Interaction of Genome-linked Protein (VPg) of Turnip Mosaic Virus with Wheat Germ Translation Initiation Factors eIFiso4E and eIFiso4F

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The interaction between VPg of turnip mosaic virus and wheat germ eukaryotic translation initiation factors eIFiso4E and eIFiso4F (the complex of eIFiso4E and eIFiso4G) were measured and compared. The fluorescence quenching data showed the presence of one binding site on eIFiso4E for VPg. Scatchard analysis revealed the binding affinity (K0) and average binding sites (n) for VPg were (8.51 ± 0.21) × 103 M−1 and 1.0, respectively. The addition of eIFiso4G to the eIFiso4E increased the binding affinity 1.5-fold for VPg as compared with eIFiso4E alone. However, eIFiso4G alone did not bind with VPg. The van't Hoff analyses showed that VPg binding is enthalpy-driven and entropy-favorable with a large negative ΔH° (−29.32 ± 0.13 kJmol−1) and positive ΔS° (36.88 ± 0.25 Jmol−1 K−1). A Lineweaver-Burk plot indicates mixed-type competitive ligand binding between VPg and anthraniloyl-7-methylguanosine triphosphate for eIFiso4E. Fluorescence stopped-flow studies of eIFiso4E and eIFiso4F with VPg show rapid binding, suggesting kinetic competition between VPg and m7G cap. The VPg protein binds much faster than cap analogs. The activation energies for binding of eIFiso4E and eIFiso4F with VPg were 50.70 ± 1.27 and 75.37 ± 2.95 kJmol−1 respectively. Enhancement of eIFiso4F-VPg binding with the addition of a structured RNA derived from tobacco etch virus suggests that translation initiation involving VPg occurs at internal ribosomal entry sites. Furthermore, the formation of a protein-RNA complex containing VPg suggests the possibility of direct participation of VPg in the translation of the viral genome.

Viral RNAs share characteristics with the host cell mRNAs but must employ different strategies for preferential translation. Recently attention has focused on the possible role of the viral protein linked to the genome (VPg)1–3. Turnip mosaic virus (TuMV) belongs to the potyvirus group, a member of the super group of picorna-like viruses (4). The viral genome is a positive single-stranded RNA molecule of about 10 kilobases in length, a poly(A) tail at the 3′-end, and a VPg of molecular mass 22–24 kDa (5–7) covalently linked to the 5′-end. The potyviral VPg is multifunctional, and interaction between VPg and eIF4E has been reported for TuMV (2, 8), but the consequences of this interaction with respect to translation initiation are not clear because elements in the 5′-untranslated region of the potyviral RNA direct cap-dependent translation (9). Viral proteins are likely to participate in the regulation of viral genome translation (10, 11). VPg has several suggested roles in the virus life cycle. Interactions of VPg with the viral RNA polymerase in yeast (12, 13) and in vitro (14) support a role in viral RNA synthesis. Additionally, VPg has been implicated in overcoming resistance in plants (15–19). VPg-Pro of tobacco etch potyvirus also interacts with eIF4E from tomato and tobacco, and the interaction was shown to enhance genome amplification (20).

In wheat germ (Triticum aestivum) and other plants, an isoform of eIF4F called eIFiso4F has been found (21, 22). eIFiso4F functions like eIF4F in supporting cap-dependent translation (22). However, eIF4F and eIFiso4F show preferences for translation of structured and unstructured non-coding regions, respectively. eIFiso4F contains two subunits, a 28-kDa eIFiso4E and an 86-kDa eIFiso4G. The eIFiso4E acts as the binding site of m7GpppN cap. eIFiso4G binds to mRNA in an ATP-dependent manner, may interact with eIF4A and eIF4B (23), and more interestingly, may interact with poly(A)-binding protein (24, 25). Wheat germ eIF4F consists of only two subunits, a 26-kDa eIF4E and a 220-kDa eIF4G in a 1:1 molar ratio. Some structural and functional similarity exists between eIF4F and eIFiso4F. Functionally, they are very similar even though these two proteins are antigenically distinct (26). Wheat germ eIFiso4F can substitute for mammalian eIF4F in an RNA-dependent ATPase activity and in cross-linking of mammalian eIF4A to the cap of oxidized mRNA (27).

