEK 293 cells, protein concentrations were determined with the protein assay reagent from Bio-Rad, in which bovine serum albumin was used as standard. For kinetic experiments, protein concentrations were determined spectrophotometrically, assuming ε$_{280}$ = 53,400 M$^{-1}$ cm$^{-1}$ at pH 7.2.

**eIF4E Phosphorylation**—Recombinant mouse Mnk kinase was produced as described previously (42) but with modifications. Plasmids pEBG-Mnk2 and pEBG-Mnk1T2A2 were generously provided by Christopher Proud (University of British Columbia). These encode, respectively, wild-type Mnk2 fused to glutathione S-transferase (GST::Mnk2) and an inactive variant, GST::Mnk1T2A2 (41). HEK 293T cells, grown in 10-cm dishes in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, were transfected with plasmids in complex with Lipofectamine 2000 (Invitrogen). After 44 h, cells were harvested and lysed at 4 °C by incubation with buffer C (10 mM HEPES/KOH, pH 7.4, 50 mM NaF, 2 mM EDTA, 2 mM sodium orthovanadate, 0.1% 2-mercaptoethanol, 1% Triton X-100, and protease inhibitor mixture) at a ratio of 3.5 ml of cell pellet to buffer C of 1:8 (w/v) over 30 min with rotation. The suspension was centrifuged for 30 min at 20,000 × g and GST::Mnk2 was purified from the supernatant on glutathione-Sepharose 4B equilibrated with buffer D (20 mM MOPS/KOH, pH 7.4, 20 mM KCl, 15 mM MgCl$_2$, 0.5 mM EDTA, 1 mM DTT, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 5% glycerol) at a supernatant-Sepharose ratio of 10:3 (v/v). Glutathione-Sepharose beads with bound GST::Mnk2 and GST::Mnk1T2A2 were stored at −80 °C.

Recombinant eIF4E was phosphorylated in vitro essentially as described (37) but with some modifications. Recombinant eIF4E (225 μg) was incubated at room temperature (23 °C) in a 0.5-mL reaction mixture containing 150 μl (packed volume) of glutathione-Sepharose-bound GST::Mnk2 and 500 μM ATP in buffer D for 6 h with rotation. The kinase was removed by centrifugation, and then eIF4E(P) was passed over a PD-10 desalting column (Amersham Biosciences) to separate it from ATP and to transfer it into an appropriate buffer for kinetic experiments. In parallel experiments, eIF4E was mock-phosphorylated with GST::Mnk1T2A2. The efficiency of eIF4E phosphorylation was determined by isoelectric focusing on CleanGel IEF polyacrylamide gels (Amersham Biosciences) in the presence of 8 M urea, 30 mM CHAPS, and 2% Pharmalyte 3–10 according to the manufacturer’s instructions. IEF was conducted at 15 °C on a Multiphor II apparatus for horizontal separation equipped with a cooling platform and MultiDrive XL power supply (Amersham Biosciences). Protein bands were stained with Coomassie Blue. In pre-
liminary experiments to determine the optimal time for enzymatic phosphorylation, we also used [γ-32P]ATP.

Synthesis of Oligoribonucleotides—T7 RNA polymerase was prepared as previously described (67). The template consisted of two complementary oligodeoxyribonucleotides (Integrated DNA Technology Inc., Coralville, IA), 5′-TAATACGACTCACTATAG-3′ and 5′-TTTTTTTTTTGCGCTATAGTGATCGTGTTA-3′, which contain the T7 promoter followed by a single-stranded 12-nucleotide 5′-overhang. This sequence was chosen because it gave the highest yield of several tested (68). The oligodeoxyribonucleotides, each at 183 μM, were annealed by heating at 90°C and slow cooling to room temperature. Transcription reactions were performed as described previously (68) with some modifications. Reaction mixtures (1 ml) contained 40 mM Hepes/KOH, pH 7.9, 10 mM MgCl2, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 0.1 mg/ml bovine serum albumin, 1000 units/ml RNA Guard (Amersham Biosciences), 2.5 mM each of ATP, CTP, UTP, and GTP, 40 μg/ml DNA template, and 100 μg of T7 RNA polymerase. Capped oligoribonucleotides were produced by lowering the GTP concentration to 0.5 mM and including m7GpppG, an “anti-reverse cap analog” (ARCA), at 3 mM, which prevents heterogeneity in synthesized oligoribonucleotides due to reverse incorporation (63). The binding affinities of m7GpppG and m5,7,8,10-GpppG for eIF4E are the same within 0.6% (69). Reaction mixtures were incubated at 35 °C for 2 h, after which the RNA template was digested with DNase RQ1 (Promega). Oligoribonucleotides were purified by size-exclusion chromatography on Bio-Gel P6 columns with diethylpyrocarbonate-treated water as eluent. The purity of oligoribonucleotides was determined by PAGE on 20% gels containing 8 M urea (70). The A260/A280 ratio for oligoribonucleotides was 1.85. Concentrations (in μg/ml) were determined as 33 × A260 nm.

Steady-state Fluorescence Measurements—A StrobeMaster lifetime spectrometer (Photon Technology Inc., South Brunswick, NJ) with the SE-900 steady-state fluorescence option was used to obtain fluorescence emission spectra of eIF4E and eIF4E(P) in the presence or absence of m7GTP. Fluorescence was monitored at an excitation wavelength of 295 nm (2-nm bandwidth) and emission bandwidth of 5 nm. Spectra were recorded with a photon counting detector (Photon Technology Inc., model 710) from 305 to 400 nm at 25 °C. Samples were maintained in a quartz cuvette (1-cm path length) with constant stirring at concentrations of both protein and m7GTP of 1 μM in buffer E (50 mM Hepes/KOH, pH 7.2, 1 mM DTT, 0.5 mM EDTA, and 50 mM KCl). Under these conditions, the Kd for the m7GTP-eIF4E interaction is ~9 nm (26). Thus, essentially all of the m7GTP is bound to eIF4E. The degree of quenching of intrinsic Trp fluorescence (Q) is an indicator of both purity and native conformation of eIF4E. For mammalian eIF4E, Q is ~65% (26). The preparations used in this study had Q = 64 ± 4% for eIF4E and Q = 59 ± 5% for eIF4E(P), indicating that both proteins were essentially native.

