Interaction of Wheat Germ Protein Synthesis Initiation Factor eIF-(iso)4F and Its Subunits p28 and p86 with m7GTP and mRNA Analogues*

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The binding of p28, p86, and native wheat germ eIF-(iso)4F with m7GTP and oligonucleotides was measured and compared. The purified subunits (p28, 28 kDa and p86, 86 kDa) of wheat germ protein synthesis initiation factor eIF-(iso)4F have been obtained from Escherichia coli expression of the cloned DNA (van Heerden, A., and Browning, K. S. (1994) J. Biol. Chem. 269, 17454–17457). The binding of the 5′-terminal cap analogue m7GTP to the small subunit (p28) of eIF-(iso)4F as a function of pH, temperature, and ionic strength is described. The mode of binding of p28 to cap analogues is very similar to the intact protein. Assuming that all tryptophan residues contribute to p28 and eIF-(iso)4F fluorescence, iodide quenching shows that all 9 tryptophan residues in p28 are solvent-accessible, while only 6 out of 16 tryptophan residues are solvent-accessible on the intact eIF-(iso)4F. The fluorescence stopped-flow studies of eIF-(iso)4F and p28 with cap show a concentration-independent conformational change. The rate of this conformational change was approximately 10-fold faster for the isolated p28 compared with the native eIF-(iso)4F. From these studies it appears that cap recognition resides in the p28 subunit. However, p86 enhances the interaction with capped oligonucleotides and probably is involved in protein-protein interactions as well. Both subunits are required for helicase activity.

Recognition of the m7-cap structure of mRNA by eucaryotic initiation factors and formation of 48 S initiation complex are important steps in initiation of protein synthesis in eucaryotic cells. In mammalian cells, formation of the 48 S initiation complex is catalyzed by initiation factors eIF-1, eIF-4A, eIF-4B, eIF-4E, and eIF-4G. The complex termed eIF-4F contains polypeptides eIF-4A, eIF-4B, and eIF-4G (Etchison and Milburn, 1987; Buckley and Ehrenfeld, 1987). In addition, eIF-4E can be isolated as a separate polypeptide (Rinker et al., 1992). Recent evidence suggests that eIF-4E and eIF-4G form a complex concurrent with the binding of mRNA to the 40 S ribosomal subunit (Ioshi et al., 1994).

Such a stepwise addition suggests specific functional roles for the separated polypeptides. In contrast to the mammalian eIF-4F complex, the individual polypeptides of the eIF-4F complex from wheat germ cannot be separated except under denaturing conditions. Wheat germ eIF-(iso)4F is one of the two wheat germ initiation factors that has cap binding ability, the other is wheat germ eIF-4F. Wheat germ eIF-(iso)4F consists of two subunits (p28 and p86) in a 1:1 molar ratio (Lax et al., 1985), while wheat germ eIF-4F consists of a 26-kDa and a 220-kDa subunit in a 1:1 molar ratio. Some structural and functional similarity exists between these two factors. Both wheat germ eIF-4F and eIF-(iso)4F have RNA-dependent ATPase activity. Only one wheat germ factor is required for ATP hydrolysis and stimulation of protein synthesis in an eIF-4F or eIF-(iso)4F deficient translation system (Lax et al., 1985, 1986b). Wheat germ eIF-(iso)4F can substitute for mammalian eIF-4F in an RNA-dependent ATPase activity and in cross-linking of mammalian eIF-4A to the cap of oxidized mRNA (Abramson et al., 1988).

We have obtained purified subunits of wheat germ eIF-(iso)4F from expression in E. coli and examined the binding and functional properties of the separated subunits. Separate subunits of eIF-(iso)4F were not functionally active as measured by the ability to stimulate polypeptide synthesis. However, when both subunits were added, activity was equal to native eIF-(iso)4F (van Heerden et al., 1994). These results indicate that the two subunits are able to associate to form an active complex. In addition, both subunits are required for activity. In order to understand the RNA binding ability of the intact protein and the function of the individual subunits, we have studied the binding and physical properties of the separated subunits, reconstituted protein, and native eIF-(iso)4F.