The interaction between the VPg of TuMV and the translation eukaryotic initiation factor eIFiso4E and eIFiso4F of Arabidopsis thaliana has previously been reported (2, 8). These observations suggest VPg is important in initiation of protein

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2 The abbreviations used are: VPg, viral protein linked to the genome; TuMV, turnip mosaic virus; eIF, eukaryotic initiation factor; IRES, internal ribosomal entry sites; Ant, anthraniloyl; m7GTP, 7-methylguanosine triphosphate; DTT, dithiothreitol; TEV, tobacco etch virus; PK1, Pseudoknot 1; S1-3, stem mutation on PK1 of TEV.
EXPERIMENTAL PROCEDURES

Materials—The TuMV full-length cDNA clone and construction of the expression vector for TuMV VPg was described previously (30, 31). Glutathione-Sepharose 4B and m7GTP-Sepharose were purchased from Amersham Biosciences. Nickel-nitrilotriacetic acid Superflow was purchased from Qiagen by Herbert and co-workers (28). Michon et al. (29) characterized the interaction of lettuce elf4E and VPg from lettuce mosaic virus. Earlier studies (30) have shown that interaction between VPg and plant elf4E is sufficiently strong so that VPg competes with cap binding. In this study we further characterize this interaction, determine the effects of elf4G on the VPg and elf4E interaction, and demonstrate an interaction with viral RNA. The kinetics of binding suggest that VPg binding to elf4E is favored. We propose a mechanism where VPg substitutes for the cap analog and enhances formation of an elf4E complex with viral internal ribosomal entry sites (IRES).

Expression and Purification of Recombinant Proteins—elf4E and elf4G were expressed in Escherichia coli containing the constructed pET3d vector in BL21 (DE3) pLysS as described elsewhere (32). A HiTrap Mono Q ion exchange column and an m7GTP-Sepharose column were used for the purification of elf4E. E. coli cells were disrupted by sonication, suspended in 20 mM HEPES/KOH, pH 7.6, 100 mM KCl, 1.0 mM MgCl2, 1.0 mM dithiothreitol, 0.1 mM EDTA, 5% glycerol, 600 mM KCl) containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 ml of aprotinin, and 100 μg/ml soybean trypsin inhibitor. The lysed cells were centrifuged at 45,000 rpm for 2 h. The supernatant was dialyzed against buffer B-50, loaded onto a 5-ml HiTrap SP column, and washed with 50 mM Tris-HCl buffer until the optical density returned to the base line. A 50–400 mM KCl linear gradient (total volume 100 ml) was used to elute the elf4G, and 1.0-ml fractions were collected. The elf4G appeared in the 200–300 mM KCl fractions. After purification, the purity was confirmed by 10% SDS-polyacrylamide gel electrophoresis. All steps were carried out in a cold box at −5°C.

For purification of TuMV VPg (30) E. coli BL21(DE3)pLysS cells were transformed with pETVPg1. Cells were cultured in LB medium containing 100 μg/ml ampicillin and 20 μg/ml chloramphenicol at 25 °C. At an A600 of 0.5, expression was induced for 6 h with 50 μM isopropyl-1-thio-β-D-galactopyranoside, after which cells were harvested by centrifugation. Subsequent steps were performed at 4 °C. Cell pellets were resuspended into buffer A and disrupted by sonication, and the lysates were centrifuged. Supernatant was applied to a 1-ml nickel-nitrilotriacetic acid Superflow column equilibrated with buffer A. The column was washed 3 times with 10 ml of buffer A, and the bound protein was eluted with 5 ml of buffer B. The protein was dialyzed against buffer C, further purified to homogeneity on Mono Q and HiLoad 16/60 Superdex 75 pg columns, and finally dialyzed against buffer D. The purified VPg-His6 was confirmed by 15% SDS-PAGE.

All samples were dialyzed against buffer B (20 mM HEPES/KOH, pH 7.6, 100 mM KCl, 1.0 mM MgCl2, 1.0 mM dithiothreitol, 1.0 mM EDTA) and passed through a 0.22 μm filter (Millipore) before the spectroscopy measurements were performed. The fractions were concentrated to a Centricon 10 (Amicon Co.) as necessary. The concentrations of protein were determined by a Bradford assay with bovine serum albumin as standard (33) using a Bio-Rad protein assay reagent (Bio-Rad).