Kinetic Methods—Rapid kinetic measurements of eIF4E and eIF4E(P) interaction with free dinucleotide cap analogs (m7GpppG and m5,7,8,10-GpppG) and an ARCA-capped oligoribonucleotide were made with a stopped-flow spectrometer with fluorescence detection (Applied Photophysics Ltd., Leatherhead, UK, model SX.18MV). The dead time of the instrument was 1.2 ms. The excitation wavelength was 295 nm with 2.3-nm bandwidth, and stray excitation was eliminated with a 320-nm Oriel long-pass filter. Excitation occurred through a 2-mm path in the stopped-flow optical cell, and emission was measured through a 10-mm path. The instrument time constant was 0.5% of the reaction half-time. For very fast reactions, no in-line filtering was used. An oversampling option of the instrument permitted us to collect 1000 data points throughout the reaction (50–200 ms). Fluorescence changes were examined up to 200 s.

The binding reaction was investigated under pseudo first-order conditions ([protein] << [ligand]) unless otherwise noted. For most experiments, concentrations of protein and ligand in the syringes of the stopped-flow instrument were 0.1–0.2 and 1–20 μM, respectively. Samples were degassed prior to loading into the syringes. Reactions were initiated by mixing equal volumes of protein and ligand at 25 °C in buffer E except containing 50, 100, 150, or 350 mM KCl as noted. The temperature was controlled with a circulating external water bath (AT ± 0.2 °C). The stopped-flow traces shown under “Results” are the average of 5–15 individual traces. Control experiments on specificity of binding were conducted with GpppG and with the uncapped form of the oligoribonucleotide. For stopped-flow experiments where the m7GpppG concentration was limiting (0.2–0.4 μM), the protein concentration was varied in the range of 1–10 μM. Dissociation of the pre-formed eIF4Em7GpppG complex was followed by measuring the increase in intrinsic Trp fluorescence when equal volumes of the complex and buffer were mixed in the stopped-flow cell at 150 mM KCl. In this case, eIF4E and m7GpppG concentrations in the syringe were equal (2 μM).

Curve Fitting and Error Analysis—Stopped-flow traces representing binding of cap analogs or a capped oligoribonucleotide to eIF4E or eIF4E(P) were analyzed using Curfit, a curve-fitting program that uses a Marquardt algorithm (71). Data were fit to both single- and double-exponential functions. For single-exponential fits,

\[ F(t) = \Delta F \exp(-k_{obs} t) + F_\infty \]  

(Eq. 1)

where \( k_{obs} \) is the observed first-order rate constant, \( \Delta F \) is the amplitude, and \( F_\infty \) is the final value of fluorescence. For double-exponential fits,

\[ F(t) = \Delta F_1 \exp(-k_{obs1} t) + \Delta F_2 \exp(-k_{obs2} t) + F_\infty \]  

(Eq. 2)

where \( k_{obs1} \) and \( k_{obs2} \) are the observed rate constants for the first and second components of a double-exponential reaction, respectively, and \( \Delta F_1 \) and \( \Delta F_2 \) are the amplitudes for the first and second components of a double-exponential reaction, respectively. An assessment of each fit was made from the residuals, which measure the differences between actual data and the calculated fit. These are designated \( \delta_i \) in the case of single-exponential fits and \( \delta_i \) in the case of double-exponential fits. The program 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. Equilibrium constants are reported either as dissociation constants (Kd) or association constants (\( K_a = 1/K_d \)).

RESULTS

Preparation of eIF4E and eIF4E(P)—The objective of the current study was to apply fast kinetic techniques to elucidate the mechanism of eIF4E-cap interactions, with particular emphasis on the effect of three parameters: phosphorylation of eIF4E, ionic strength, and linkage of the cap to an oligoribonucleotide chain. This required preparation of milligram quantities of highly purified, native eIF4E and eIF4E(P). In previous reports in which recombinant eIF4E was used for kinetic studies, the protein was purified from inclusion bodies solubilized with either urea (37) or guanidine hydrochloride (35, 36) and then renatured by dialysis. It is possible to calculate what fraction of a given eIF4E preparation is in the native state by determining the quenching of intrinsic Trp fluorescence (Q) by steady-state fluorescence spectroscopy (see “Experimental Procedures”). In the only case where this was reported, 57 ± 16% of the renatured protein preparation was found to be active, presumably due to
imperfect refolding in vitro (35). To avoid this problem, we purified human eIF4E exclusively from the soluble fraction of *E. coli* lysates. The protein appeared as a homogeneous band by SDS-PAGE (Fig. 1A). eIF4E prepared in this way had a value of Q indicating that it existed entirely in the native conformation (see “Experimental Procedures”). Another possible source of inactive protein is the presence of contaminating cap analog in complex with eIF4E (66), due to the conventional method of eluting eIF4E from m⁷GTP-Sepharose with m⁷GTP (65). Such eIF4E preparations contain up to 60% bound m⁷GTP, which cannot be removed by dialysis or ion exchange chromatography (26). To avoid this problem, we eluted the protein from m⁷GTP-Sepharose with 0.5 M KCl rather than m⁷GTP.