MATERIALS AND METHODS

Buffer A, used for fluorescence measurements, consisted of 20 mM HEPES-KOH, 100 mM KCl, 1 mM dithiothreitol, and 1 mM MgCl2 and was adjusted to the appropriate pH. Buffer B, used in isolation of the wheat germ factors, consisted of 20 mM HEPES-KOH, pH 7.6, 0.1 mM EDTA, 1 mM diithiothreitol, 10% glycerol, and KCl as indicated. All chemicals were reagent grade or better. M7GTP and M7GpppG were purchased from Sigma (St. Louis, MO). M7GTP-Sepharose was obtained from Pharmacia Biotech Inc. mRNA analogues (capped and uncapped oligonucleotides) were transcribed from partially double-stranded deoxyoligonucleotide templates containing a T7 RNA polymerase primer according to the procedures of Milligan et al. (1987) and were purified according to the method of Draper et al. (1988); the oligonucleotides used for the binding study, I and II, are shown in Fig. 1. The oligonucleotides used for the helicase assay, III and IV, are shown below.

Wheat germ initiation factor eIF-(iso)4F was purified according to the protocol of Lax et al. (1986a, 1986b) with the following modifications. The 40% ammonium sulfate fraction was loaded onto a DE52 column, equilibrated with buffer B containing 40 mM KCl (B-40). The column was washed until the OD was less than 0.2. The buffer was then

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‡The abbreviations used are eIF, eucaryotic initiation factor; m7G, 7-methylguanosine.
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Fig. 1. Structures of oligonucleotides I and II.

Oligonucleotide I: 5'-GGCGCUCGAUACCACCACUAAGGUAUA-

Oligonucleotide II: 3'-ACCACUAAGGUAUACCACUCGCAGCCGAGCCGAGCCGAGG

The unwinding reaction for the helicase assay of m7GTP was performed by incubating partially double-stranded RNA with eucaryotic initiation factors (5 μM eIF-4A, 2 μM p28, p86, and eIF-(iso)4F were used) in 20 mM Tris-HCl buffer (pH 7.5) containing 2 mM ATP, 1 mM magnesium acetate. The reaction mixture was incubated at 37°C for 20 min and then transferred to an ice bath and 2 ml of 5 × stopping solution containing 50% glycerol and 10% SDS was added. SDS was used to separate the protein from the RNA and to avoid the formation of a large molecular weight complex. The sample was loaded onto a 15% nondeaturing polyacrylamide gel. The double- and single-stranded RNA on the gel was silver-stained (Bassam et al., 1991), and the bands were quantitatively determined by a laser scanning densitometer.

Stopped-flow fluorescence experiments were performed on a Hi-Tech SF-51 stopped-flow spectrophotometer equipped with a Berger-type mixing chamber and a 2 × 2 × 10-mm flow cell with a dead time of 1.5 ms. The excitation wavelength was 280 nm, and the slit width was 2 mm. Light emitted from the reaction mixture was monitored after passage through a cut-off emission filter (WG-320 provided by Hi-Tech). A series of stopped-flow experiments were carried out at 23°C in buffer A, pH 7.05, under different concentrations of cap analogue m7GpppG.

Fluorescence measurements were carried out at 23°C, unless otherwise noted, and data were collected and analyzed as described previously in detail (Carberry et al., 1989, 1990). Background fluorescence emission was subtracted.

The double-stranded RNA (Tm = 65°C) for the helicase assay of eIF-(iso)4F was prepared by annealing the partially complementary III and IV single-stranded RNA. Equal molar amounts of RNA III and IV were mixed and heated to 80°C for 10 min, slowly cooled to 50°C for 30 min, and then cooled to 40°C and kept overnight. The double-stranded RNA is shown as follows.