The Tobacco Etch Virus (TEV) mRNA Synthesis in Vitro—The (TEV PK1 and S1-3 of PK1) clones were described previously (34). DNA was linearized with Sall, a site immediately upstream of the luc open reading frame. The linearized DNA was treated with 50 mM Tris-HCl, pH 7.5, containing proteinase K (100 μg/ml), 0.5% SDS, and 5 mM CaCl2 for 30 min at 37 °C. DNA was further purified by phenol:chloroform:isoamyl alcohol (25:24:1) at pH 8.0 and ethanol precipitation. Purity was confirmed by 1% agarose gel electrophoresis. All steps were carried out in a cold box at −5°C.

Fluorescence Titration Measurements—Fluorescence measurements were performed using a Spex Fluorolog r2 spec-
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trofluorimeter equipped with excitation and emission polarizers. The formation of the binary protein-protein and protein-RNA complexes was studied by direct fluorescence titration. The excitation and emission slits were set on 4 and 5 nm, respectively. The excitation wavelength for eIFiso4E was 280 nm, and emission was monitored at 332 nm. The excitation slits were chosen to avoid photobleaching, and the absorbance of the sample at the excitation wavelength was less than 0.02 to minimize the inner-filter effect. Emission spectra were corrected for the wavelength-dependent lamp intensity and monochromator sensitivities.

The samples were thermostatted at the different temperatures, i.e. 5, 10, 15, 22, 25, and 32 ± 0.5 °C. The temperature was monitored by a thermocouple inside the cuvette. Titration were performed in 20 mM HEPES/KOH, pH 7.6, 100 mM KCl, 1.0 mM MgCl$_2$, and 1.0 mM DTT. The normalized fluorescence difference (ΔF/ΔF$_{max}$) between the protein-protein complex and the sum of the individual fluorescence spectra was used to determine the equilibrium dissociation constant (K$_d$). The details of the data fitting are described elsewhere (35). Fluorescence intensities, when necessary, were corrected for dilution and for inner filter effect. Nonlinear least squares fitting of the data were performed using KaleidaGraph software (Version 2.1.3; Abelbeck Software).

Determination of the Number of Binding Sites—Protein-protein interaction of genome-linked protein (VPg) of turnip mosaic virus with wheat germ eIFiso4E and eIFiso4F (complex of eIFiso4E and eIFiso4G) were studied by direct fluorescence titration. A solution of purified eIFiso4E (0.5 μM) was titrated with increasing amounts of VPg protein. The fluorescence intensity at emission maximum (332 nm) was used for calculating the relative fluorescence, considering the fluorescence intensity of control untreated eIFiso4E as 100. The fractional quench, Q, was determined at each VPg/eIFiso4E molar ratio (R). For an observed fluorescence intensity F, the fractional quench, Q, was obtained from the equation Q = (F$_0$ - F)/m (maximal quench). Fractional quench, Q, is linearly related to VPg binding. [VPg-eIFiso4E]/[eIFiso4E]$_T$ = Q, where [eIFiso4E]$_T$ represents the total protein concentration. The average number of binding sites (n) were determined from intercept on the x axis of the Scatchard plot Q versus Q/(R - Q)[eIFiso4E]$_T$ (36, 37). The slope also gives K$_d$, which is in agreement with K$_d$ obtained from nonlinear least square fitting.

Thermodynamic Parameters for eIFiso4E and eIFiso4F Binding to VPg—The temperature dependence of K$_d$ for VPg was analyzed according to the van’t Hoff isobaric equation assuming the entropy change, ΔS, and the enthalpy change, ΔH, as constants over the range of temperatures studied.

$$\ln K_d = \frac{1}{R} \left( \frac{\Delta H}{T} - \Delta S \right)$$  

(Eq. 1)

Competitive Binding Experiments—The fluorescent cap analogue, anthraniloyl (Ant)-m$^7$GTP, was synthesized as described previously (38). The competitive substitution reactions were performed at constant Ant-m$^7$GTP concentration (0.1 μM) and increasing amounts of VPg. The fluorescence measurements were made at 25 °C in 20 mM HEPES/KOH, pH 7.6, 100 mM KCl, 1.0 mM MgCl$_2$, and 1.0 mM DTT. An excitation wavelength of 332 nm was used to monitor the Ant-m$^7$GTP fluorescence emission at 420 nm.