eIF4E(P) was prepared by *in vitro* phosphorylation with activated mouse Mnk2. It has previously been shown that this kinase phosphorylates human eIF4E exclusively at Ser-209 in vitro (41, 42), which is the same as the *in vivo* site (38, 39). The degree of phosphorylation was measured by two methods: IEF and calculating the moles of phosphate incorporated per mole of eIF4E from the specific activity of [γ-³²P]ATP in the kinase reaction. Both methods revealed that phosphorylation was essentially stoichiometric (Fig. 1B and data not shown). Both untreated and kinase-treated proteins appeared on the IEF gels as doublets, the minor form constituting ~11% of the total. The minor form has been observed previously in preparations of both phosphorylated and unphosphorylated eIF4E (72, 73). The structural basis for the heterogeneity is unknown. It may represent post-translational modification, e.g. incomplete N-terminal acetylation (74), or it may be an artifact of IEF. For instance, the concentration of CN⁻ in the 8 M urea used in the IEF gel can reach 20 mM and can carbamylate NH₂ groups in proteins (75), removing one positive charge. Regardless of the sources of microheterogeneity, both forms had nearly the same molecular mass (Fig. 1A), were able to bind m⁷GTP-Sepharose, and were stoichiometrically phosphorylated (Fig. 1B). eIF4E(P) prepared in this way had a value of Q indicating that it existed entirely in the native conformation (see “Experimental Procedures”).

**FIGURE 1.** Characterization of purified recombinant human eIF4E and eIF4E(P). A, SDS-PAGE (10% gel) of eIF4E after column chromatography on m⁷GTP-Sepharose. The mobilities of molecular mass markers are indicated on the left. B, phosphorylation of eIF4E as monitored by IEF. Recombinant human eIF4E was treated with *in vivo* activated GST::Mnk2 and analyzed by IEF in the range of pH 3–10, as described under “Experimental Procedures.” The polarity during IEF is indicated by + and −. In both A and B, protein is visualized by Coomassie Blue staining.

**FIGURE 2.** Kinetics of the m⁷GpppG-induced decrease in intrinsic Trp fluorescence of eIF4E and eIF4E(P) as monitored by stopped-flow fluorescence. A, reaction of 0.1 μM eIF4E with m⁷GpppG at 350 mM KCl and 25 °C (upper panel). Solid lines represent fitting of the data with Equation 1. Fluorescence is measured in volts (V). Traces are offset vertically to allow each to be seen individually. For m⁷GpppG at 1, 3, 5, and 9 μM, *k_*obs was 118, 176, 229, and 362 s⁻¹, respectively. Residuals for fits to Equation 1 are shown in the lower panel. B, same as A except that eIF4E(P) was used. For m⁷GpppG at 1, 3, 5, and 9 μM, *k_*obs was 112, 164, 236, and 376 s⁻¹, respectively. A control reaction for both proteins was performed with 9 μM GpppG (top trace), which binds eIF4E with an affinity that is ~10⁵-fold lower than that of m⁷GpppG (26).
Association of eIF4E with m⁷GpppG—We initially attempted to measure kinetic parameters for the interaction of m⁷GTP, the most commonly used cap analog, with eIF4E and eIF4E(P) by stopped-flow experiments but found that rate of fluorescence change was too fast to be followed. We therefore turned to m⁷GpppG, which binds eIF4E with only 7% the affinity of m⁷GTP (26). However, m⁷GpppG is more similar to the natural mRNA cap than m⁷GTP because of the second nucleoside moiety. We measured the reaction over a range of m⁷GpppG concentrations under pseudo first-order reaction conditions, where [eIF4E] ≪ [cap analog]. The first stopped-flow study of eIF4E-m⁷GpppG interactions (34), which utilized wheat eIF(iso)4F, was also conducted under pseudo first-order conditions. A later study of mouse eIF4E-(28–217) (35) used second-order reaction conditions, i.e. the concentrations of eIF4E and cap analog were approximately equal, and both were varied.

The latter experimental approach makes treatment of the data more complicated and can potentially cause artifacts (see below). Under pseudo first-order conditions, if the association reaction is a simple one-step process, $k_{obs}$ is predicted to vary linearly with cap analog concentrations (76) (Equation 3).

$$k_{obs} = k_{on}[m⁷GpppG] + k_{off}$$  
(Eq. 3)

Also, under pseudo first-order conditions, the binding reaction is not sensitive to small amounts of inactive protein in the preparation.

The rapid mixing of eIF4E with m⁷GpppG resulted in a decrease in intrinsic Trp fluorescence that was dependent on m⁷GpppG concentration (Fig. 2A, upper panel), the apparent rate constant of the decrease being $k_{off}$. By contrast, there was no decrease in fluorescence with GpppG (top trace), which binds eIF4E with an affinity that is ~10⁻³-fold lower than that of m⁷GpppG (26). The data were fit with a single-exponential function (Equation 1) (solid lines). The residuals, representing the deviation between the calculated and actual data, indicate that the single-exponential function fit the points over the entire time of the measurement (lower panels). Treatment of the data using a double-exponential function (Equation 2) did not improve the fit (not shown). We followed the reaction over 200 s to test whether there was a second slow phase but saw no evidence for one (not shown).

The binding reaction of eIF4E(P) also was probed over the same range of m⁷GpppG concentrations (Fig. 2B, upper panel). The traces again followed a single-exponential function over all cap analog concentrations. The residuals did not vary over the time course (lower panels) nor were they diminished by a double-exponential fit (not shown). To ensure that any differences between eIF4E and eIF4E(P) were not an artifact due to nonspecific changes occurring during the 6-h kinase reaction, we also conducted a mock kinase reaction in which the active GST::Mnk2 was replaced with GST::Mnk1T2A (77) (see “Experimental Procedures”). The $k_{obs}$ values for binding of m⁷GpppG were indistinguishable between unphosphorylated eIF4E and mock-phosphorylated eIF4E (data not shown).