Oligonucleotide III: 5'-m7GpppGGCCGCUCGAUACCACCACUAAGGUAUA-

Oligonucleotide IV: 3'-ACCACUAAGGUAUACCACUCGCAGCCGAGCCGAGG

The pH dependence of m7GTP binding to the p28 subunit of eIF-(iso)4F is shown in Fig. 3. The pH optimum for m7GTP binding was 6.67 for p28, compared with the pH optimum of 7.6 for eIF-(iso)4F-m7GpppG complex (Carberry et al., 1991).

Temperature Effects—A Van't Hoff plot of −ln(Km) versus the reciprocal of temperature (T−1) can be used to calculate the thermodynamic parameters of entropy (ΔS) and enthalpy (ΔH). Fig. 4 shows the Van't Hoff plot based on m7GTP binding to p28; the values of ΔH and ΔS were obtained from the intercept and slope, respectively. The values of 5.8 ± 0.4 kcal/mol and 44.7 ± 1.9 cal/(mol °C) were obtained for ΔH and ΔS, respectively. These results are similar to the eIF-4F-m7GTP interaction, where ΔH was 6.25 ± 0.25 kcal/mol, and ΔS was 46.1 ± 1.8 cal/(mol °C) (Carberry et al., 1989). Similar values were also obtained for the eIF-4F-m7GTP interaction, where ΔH was 6.84 ± 0.7 kcal/mol, and ΔS was 47.4 ± 5.0 cal/(mol °C) (Carberry et al., 1991). An interpretation has been given by Ross and Sumanian, 1981) that positive ΔH and ΔS values suggest either hydrophobic (Gill et al., 1967, 1976) or ionic (Pimentel and McClellan, 1971) interactions. The ionic strength dependence of m7GTP binding with p28 was investigated in order to determine if the interaction was hydrophobic or ionic.
Ionic Strength Dependence—Debye-Huckel theory predicts that for charge-charge interactions, a plot of log $K_{eq}$ versus the square root of the ionic strength will give a linear plot. The slope equals 1.02$Z_A Z_B$ for ionic interactions where $Z_A$ and $Z_B$ are the charges of the reactants. The binding of m$^7$GTP to p28 as a function of the concentration of KCl and KC$_2$H$_3$O$_2$ was measured. The treatment of these data according to Debye-Huckel theory is shown in Fig. 5. For the interaction of a single positive and negative charge, $Z_A Z_B = -1$. For m$^7$GTP binding with p28 in KC$_2$H$_3$O$_2$, $Z_A Z_B = 0.008$, demonstrating that the binding of m$^7$GTP to p28 has little dependence on ionic strength. There are no significant ionic interactions involved in the binding of m$^7$GTP to p28. Using KCl to vary ionic strength, the value of $Z_A Z_B$ was $-0.47$. This difference between KCl and KC$_2$H$_3$O$_2$ suggests a possible uptake of Cl$^-$ by the p28. The special effect of anions on protein-RNA binding was reported earlier with the wheat germ eIF-4B protein (Sha et al., 1994).

Iodide Quenching Effect—Iodide quenching to determine the solvent accessible tryptophan residues was described previously (Sha et al., 1994). The modified Stern-Volmer plot (Fig. 6) shows quantitative results for quenching of p28 and eIF-(iso)4F. The reciprocal of the y intercept represents the fraction of accessible tryptophan residues among all tryptophan in the protein which contribute to the fluorescence (Lakowicz, 1983). A Y intercept of $1.02 \pm 0.05$ and $2.65 \pm 0.50$ was obtained for p28 and eIF-(iso)4F, respectively. Reciprocal values gave the fraction of accessible tryptophan residues as 98.0% ± 4.5% and 37.7% ± 6.0% for p28 and eIF-(iso)4F. Thus, if all tryptophan residues contribute to the protein fluorescence, all 9 tryptophan residues in p28 are solvent accessible and only 6 tryptophan residues from a total of 16 in eIF-(iso)4F are solvent-accessible, indicating some tryptophan residues on p28 are buried during binding with the large subunit, p86, to form eIF-(iso)4F.