Stopped-flow Fluorescence Kinetics—Stopped-flow fluorescence experiments were performed on an OLIS RSM 1000 stopped-flow system with a 1-ms dead time. The excitation wavelength was 280 nm, and the cut-on filter was 324 nm for eIFiso4E and eIFiso4F with VPg interactions. A reference photomultiplier was used to monitor fluctuations in the lamp intensity. The temperature of the flow cell and solution reservoirs was maintained using a temperature-controlled circulating water bath. VPg binding induced a decrease in eIFiso4E fluorescence. After rapid mixing of 1 μM (0.5 μM after mixing) eIFiso4E or eIFiso4F with 5 μM (2.5 μM after mixing) VPg, the time course of the fluorescence intensity change was recorded by computer data acquisition. In each experiment, 1000 pairs of data were recorded, and sets of data from 3 experiments were averaged. Each averaged set of stopped-flow data was then fitted to nonlinear analytical equations using Global analysis software provided by OLIS. Data were fitted to the single and double exponential functions. Fitted curves correspond to the single exponential equation (39),

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

(Eq. 2)

where $F_t$ is that fluorescence observed at any time, t, ΔF is the amplitude, $F_\infty$ is the final value of fluorescence, and $k_{obs}$ is the observed first-order rate constant. The kinetic data for double exponential fits

$$F_t = \Delta F_1 \exp(-k_{obs1} t) + \Delta F_2 \exp(-k_{obs2} t) + F_\infty$$

(Eq. 3)

where $\Delta F_1$ and $\Delta F_2$ are the amplitudes of two exponentials with rate constants $k_{obs1}$ and $k_{obs2}$ respectively. The residuals were measured by the differences between the calculated fit and the experimental data. The reaction was consistent with a single exponential process. The derived rate constants were used to construct an Arrhenius plot according to the equation,

$$\ln k = \frac{E_a}{RT} + \ln A$$

(Eq. 4)

where k is the rate constant, $E_a$ is the activation energy, and A is the Arrhenius pre-exponential term. The activation energy was calculated from the slope of the fitted linear plot of ln k versus 1/T (Kelvin).

Dissociation Rate Constants—To measure the dissociation rate constants, a complex of eIFiso4E or eIFiso4F with VPg was rapidly diluted 15-fold in a spectrofluorimeter cuvette, and the resulting increase in fluorescence was measured. Because of the high binding affinity of the protein-protein complex, a large dilution, which could not be accomplished by stopped-flow, was necessary. The concentration of the reactants before mixing was 10 μM VPg and 2 μM (each) eIFiso4E and eIFiso4F, respectively. The dissociation rates were determined from fits of the appropriate equations to the data using nonlinear least-square fitting program KaleidaGraph software (Version 2.1.3, Abelbeck software).
RESULTS

Fluorescence Titration of eIFiso4E and eIFiso4F with VPg—
To determine the binding constants for the viral protein linked to the genome (VPg) of TuMV and wheat germ eukaryotic translation initiation factors eIFiso4E and eIFiso4F, direct fluorescence titration studies were performed as shown in Fig. 1. After the inner-filter effect corrections, we observed a total percent quench 58 and 50% for eIFiso4E and eIFiso4F fluorescence upon the addition of VPg at the highest molar ratios. The inset in Fig. 1 shows the corresponding Scatchard analyses. The slope and intercept of the straight line obtained on the plot $Q/[VPg]$ vs. $Q$ provided the binding constant ($K_b$) and binding capacity ($n$) of translation initiation factors for viral genome-linked protein (VPg). $K_b$ values were obtained using a non-linear least squares analysis described elsewhere (35). eIFiso4F exhibited almost 1.5 times stronger binding affinity than eIFiso4E (eIFiso4F, $K_d = 81.31 \pm 1.9$ nM; eIFiso4E, $K_d = 117.48 \pm 2.7$ nM). Similarly, the average binding sites ($n$) of eIFiso4E and eIFiso4F for VPg were determined to be 0.98 ± 0.15. Scatchard analysis of the binding data (Fig. 1) suggests that eIFiso4E and eIFiso4F each bind a single VPg.