Plots of $k_{obs}$ versus m⁷GpppG concentration for eIF4E and eIF4E(P) were linear (Fig. 3). The slope and y intercept are $k_{on}$ and $k_{off}$ respectively (Equation 3). The values obtained for $k_{on}$ for both eIF4E and eIF4E(P) were in the range of $33–292 \times 10⁻³\ M⁻¹\ s⁻¹$, depending on the ionic strength, and those obtained for $k_{off}$ were $70–87\ s⁻¹$ (Table 1). Such high values indicate that equilibrium is reached rapidly and that slower methods of obtaining kinetic constants may not yield accurate results (see “Discussion”). $K_d$ values calculated from these kinetic constants ranged from 0.24 to 2.48 μM, again depending on the ionic strength (Table 1). These are somewhat higher than $K_d$ values obtained by equilibrium methods (26, 32). For instance, at 100 mM KCl, the kinetic approach yielded $K_d = 0.45\ μM$ for eIF4E (Table 1), whereas the equilibrium approach yielded 0.135 μM in one study (26) and 0.08 μM in another (32). For eIF4E(P) at 100 mM KCl, kinetic measurements gave $K_d = 1.06\ μM$, whereas equilibrium measurements gave $K_d = 0.172\ μM$ (32). The reason for these discrepancies is not known, but it should be noted that, besides the method of $K_d$ determination, the protein preparations were differ-

![FIGURE 3. Dependence of $k_{obs}$ on m⁷GpppG concentration as a function of KCl concentration. $k_{obs}$ obtained in experiments similar to those shown in Fig. 2 at the indicated KCl concentrations is plotted versus (m⁷GpppG) for eIF4E (panel A) and eIF4E(P) (panel B). Data are fit with Equation 3. Numerical values for $k_{on}$, $k_{off}$ and $K_d$ are given in Table 1.]

TABLE 1 Kinetic constant for association and dissociation of eIF4E and eIF4E(P) with m⁷GpppG obtained from pre-steady-state experiments

| KCl concentration (mM) | $k_{on} \times 10⁻³$ | $k_{off}$ (s⁻¹) | $K_d$ (μM) |
|------------------------|----------------------|-----------------|-------------|
| 50                     | 292 ± 16             | 138 ± 8         | 0.24 ± 0.06 |
| 100                    | 184 ± 10             | 80 ± 4          | 0.45 ± 0.13 |
| 150                    | 102 ± 7              | 56 ± 2          | 0.85 ± 0.16 |
| 350                    | 33 ± 2               | 34 ± 2          | 2.48 ± 0.39 |

Data are obtained from experiments similar to Fig. 3, with application of Equation 3.
rate of caps with eIF4E but not the dissociation rate. This contradicts results obtained by SPR (37) (see "Discussion").

The KCl concentration dependence of $K_d$, as calculated from these kinetic parameters, closely resembled that of $k_{on}$ (Fig. 4B). $K_d$ values decreased $\sim$10-fold for eIF4E and $\sim$4-fold for eIF4E(P) from 50 to 350 mM KCl. The difference in $K_d$ values between eIF4E and eIF4E(P) was most pronounced ($2.2–2.4$-fold) at 50 and 100 mM KCl. This was progressively eliminated at higher KCl concentrations, and by 350 mM, there was no difference in $K_d$ values between eIF4E and eIF4E(P).

The Apparent Kinetic Mechanism of $m^7$GpppG Binding Depends on eIF4E Concentration—The linearity of the $k_{on}$ versus $m^7$GpppG plots (Fig. 3) is consistent with a one-step, second-order reaction mechanism,

$$
{\text{eIF4E}} + m^7\text{GpppG} \rightleftharpoons eIF4Em^7\text{GpppG}^*,
$$

SCHEME 1

where the asterisk (*) denotes a reduction in Trp fluorescence of eIF4E in complex with $m^7$GpppG. This conflicts with published studies indicating that cap binding to eIF4E occurs in a two-step process (26, 34, 35). The authors of one of these reports (35) found that the experimental data could be fit better with a double- than a single-exponential function. They attributed the slow phase to a second step in the binding reaction, in which eIF4E fluorescence is further reduced,

$$
{\text{eIF4E}} + m^7\text{GpppG} \rightleftharpoons eIF4Em^7\text{GpppG}^* \rightleftharpoons eIF4Em^7\text{GpppG}^{**},
$$

SCHEME 2

where the asterisk (*) denotes a complex with reduced fluorescence relative to free eIF4E, and the double asterisk (**) denotes a different complex with even lower fluorescence. The mechanisms represented by Schemes 1 and 2 can be distinguished experimentally (see below).

Under pseudo first-order conditions, the kinetic mechanism and parameters for a simple association between eIF4E and $m^7$GpppG (Scheme 1) theoretically do not depend on the choice of which component is limiting. For the experiments shown in Figs. 2–4, the eIF4E concentration was limiting and the $m^7$GpppG concentration was varied. We measured the same association reaction but at a limited value of $m^7$GpppG concentration (0.1 mM) and variable eIF4E concentrations, so that $[m^7\text{GpppG}] < [\text{eIF4E}]$. To our surprise, the kinetics of the reaction under these conditions were different. As the eIF4E concentration was increased from 0.5 to 5 mM, the stopped-flow traces became increasingly biphasic. Representative results for 0.5 and 2 mM eIF4E are shown in Fig. 5, A–D. The same data for [eIF4E] = 0.5 mM are presented in Fig. 5, A and C, but with either single- or double-exponential fits, respectively. The points were fit well by a single-exponential function (Equation 1), with $k_{obs} = 71 \pm 4 s^{-1}$ and $\Delta F = 0.0155 V$ (Fig. 5A). Application of a double-exponential function (Equation 2) did not improve the fit (Fig. 5C). By contrast, at [eIF4E] = 2.0 mM, the data were fit poorly by a single-exponential function (Fig. 5B), whereas they were fit well by a double-exponential function (Fig. 5D), with $k_{obs1} = 205 \pm 20 s^{-1}$ and $k_{obs2} = 8.1 \pm 4 s^{-1}$. The amplitudes for the fast and slow phases were similar, 0.080 and 0.078 $V$, respectively. The relative improvement of the fit with a double-versus single-exponential function, $(\delta_2 – \delta_1)/\delta_1$, was 4. As the eIF4E concentration was raised, the amplitude of the slow phase

FIGURE 4. Dependence of $k_{on}$ and $K_d$ for binding of $m^7$GpppG to eIF4E and eIF4E(P) on KCl concentration. Data for eIF4E (open circles) and eIF4E(P) (filled circles) are derived from Table 1.