Comparison of Oligonucleotide Binding to Wheat Germ eIF-(iso)4F and Its Subunits—The binding of p28, p86, and wheat germ eIF-(iso)4F with m$^7$GTP and mRNA analogues was measured and compared in Table I. The association of p28 with p86 to form eIF-(iso)4F did not significantly increase the $K_{eq}$ for m$^7$GTP and capped RNA binding, yet both subunits were required for activity.

Helicase Activity—the eIF-4A-dependent helicase activity of eIF-(iso)4F and its subunits was measured by unwinding the partially double-stranded RNA annealed from oligonucleotide III and IV. It was found that neither the p28 nor the p86 subunit alone had helicase activity, while the combination of p28 and p86 gave full helicase activity as compared with eIF-(iso)4F (Table II). These data demonstrated that p86, the large subunit of eIF-(iso)4F, although it did not significantly enhance the RNA binding, was necessary for mRNA secondary structure unwinding during the translation process.
Stopped-flow Fluorescence Kinetics—The stopped-flow data for the binding of m7GpppG1 to eIF-(iso)4F and p28 were plotted as ΔF versus time as shown in Fig. 7. Fitted curves correspond to the following single exponential equation (Olsen et al., 1993),

\[ \Delta F = \Delta F_0 (1 - \exp(-k_{obs}t)) \]  

(Eq. 1)

where k_{obs} is the observed first-order rate constant, and ΔF_{\text{eq}} is the maximum fluorescence change.

Mechanisms for eIF-(iso)4F-cap Interaction—Stopped-flow experiments were conducted using high concentrations of m7GpppG1 and limiting concentrations of eIF-(iso)4F to ensure that the bimolecular combination of mRNA cap analogue with protein was pseudo first-order. The mechanisms considered involved a one- and a two-step binding process (Garland, 1978).

The one-step reaction is as follows,

\[ k \]

\[ P + C \rightarrow P \cdot C \]

\[ k_1 \]

**MECHANISM i**

where k_1 and k_1 are forward and reverse rate constants, respectively; P and C refer to eIF-(iso)4F and m7GpppG1, respectively. Under the pseudo first-order condition, the observed rate constant is predicted to be a linear function of substrate concentration, i.e. k_{obs} = k_1[C] + k_1. The two-step reaction is as follows,

\[ \frac{k_1}{k_2} \]

\[ P + C \rightarrow (P + C)^* \rightarrow P \cdot C \]

\[ k_1 \]

\[ k_2 \]

**MECHANISM ii**

which involves a fast association of protein, eIF-(iso)4F, and m7GpppG1.

**TABLE I**

Equilibrium binding constants for the interaction of p28, p86, and wheat germ eIF-(iso)4F with m7GTP and oligonucleotides

| Initiation factors | Oligonucleotides | K_{eq} |
|--------------------|------------------|--------|
| p28                | m7GTP (5.06 ± 0.08) \times 10^6 |        |
|                    | GTP (1.92 ± 0.23) \times 10^6 |        |
|                    | Capped RNA (II) (1.24 ± 0.04) \times 10^6 |        |
|                    | Uncapped RNA (II) (0.17 ± 0.02) \times 10^6 |        |
| p86                | m7GTP (3.60 ± 0.11) \times 10^6 |        |
|                    | GTP (3.30 ± 0.17) \times 10^6 |        |
|                    | Capped RNA (II) (1.22 ± 0.05) \times 10^6 |        |
|                    | Uncapped RNA (II) (1.11 ± 0.06) \times 10^6 |        |
| p28 + p86          | m7GTP (5.59 ± 0.21) \times 10^6 |        |
|                    | Capped RNA (II) (1.44 ± 0.07) \times 10^6 |        |
| eIF-(iso)4F        | m7GTP (5.40 ± 0.08) \times 10^6 |        |
|                    | Capped RNA (II) (1.55 ± 0.05) \times 10^6 |        |

A plot of 1/k_{obs} versus 1/[C] will give an intercept of 1/k_2 (Fig. 8).