Fig. 2 shows a representative plot of the fluorescence intensity measurements on binding of eIFiso4E and eIFiso4F with VPg. The titration curve shows the difference in fluorescence intensity between the protein–protein complex and the sum of the individual fluorescence intensities. From such analysis, the equilibrium binding constant can be calculated for the interactions between eIFiso4E or eIFiso4F with VPg (Table 1). eIFiso4F showed a strong interaction with VPg in the presence of eIFiso4G. No interaction was observed between eIFiso4G and VPg. A comparison of the dissociation constants at different temperatures is shown in Table 1. eIFiso4F has a higher affinity for VPg with a dissociation constant of 62.43 ± 0.61 nM as compared with eIFiso4E (97.47 ± 1.6 nM) at 22 °C. From the temperature dependence of these dissociation constants, the thermodynamic parameters for eIFiso4E and eIFiso4F with VPg were calculated (Table 2). A von’t Hoff plot of $-\ln K_{eq}$ versus the reciprocal of temperature ($T^{-1}$) was used to calculate the thermodynamic parameters, entropy ($\Delta S^\circ$), and enthalpy ($\Delta H^\circ$). Fig. 3 shows the van’t Hoff plot based on VPg binding to eIFiso4E or eIFiso4F; the values of $\Delta H^\circ$ and $\Delta S^\circ$ were obtained from the intercept and slope, respectively (correlation coefficient of >0.95). The van’t Hoff analyses showed that the VPg binding is enthalpy-driven and entropy-favorable with a large negative $\Delta H^\circ$ ($-29.32 \pm 0.13$ kJ mol$^{-1}$) and positive $\Delta S^\circ$ ($36.88 \pm 0.25$ kJ mol$^{-1}$ K$^{-1}$) for eIFiso4E. The $\Delta G^\circ$ values at 25 °C were calculated from equation, $\Delta G^\circ = -RT \ln K_{eq}$. The $\Delta G^\circ$ values for eIFiso4E ($-39.53 \pm 0.32$ kJ mol$^{-1}$) and eIFiso4F ($-40.45 \pm 0.51$ kJ mol$^{-1}$) with VPg were shown in Table 2. Although the $\Delta G^\circ$ value for the binding of VPg to eIFiso4E and eIFiso4F was almost the same, the data suggest different forces driving the interaction. The binding of eIFiso4E-VPg and eIFiso4F-VPg occurs as enthalpy-driven with large negative $\Delta H^\circ$ and large positive $\Delta S^\circ$, leading to a negative $\Delta G^\circ$ (Table 2). However, the activation energy for eIFiso4F is substantially larger, suggesting an energetically less favorable transition state.

To determine whether binding of VPg for eIFiso4E is competitive or noncompetitive for cap association, the fluorescent cap analog Ant-m7GTP was used. The fluorescence change of the Ant-m7GTP cap was measured with increasing concentrations of eIFiso4E in the absence and presence of VPg. Fluorescence data were represented as a Lineweaver-Burk plot as shown in Fig. 4. Data points were fitted using least square analysis. Lineweaver-Burk plots meet at the left
TABLE 1
Equilibrium dissociation constants for the interaction of eIFiso4E and eIFiso4F (the complex of eIFiso4E and eIFiso4G) with VPg, determined by fluorescence titration

| Complex          | $K_d$ at 5 °C (nM) | $K_d$ at 10 °C (nM) | $K_d$ at 15 °C (nM) | $K_d$ at 22 °C (nM) | $K_d$ at 25 °C (nM) | $K_d$ at 32 °C (nM) |
|------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|
| eIFiso4E-VPg     | 47.09 ± 0.65       | 58.16 ± 0.51       | 70.89 ± 0.73       | 97.47 ± 1.6        | 117.48 ± 2.7       | 137.59 ± 0.93      |
| eIFiso4F-VPg     | 30.52 ± 0.36       | 39.19 ± 0.29       | 47.87 ± 0.44       | 62.43 ± 0.6        | 81.31 ± 1.9        | 97.76 ± 0.58       |
| eIFiso4E-Ant-m7GTP $^a$ | 10.5               | 20.95              | 33.7               | 56.69              | ND                 | 112.2              |
| eIFiso4F-Ant-m7GTP $^a$ | 9.2                | 16.34              | 23.02              | 36.9               | ND                 | 76.6               |

$^a$ Values were obtained from Khan and Goss (35). ND, not determined. Dissociation constants were obtained by titrating eIFiso4E or eIFiso4F (500 nM) with VPg (0–1000 nM). The excitation wavelength was 280 nm, and fluorescence emission was 332 nm.