The Effect of eIF4E Phosphorylation on $k_{on}$ Is Diminished at High Ionic Strength—It is known that electrostatic forces play an important role in determining $K_d$ for eIF4E-cap analog interactions. Specifically, the affinity for cap analogs of natural rabbit reticulocyte eIF4E (16) and recombinant mouse eIF4E (28–217) (26, 32) decreases with increasing ionic strength. To determine the basis for the effect of KCl on $K_d$, we plotted $k_{on}$ and $k_{off}$ from Table 1 against KCl concentrations (Fig. 4A). Over a range of KCl concentrations from 50 to 350 mM, $k_{on}$ decreased $\sim$9-fold for eIF4E and $\sim$4-fold for eIF4E(P), whereas $k_{off}$ did not change significantly (inset). Furthermore, there was no statistical difference in $k_{off}$ between eIF4E (open symbols) and eIF4E(P) (filled symbols). Thus, phosphorylation diminishes the association

ent: full-length human eIF4E phosphorylated enzymatically (Table 1) versus truncated mouse eIF4E phosphorylated by intein technology (26, 32). Despite these differences in $K_d$ values, the change in $K_d$ for eIF4E versus eIF4E(P) was similar for kinetic and equilibrium determinations: a 2.35-fold increase in $K_d$ by kinetic methods (Table 1) and a 2.15-fold increase by equilibrium methods (32).
progressively increased in relation to that of the fast phase (Table 2).

Our observation that the mechanism appears to change from a one-step to a two-step process as the eIF4E concentration is increased might be explained by formation of inactive eIF4E dimers (or multimers). We suggest that there is a pre-existing equilibrium that becomes important at high protein concentrations.

\[
\text{eIF4E} \rightleftharpoons (\text{eIF4E})_n
\]

**SCHEME 3**

When the concentration of active eIF4E is lowered due to interaction with m'GpppG during the stopped-flow experiment, the equilibrium...
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shifts and the dimers slowly dissociate, yielding active monomers that can then bind ligand.

\( k_{\text{on}} \) \( \Rightarrow \) eIF4E + m'GpppG \( \Rightarrow \) eIF4E-m'GpppG* \( k_{\text{off}} \)

SCHEME 4

This dissociation reaction produces a second, slow phase of eIF4E-m'GpppG interaction. Scheme 4 could possibly explain our results (Fig. 5D and Table 2) as well as those of previous studies, which were conducted at molar concentrations of either wheat eIF(iso)4F (34) or mouse eIF4E (28–2–17) (35) that were at least 5-fold higher than in Figs. 2–4. Thus, there are two alternative hypotheses to explain the double-exponential kinetics observed at high eIF4E concentrations: dissociation of inactive dimeric eIF4E (Scheme 4) and a two-step binding reaction (Scheme 2).

At 50 mM KCl, the authors of one study (35) calculated \( k_{-1} = 30 \) s\(^{-1}\) and \( k_{-2} = 1 \) s\(^{-1}\). At 150 mM KCl, they obtained \( k_{-1} = 50 \) s\(^{-1}\) and \( k_{-3} = 6 \) s\(^{-1}\). Thus, the step defined by \( k_{-2} \) in Scheme 2 is rate-limiting for the reverse direction (dissociation of the eIF4E-m'GpppG complex) in the two-step model. This provides a means to distinguish experimentally between the one- and two-step models. The dissociation kinetics of the eIF4E-m'GpppG complex can be followed by diluting the complex with buffer in the stopped-flow cell. A first-order rate constant is obtained from the increase in fluorescence upon dissociation of the complex. For the one-step model with pre-equilibrium between monomers and dimers (Scheme 4), \( k_{\text{off}} \) determined in the forward reaction is expected to be equal to \( k_{\text{off}} \) determined in the reverse reaction. For the two-step model (Scheme 2), the apparent \( k_{\text{off}} \) determined in the reverse reaction is the limiting \( k_{-2} \) and is expected to be considerably smaller than the apparent \( k_{\text{off}} \) determined in the forward reaction.

We therefore measured the rate of dissociation of the pre-formed eIF4E-m'GpppG complex by diluting the complex 2-fold with buffer.

Table 2: Effect of eIF4E concentration on the amplitudes of the first and the second phases for reaction of eIF4E with m'GpppG at limiting m'GpppG concentrations

| eIF4E Concentration | \( \Delta F_{\text{on}}^* \) | \( \Delta F_{\text{off}}^* \) |
|---------------------|---------|---------|
| \( \mu\)M           | %       | %       |
| 1.0                 | 69 ± 6  | 31 ± 30 |
| 1.5                 | 61 ± 8  | 39 ± 6  |
| 2.0                 | 52 ± 7  | 48 ± 7  |
| 2.5                 | 34 ± 7  | 70 ± 2  |

*As noted under "Experimental Procedures," the amplitudes of fluorescence changes \( \Delta F \) were measured in \( \mu\)M. However, different stopped-flow instrument settings were used for different eIF4E concentrations, so that a direct comparison of absolute values of \( \Delta F \) is not meaningful. Instead, the relative amplitudes at each eIF4E concentration are presented.