TABLE 2
Thermodynamic parameters of eIFiso4E and eIFiso4F (the complex of eIFiso4E and eIFiso4G) with VPg

| Complex          | $\Delta H^\circ$ (kJ mol$^{-1}$) | $\Delta S^\circ$ (kJ mol$^{-1}$) | $\Delta G^\circ$ (kJ mol$^{-1}$) | $E_a$ (kJ mol$^{-1}$) |
|------------------|---------------------------------|---------------------------------|---------------------------------|----------------------|
| eIFiso4E-VPg     | $-29.32 \pm 0.13$               | $36.88 \pm 0.25$               | $-39.53 \pm 0.32$              | 50.70 ± 1.27         |
| eIFiso4F-VPg     | $-31.03 \pm 0.19$               | $32.32 \pm 0.43$               | $-40.45 \pm 0.51$              | 75.37 ± 2.95         |

$^a$ Values were obtained from Khan and Goss (35). ND, not determined. Dissociation constants were obtained by titrating eIFiso4E or eIFiso4F (500 nM) with VPg (0–1000 nM). The excitation wavelength was 280 nm, and fluorescence emission was 332 nm.

FIGURE 3. van’t Hoff plots for the interaction of VPg with eIFiso4E (C) and eIFiso4F (D).

The effect of TEV PK1 on the binding of eIFiso4E with VPg was measured. TEV PK1 is an internal ribosome entry site that confers translational enhancement to uncapped RNA. The stem mutant S1-3 increases translation of uncapped RNA by about 10% of the level of PK1. The binding affinity of eIFiso4F-VPg ($K_d = 74.80 \pm 1.9$ nM) increased about 1.6-fold in the presence of 1 μM TEV PK1 ($K_d = 46.31 \pm 1.6$ nM) at 25 °C (Fig. 4). However, the addition of 1 μM S1-3 of PK1 in eIFiso4F-VPg increased binding affinity ($K_d = 58.2 \pm 2.1$ nM) about 1.3-fold.

The presence of TEV PK1 positively affects the binding of VPg with eIFiso4F. The experiments with TEV PK1 suggest that cooperativity between VPg and TEV PK1 for eIFiso4F binding sites is possible. This is the first demonstration that IRES can affect eIFiso4E-VPg interactions. These data suggest both ligands are bound at the same time and support the idea that VPg is involved in at least the initial stages of translation.

Stopped-flow Fluorescence Kinetics—The stopped-flow data for the binding of VPg to eIFiso4E and eIFiso4F were plotted as the fluorescence intensity versus time as shown in Fig. 6. Time course data were fit by nonlinear regression analysis (39) assuming a single-exponential change. The addition of eIFiso4G to the eIFiso4E increased the rate constant ($k_2$) for VPg binding about 1.6-fold (eIFiso4E, $k_2 = 43.88 \pm 0.81$ s$^{-1}$; eIFiso4F, $k_2 = 69.94 \pm 1.1$ s$^{-1}$).

Under the pseudo-first order conditions, where VPg was in excess, the observed rate constant is predicted to be a linear function of the concentration of VPg. The results show that the observed binding rates had little dependence on VPg concentration over a concentration range of 2.5–10 μM, a 4–19-fold excess. The observed rate varied from 43.88 to 45.26 s$^{-1}$ for eIFiso4E and from 69.94 to 71.63 s$^{-1}$ for eIFiso4F (eIFiso4E-eIFiso4G complex) with VPg. Stopped-flow experiments were conducted using high concentrations of VPg and limiting concentrations of eIFiso4E and eIFiso4F to ensure that binding is limited by VPg.
the bimolecular combination of VPg with eIFiso4E was pseudo-first order. The mechanisms considered involved a one- and two-step binding process (40, 41). The one-step reaction is

\[
\text{eIFiso4E} + \text{VPg} \rightleftharpoons \text{eIFiso4E-VPg}
\]

where \( k_1 \) and \( k_{-1} \) are forward and reverse rate constants, respectively. Under the pseudo-first order condition, the observed rate constant is predicted to be a linear function of substrate concentration, i.e. \( k_{\text{obs}} \left( k_1 + k_{-1} \right) \). The two-step reaction is,

\[
\text{eIFiso4E} + \text{VPg} \rightleftharpoons (\text{eIFiso4E-VPg})^* \rightleftharpoons \text{eIFiso4E-VPG}
\]

which involves a fast association of eIFiso4E and VPg followed by a slow change of conformation of the first association complex (eIFiso4E-VPg)* to the stable complex, eIFiso4E-VPG, giving rise to the fluorescence change.