Table 3: Kinetic constant for association and dissociation of eIF4E and eIF4E(P) with an ARCA and an ARCA-capped oligoribonucleotide

| Protein | Ligand | 100 mM KCl Concentration | 350 mM KCl Concentration |
|---------|--------|--------------------------|--------------------------|
|         |        | \( k_{\text{on}} \times 10^6 \) | \( k_{\text{off}} \) | \( K_d \) | \( k_{\text{on}} \times 10^6 \) | \( k_{\text{off}} \) | \( K_d \) |
| eIF4E   | ARCA   | 205 ± 24                 | 76 ± 27                  | 0.37 ± 0.14 | 41 ± 4 | 107 ± 11          | 2.61 ± 0.37 |
| eIF4E   | Capped oligo | ND* | ND | ND | 101 ± 4 | 11 ± 10 | 0.11 ± 0.10 |
| eIF4E(P) | ARCA   | 84 ± 7                   | 74 ± 20                  | 0.88 ± 0.25 | 32 ± 3 | 106 ± 13          | 3.31 ± 0.51 |
| eIF4E(P) | Capped oligo | 394 ± 54                 | 103 ± 50                 | 0.26 ± 0.13 | 37 ± 3 | 18 ± 6           | 0.49 ± 0.17 |

*ND, not determined because the rate was too fast to be measured.
Phosphorylation of eIF4E decreased $k_{\text{off}}$ for the capped oligoribonucleotide sufficiently that it could be measured at both 100 and 350 mM KCl (Table 3). The capped oligoribonucleotide associated with eIF4E(P) 4.7-fold faster than the cap analog at 100 mM KCl ($k_{\text{on}} = 394 \pm 54 \times 10^6$ versus $84 \pm 7 \times 10^6$ M$^{-1}$ s$^{-1}$). At 350 mM KCl, the effect of the oligoribonucleotide chain was eliminated, because $k_{\text{on}}$ values were statistically the same for capped oligoribonucleotide and cap analog. Because this was not the case for unphosphorylated eIF4E, it appears that Ser-209 phosphorylation negates the increase in association rate contributed by the oligoribonucleotide chain, perhaps because of charge repulsion. No statistical difference in $k_{\text{off}}$ could be detected between capped oligoribonucleotide and cap analog at 100 mM KCl but at 350 mM, the capped oligoribonucleotide dissociated 5.9-fold more slowly than the cap analog. For both eIF4E and eIF4E(P) at 350 mM KCl, the decrease in $k_{\text{off}}$ is the major determinant for the higher affinity of capped oligoribonucleotide compared with cap analog.

**DISCUSSION**

Protein synthesis is a dynamic process in which cap binding and release presumably occur at least once for each round of initiation. We therefore sought to compare kinetic rather than equilibrium parameters for cap interaction with eIF4E versus eIF4E(P). Because the binding and dissociation reactions are fast, an appropriate method is to measure pre-steady-state kinetics in rapid mixing experiments. Whereas eIF4E and eIF4E(P) have been compared by equilibrium techniques, and eIF4E interactions with cap analogs have been studied by stopped-flow techniques, eIF4E and eIF4E(P) have not previously been compared by stopped-flow techniques. Furthermore, the binding of eIF4E or eIF4E(P) to capped oligoribonucleotides, which are structurally more similar to mRNA than cap analogs, has not previously been studied by stopped-flow techniques.

**Magnitude of Kinetic Constants**—Our stopped-flow data for cap analogs are in reasonable agreement with those of Blachut-Okrasinska et al. (35), who found $k_{\text{off}} = 24–35$ s$^{-1}$ for the interaction between m$^7$GpppG and mouse eIF4E-(28–217) in stopped-flow experiments conducted from 50 to 350 mM KCl, compared with our values of 72–85 s$^{-1}$ (Table 1). Our ~2-fold higher $k_{\text{off}}$ values may be because of human versus mouse eIF4E (although they are 99% identical), the presence of the N-terminal 27 amino acid residues in our eIF4E preparations, or the fact that the natural Thr-205 is replaced with Cys-205 in eIF4E-(28–217) phosphorylated by intein technology. Dlugosz et al. (36) did not study m$^7$GpppG, but they did obtain $k_{\text{on}}$ values for m$^7$GTP and m$^7$GDP by stopped-flow measurements of mouse eIF4E-(28–217) that were in the range of 40–75 s$^{-1}$. Similarly, $k_{\text{on}}$ for vaccinia virus VP39 was reported to be 41–60 s$^{-1}$ (61).

Our $k_{\text{off}}$ values for eIF4E and eIF4E(P) interaction with a capped oligoribonucleotide are at variance with the findings of Scheper et al. (37), despite the fact that human eIF4E was used in both cases and the method of phosphorylation was the same. These authors determined $k_{\text{off}}$ for a capped 18-mer oligoribonucleotide at 100 mM KCl to be 0.06 s$^{-1}$ for mock-phosphorylated eIF4E, 0.078 s$^{-1}$ for untreated eIF4E, and 0.58 s$^{-1}$ for eIF4E(P), whereas our $k_{\text{off}}$ value for eIF4E(P) under similar conditions was 103 s$^{-1}$, or 186-fold greater (Table 3). This discrepancy may be because of the inability of SPR to measure kinetic parameters for reactions as fast as the binding of eIF4E to cap analogs or capped oligoribonucleotides. The upper limits for $k_{\text{on}}$ and $k_{\text{off}}$ measured by SPR are
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10^{-7} \text{ M}^{-1} \text{ s}^{-1} \text{ and } 0.1 \text{ s}^{-1}, \text{ respectively} (78), \text{ which are several orders of magnitude lower than with stopped-flow techniques. This conclusion is further supported by the low analyte response observed by Schaper et al. (37). From the 1:1 stoichiometry of the binding reaction, the molecular masses of a capped 18-mer oligoribonucleotide and eIF4E, and the ligand response (79), one can calculate a theoretical analytic response of 586 RU. The analyte responses reported by Schaper et al. (37) do not exceed 200 RU for eIF4E or 50 RU for eIF4E(P). One of the causes for a low maximum analytic response is when reaction rates exceed the range measurable by the SPR technique (78, 80).

Mechanism of eIF4E Association with Cap Analogs as Inferred from Kinetics Measurements—Cap binding to eIF4E has been studied extensively by both equilibrium and kinetic methods (see Introduction). Most of these studies have utilized a decrease in intrinsic Trp fluorescence upon cap binding, although some have utilized SPR (37), isothermal titration calorimetry (87), or NMR (12). Results from some of these studies have led the authors to propose a two-step binding mechanism (26, 34, 35). The first step is envisioned as being the ligand entering the cap-binding slot and anchoring, via the triphosphate moiety, to basic amino acid residues. The second step is a change within the m7G-binding slot that leads to a further fluorescence decrease. Molecular dynamics simulations have also supported a model in which there is a conformational change in eIF4E upon cap binding (13) (although this does not per se constitute proof for two kinetically distinct steps). By contrast, a one-step binding model has been proposed for another cap-binding protein, the vaccinia virus VP39 (61). Cap binding by VP39 involves a cation-π sandwich of m7G between two aromatic amino acid residues, similar to the cap-eIF4E interaction. However, in this case, phosphate groups do not contribute to the binding process.

Our data also show that association between eIF4E and cap analogs as well as oligoribonucleotides behave kinetically as a simple one-step process at all salt concentrations investigated and regardless of whether eIF4E is phosphorylated. The discrepancy between our results and those of others (26, 34, 35) could be explained by the existence of a rate-limiting, parallel reaction resulting from dissociation of pre-formed eIF4E dimers or oligomers. We postulate an equilibrium between the reactive monomer and unreactive dimers or higher order oligomers (Scheme 3). The fast phase of fluorescence change is because of m7GpppG binding to monomeric eIF4E according to Scheme 1. The slow phase is a result of the rate-limiting dissociation of unreactive oligomers to yield reactive monomers, which in turn react with the cap analog according to Scheme 4. However, the cap-binding reaction per se is still a one-step process.

The evidence supporting this alternative mechanism is as follows. First, under pseudo first-order reaction conditions (with [eIF4E] limiting and <0.5 μM), the experimental data were fit by a single-exponential function and dependence of $k_{\text{on}}$ on m7GpppG concentrations was linear. Second, the slower second phase was observed only at elevated eIF4E concentrations, which is consistent with concentration-dependant protein self-association. Whereas most of our experiments were conducted at eIF4E concentrations of 0.1–0.2 μM, those of Sha et al. (34) were conducted at 0.5 μM and those of Blachut-Okrasinska et al. (35) were conducted at 0.1–4.1 μM. Examination of the latter data (35) reveals that the double-exponential function improves the fit only when the eIF4E concentration is increased (Tables A1, A2, and A3). We did not observe biphasic kinetics until eIF4E concentrations exceeded 0.5 μM (Table 2). Third, the value of $k_{\text{off}}$ determined from the reverse reaction is the same as $k_{\text{off}}$ determined from the forward reaction.

Effect of eIF4E Phosphorylation on Cap Binding—Two similar models have been proposed for the role of eIF4E phosphorylation in translation initiation. Marcotrigiano et al. (11) suggested a “clamping” mechanism in which the generation of a salt bridge of Ser-209 with Lys-159 acts as a clamp that stabilizes the cap in the binding slot. Tomoo et al. (13) used molecular dynamics simulations to suggest that a hydrogen-bonded cluster of water molecules or polar amino acid residues could form around the diatomic form of Ser(P)-209. This could potentially block the release of the cap from the binding slot. In both cases, stabilization is envisioned to facilitate the assembly of initiation complexes loaded with mRNA. The principal prediction from both hypotheses is a significant decrease in $k_{\text{off}}$ upon phosphorylation of eIF4E because of closure of the cap-binding pocket. Also, in such a mechanism, $k_{\text{on}}$ is expected to be significantly impaired if mRNA were to interact with eIF4E in which a salt bridge had already been formed between Ser-209 and Lys-159. A different type of model was proposed by Schaper et al. (37) on the basis of their results from SPR experiments showing that phosphorylation of eIF4E accelerates the rate of cap dissociation. They speculated that phosphorylation facilitates the release of eIF4E and other initiation factors from the 5′-end of the mRNA.

Our determination of $k_{\text{on}}$ and $k_{\text{off}}$ for eIF4E and eIF4E(P) allows us to test these predictions directly. We did not find any significant difference in $k_{\text{off}}$ values between eIF4E and eIF4E(P) at any salt concentrations investigated, either for cap analogs or capped oligoribonucleotide (Tables 1 and 3). By contrast, Schaper et al. (37) found a ~10-fold increase in $k_{\text{off}}$ for eIF4E(P) interaction with capped oligoribonucleotide compared with eIF4E. Furthermore, $k_{\text{on}}$, from that study (calculated from $K_g$ and $k_{\text{on}}$) is slightly higher for eIF4E(P) binding to capped oligoribonucleotide compared with eIF4E, although we find $k_{\text{on}}$ to be 2–3-fold lower for eIF4E(P) compared with eIF4E, as measured with both cap analogs (Tables 1 and 3) and the capped oligoribonucleotide (at 350 mM KCl) (Table 3). Thus, our results do not support any of the previously proposed hypotheses. Instead, we propose that phosphorylation of Ser-209, which is located at the entrance to the cap-binding slot (Fig. 8A), diminishes the rate of association by charge repulsion but has no effect on the rate of dissociation. This model is consistent with the observation that the effect of eIF4E phosphorylation is progressively eliminated as the KCl concentration is increased (Refs. 32 and 35; Fig. 4A); at high salt concentrations, the charge on Ser(P)-209 is shielded and its inhibitory effect on the association rate is masked (Fig. 4A).