The binding rates have the following relationship with the concentration of substrate: \( \frac{1}{k_{\text{obs}}} = \frac{1}{k_2} + \frac{1}{K_1} [C] \) as described previously (39), where \( k_{\text{obs}} \) is the observed first order rate constant, \( k_2 \) is the forward rate constant for the second step, \( K_1 \) is the equilibrium constant for the first step, and \([C]\) is the concentration of VPg. Fig. 7 shows \( 1/k_{\text{obs}} \) versus \( \frac{1}{[C]} \) of eIFiso4E and eIFiso4F, which shows a linear relationship. From the intercept of \( 1/k_2 \), \( k_2 \) was found to be \( 46.45 \pm 0.94 \text{ s}^{-1} \) for eIFiso4E and \( 71.70 \pm 1.5 \text{ s}^{-1} \) for eIFiso4F.

Rate constants for eIFiso4E and eIFiso4F binding to VPg at different temperatures are shown in Table 3. The values of \( k_2 \) for VPg binding to eIFiso4E and eIFiso4F at different temperatures were calculated from the data sets collected at each temperature as in the example shown in Fig. 6. The addition of eIFiso4G to eIFiso4E increased \( k_2 \) values. Similarly, the \( k_2 \) values for protein complexes increased with an increase in temperature (Table 3). The stopped-flow kinetic data were fit with a single-exponential function showed that at \( 22^\circ \text{C} \) the binding of eIFiso4F to the VPg changed the conformation \( \sim 1.6 \) times faster than the binding of eIFiso4E (\( k_2 = 69.94 \pm 1.1 \text{ s}^{-1} \) for eIFiso4F; \( k_2 = 43.88 \pm 0.81 \text{ s}^{-1} \) for eIFiso4E) (Fig. 6A). Treatment of the data using a double-exponential function did not
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**TABLE 3**

| Complex                  | $k_2$ at 5°C | $k_2$ at 10°C | $k_2$ at 15°C | $k_2$ at 22°C | $k_2$ at 32°C | $k_2$ $^a$ |
|--------------------------|-------------|--------------|--------------|--------------|--------------|------------|
| eIFiso4E-VPg             | 8.95 ± 0.72 | 13.83 ± 0.56 | 21.59 ± 0.49 | 43.88 ± 0.81 | 62.52 ± 0.79 | 34.19 ± 1.24 |
| eIFiso4F-VPg             | 7.03 ± 0.37 | 12.97 ± 0.48 | 24.96 ± 0.68 | 69.94 ± 2.51 | 125.21 ± 2.74 | 18.24 ± 0.87 |

$^a$ The dissociation rate ($k_2$) constants were measured at 22°C.

![FIGURE 8. Arrhenius plots for the interaction among eIFiso4E ( ), eIFiso4F ( ), and VPg.](Image)

![FIGURE 9. Kinetics of VPg dissociation from eIFiso4E-VPg ( ) and eIFiso4F-VPg ( ).](Image)

The rate constant values (Table 3) as a function of temperature were used to construct an Arrhenius plot according to equation $\ln k = -E_a / RT + \ln A$. Where $k$ is the rate constant, $E_a$ is the activation energy, and $A$ is the Arrhenius pre-exponential term. The activation energy was calculated from the slope of the fitted linear plot of $\ln k$ versus $1/T$ (Kelvin).

The rate constant values (Table 3) as a function of temperature were used to construct an Arrhenius plot (Fig. 8) according to Equation 4. The activation energies were calculated from the slope of the fitted linear plot of $\ln k$ versus $1/T$ (Kelvin). The activation energy for binding of VPg to eIFiso4E and eIFiso4F, determined from the temperature dependence of the rate constants, is shown in Table 2. The activation energy for binding of VPg to eIFiso4F (75.37 ± 2.95 kJ mol$^{-1}$) is 1.5-fold higher than that of eIFiso4E (50.70 ± 1.27 kJ mol$^{-1}$). The dissociation reaction was monitored as described under “Experimental Procedures.” Fig. 9 shows the results of the dilution experiment. The dissociation rate constants were obtained from the fitted curves as $(34.19 ± 1.24) \times 10^{-3}$ s$^{-1}$ and $(18.24 ± 0.87) \times 10^{-3}$ s$^{-1}$ for eIFiso4E and eIFiso4F with VPg (Table 3). The addition of eIFiso4G to eIFiso4E decreased the dissociation rate ∼2-fold for VPg.