Reaction of eIF4E and eIF4E(P) with Capped Oligoribonucleotides: an Additional Binding Site for mRNA?—We found that a capped 12-mer oligoribonucleotide interacts with both eIF4E and eIF4E(P) in a very fast reaction. The addition of an oligoribonucleotide chain to the cap structure does not change the kinetic mechanism of binding. It is still a one-step reaction, but both $k_{\text{on}}$, and $k_{\text{off}}$ are different for binding of the capped oligoribonucleotide compared with the cap analog. For eIF4E(P) at 100 mM KCl, $k_{\text{on}}$, is ~5-fold greater for capped oligoribonucleotide than cap analog (Table 3). The reaction for unphosphorylated eIF4E is too fast to be measured by stopped-flow kinetics at 100 mM KCl, but at 350 mM KCl, the reaction is slowed enough to reveal that $k_{\text{on}}$, is ~2.5-fold greater for capped oligoribonucleotide than cap analog. Interestingly, the combination of two inhibitory effects on $k_{\text{on}}$, introduction of a phosphate group on Ser-209, and shielding of positively charged amino acid residues by high ionic strength, causes $k_{\text{on}}$, to be the same for capped oligoribonucleotide and cap analog.

3 The analyte response can be calculated from the expression (80), $S = (AR/LR) \times (LM/AM)$, where $S$ is the stoichiometry, $AR$ is the analyte response, $LR$ is the ligand response, $LM$ is the ligand molecular mass, and $AM$ is the analyte molecular mass. $LR$ is not given in Ref. 37 but is given in a publication cited in that work (85) as 150 RU for SPR of eIF4E on immobilized capped oligoribonucleotides. $LM$ and $AM$ are 6,400 and 25,000 Da, respectively, and $S$ is 1.0. $AR$ is therefore calculated to be 586 RU.
is $k_{on}$, which decreases 2–3-fold. Is such a difference biologically significant? Even though 48 S complex formation is thought to be rate-limiting for initiation of protein synthesis (81, 82), the association of mRNA with eIF4E and eIF4E(P) is so fast (as inferred from measurements with a capped oligoribonucleotide) that it may not be rate-limiting for 48 S initiation complex formation, especially because $k_{on}$ is near the diffusion limit for both eIF4E and eIF4E(P). Perhaps some other process in 48 S complex formation is rate-limiting. Arguing against this suggestion is the fact that the binding affinity of various cap analogs to eIF4E is positively correlated with the translational efficiency of mRNA capped with those analogs, as measured both in vitro (63, 69, 83) and in vivo (84). This correlation would not hold if cap-eIF4E interactions did not make a significant contribution to the rate of initiation. Given that eIF4E is involved in recruitment of the unwinding machinery (1), it is possible that the effect of eIF4E phosphorylation on the rate of initiation is manifested in subsequent steps rather than cap binding per se.

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FIGURE 8. Three-dimensional models of human eIF4E in complex with m7GpppA. Data are taken from Ref. 86. A. ribbon drawing showing phosphorylation at Ser-209. B. space-filling model showing surface charge distribution. The color code for amino acid residues are: basic, green; acidic, magenta; hydrophobic, blue; and uncharged polar, yellow. The images were created using the Brookhaven Protein Data Bank data file 1WKW and RasMol.

At high salt concentrations, a difference in $k_{on}$ between capped oligoribonucleotide and cap analog also became apparent. For eIF4E, it was $\sim$10-fold lower for capped oligoribonucleotide, and for eIF4E(P), it was $\sim$7-fold lower. Thus, both $k_{on}$ and $k_{off}$ contribute to a decrease in $K_d$ when comparing capped oligoribonucleotide to the cap analog: $\sim$24-fold for eIF4E and $\sim$7-fold for eIF4E(P) at 350 mM KCl. These data are in agreement with previous equilibrium experiments where the binding of natural rabbit eIF4E to globin mRNA was found to be 5.3–5.5-fold stronger than its binding to m7GpppG (18, 22). Binding to capped oligoribonucleotides was also stronger than to m7GpppG but varied with the degree of oligoribonucleotide secondary structure (22).

The pronounced increase in affinity for capped oligoribonucleotide suggests that some additional stabilizing interactions may be formed that slow dissociation of the larger ligand. The $\Delta G^\circ$ caused by addition of the oligoribonucleotide chain to the cap structure is $\sim$1.9 kcal/mol for eIF4E and $\sim$1 kcal/mol for eIF4E(P). This is sufficient for the formation of an additional salt bridge or hydrogen bond between the body of the mRNA and amino acid residues near the entrance of the cap-binding slot. There are several basic amino acid residues located on the surface of eIF4E near the cap-binding slot (Arg-112, Arg-157, Lys-159, and Lys-162) (Fig. 8B). Additional basic residues are found on the lateral surface of a groove where the polynucleotide chain of mRNA conceivably could be bound (Lys-206, Lys-212, Lys-192, Lys-119, and Lys-52). It is possible that additional interactions occur between the polynucleotide chain and one or more of these basic side chains.

Role of eIF4E Phosphorylation in Protein Synthesis—Our results indicate that the only kinetic parameter affected by eIF4E phosphorylation is $k_{on}$, which decreases 2–3-fold. Is such a difference biologically significant? Even though 48 S complex formation is thought to be rate-limiting for initiation of protein synthesis (81, 82), the association of mRNA with eIF4E and eIF4E(P) is so fast (as inferred from measurements with a capped oligoribonucleotide) that it may not be rate-limiting for 48 S initiation complex formation, especially because $k_{on}$ is near the diffusion limit for both eIF4E and eIF4E(P). Perhaps some other process in 48 S complex formation is rate-limiting. Arguing against this suggestion is the fact that the binding affinity of various cap analogs to eIF4E is positively correlated with the translational efficiency of mRNA capped with those analogs, as measured both in vitro (63, 69, 83) and in vivo (84). This correlation would not hold if cap-eIF4E interactions did not make a significant contribution to the rate of initiation. Given that eIF4E is involved in recruitment of the unwinding machinery (1), it is possible that the effect of eIF4E phosphorylation on the rate of initiation is manifested in subsequent steps rather than cap binding per se.