**DISCUSSION**

In this study, experiments were undertaken to investigate the importance of the VPg of turnip mosaic virus (TuMV) in protein synthesis. Although it had been previously shown that VPg interacted with both eIFiso4E and eIFiso4F (2, 30), the quantitative effects of eIFiso4F on these interactions had not been determined. We have found that eIFiso4G increases the binding of eIFiso4E by 1.5-fold for VPg as compared with eIFiso4E alone. When compared with eIFiso4F binding to cap analogs, however, this is not sufficient to out-compete the cap for binding. Earlier studies using surface plasmon resonance (30) had shown that the A. thaliana eIFiso4E bound VPg more tightly than the cap analog, suggesting that it would preferentially bind to eIFiso4E. Our fluorescence data for T. aestivum (wheat germ) eIFiso4E show a weaker binding affinity for VPg than found for A. thaliana but also showed a single binding site for VPg and cap analog and predominantly competitive binding for VPg and m$^7$GTP. This is in contrast to the data of Michon et al. (29) for lettuce eIF4E and lettuce mosaic virus VPg. They found the cap analog m$^7$GDP and VPg bound eIF4E at two distinct sites with similar affinity.

The sequence homology of eIFiso4E in A. thaliana and T. aestivum eIFiso4E share 70% identity (42). The two initiation factors are mechanistically equivalent for the translation process but exhibit differences in their ability to bind m$^7$G cap (22). VPg exhibits different binding affinity for T. aestivum (97.47 ± 1.6 nM) and A. thaliana eIFiso4E (55 nM) (30). However, binding was only different by less than a factor of two. Leonard et al. (2) demonstrated the interaction of VPg with various initiation factor isomers and, moreover, identified the binding domain in a highly conserved region of the VPg indicative of interactions that play an important role in the viral life cycle. This role is...
supported by the fact that a mutation in Vpg abolishes its interaction with eIFiso4E in vitro and abolished viral infection in whole plants (2). In the presence of eIFiso4G, the binding of Vpg for eIFiso4E increases sufficiently ($K_d = 62.43 \pm 0.61$ nM) to be competitive with the cap ($K_d = 57$ nM) (35), although not to the exclusion of cap binding.

The addition of an IRES element to eIFiso4F increased Vpg binding by 1.6-fold. This interaction is perhaps the most direct evidence for the involvement of Vpg in translation. Not only does binding of IRES element increase Vpg binding, but it also indicates that both Vpg and IRES elements can be bound at the same time. This is reminiscent of poly(A)-cap binding in host mRNA where synergistic effects lead to a stable complex (24, 25). It is possible that a similar complex is formed with Vpg and viral IRES.

This binding cooperativity seems to be a feature of eIF4E to regulate its activity. For instance, binding of mammalian eIF4G to eIF4E increased the affinity of the latter for the cap analogue (43); moreover, the wheat germ poly(A)-binding protein enhanced the binding affinity of eIF4E isomers for the cap analogue (25). The increase in binding affinity of eIFiso4F-Vpg (~1.6-fold) in the presence of TEV PK1 suggested that TEV shows the positive cooperative binding with eIFiso4F-Vpg. The kinetic results lend further insight into a proposed mechanism. Cap binding to eIF4F is believed to be the rate-limiting step for protein synthesis. We have previously determined the rates of this reaction for eIFiso4F and the effects of other initiation factors. Here, we find that the binding of eIFiso4F to Vpg follows a similar mechanism. The rate of binding, however, is ~5-fold faster for Vpg binding. Therefore, the binding of Vpg to eIFiso4F is highly kinetically favored, and the additional stabilization from the IRES element to the resulting complex suggests a pathway for assembly of a complex for protein synthesis initiation. The proposed mechanism is shown in Fig. 10. This model for Vpg involvement in translation shows a fast binding step for Vpg binding to eIFiso4F. This fast reaction kinetically favors Vpg over m7GTP. The addition of an IRES element further stabilizes the eIFiso4F-Vpg interaction. It should be noted that the potyviral 5'-untranslated region without the Vpg structure is sufficient to direct cap-independent translation in vitro (9). There may be redundant features for viral translational initiation.

**Binding of eIFiso4E and eIFiso4F with Vpg**

The functional role of the Vpg-eIFiso4F-IRES interaction remains to be determined. The critical role of translation initiation factors in virus function has been demonstrated through genetic means (44), and we are now beginning to understand these mechanisms on a molecular level.

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