This model gives a k_2 value of 12.2 ± 0.5 s⁻¹. Mechanisms for p28-cap Interaction—Stopped-flow experiments of p28 binding with cap similar to those described above were also conducted. Earlier CD experiments have shown that p28 undergoes conformational changes upon binding to m7GpppG2 in agreement with Mechanism ii. The same Equation 4 was used here for the plot of Mechanism ii, the plot of 1/k_{obs} versus 1/[C], which gave a k_2 value of 123.5 ± 8.6 s⁻¹ for p28 binding with the m7GpppG cap analogue. While more complex models with additional conformational changes will also fit the data, at present there is no experimental data to suggest such a mechanism is required.

**DISCUSSION**

The general mechanism of interaction of the cap with the p28 subunit of eIF-(iso)4F is very similar to the wild type protein. The pH optimum for cap binding to p28 was found to be 7.05;

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**TABLE II**

eIF-4A-dependent helicase activity of eIF-(iso)4F and its subunits measured by densitometry scan of RNA bands on silver-stained acrylamide gel

| eIF factors | Double-stranded RNA | Single-stranded RNA IV | Single-stranded RNA III |
|-------------|---------------------|-------------------------|-------------------------|
| eIF-4A alone | Band volume 242.50 | 2.41 | 13.63 |
|             | Ratio % 93.8% | 1.0% | 5.3% |
|             | Unwinding % 6.2% |        |        |
| eIF-4A and p28 | Band volume 641.89 | 11.24 | 0 |
|             | Ratio % 98.5% | 1.7% | 0.0% |
|             | Unwinding % 6.2% |        |        |
| eIF-4A and p86 | Band volume 606.28 | -1.24 | 0.05 |
|             | Ratio % 100.2% | -0.2% | 0.0% |
|             | Unwinding % -0.2% |        |        |
| eIF-4A and p28 + p86 | Band volume 516.93 | 472.60 | 147.70 |
|             | Ratio % 45.5% | 41.6% | 13.0% |
|             | Unwinding % 54.5% |        |        |
| eIF-4A and eIF-(iso)4F | Band volume 707.41 | 566.34 | 227.72 |
|             | Ratio % 47.1% | 37.7% | 15.1% |
|             | Unwinding % 52.9% |        |        |
binding of \( m^7 \)GTP to p28, p86, and eIF-(iso)4F

compared with the pH optimum of 7.6 for eIF-(iso)4F-m\( m^7 \)GpppG complex (Carberry et al., 1991). The pK\( _a \) values of amino acids are known to be environmentally sensitive and this could account for the shift in binding profile between p28 and eIF-(iso)4F. The shift in pK\( _a \) implies the binding site has become more positively charged in the separated subunit. The previous sequence comparison (Allen et al., 1992) of wheat p28 with the cap-binding protein eIF-4E from mammals and yeast had shown about 38% sequence homology. There are three highly conserved histidine residues (position 33, 91, 193 from NH\(_2\)-terminal) and 9 tryptophan residues in p28 (positions from NH\(_2\)-terminal: 39, 42, 55, 72, 101, 112, 126, 161, 178), one more tryptophan residue than in mammalian and yeast cap-binding proteins (Trp-178). Site-directed mutagenesis of yeast eIF-4E showed that tryptophan 1, 2, and 8 were required for cap binding activity (Altmann et al., 1988). Site-directed mutagenesis of human eIF-4E showed that tryptophan 5 and the glutamic acid residue three amino acids to the carboxyl-terminal side of tryptophan 5 were involved in cap recognition. None of these studies examined the tertiary structure of the mutant protein; however, Ueda et al. (1991) proposed that the base stacking of the tryptophan and hydrogen-bond pairing by the glutamic acid are responsible for binding the \( m^7 \)G cap of mRNA. The p28 of eIF-(iso)4F has a glutamic acid residue four amino acids to the carboxyl-terminal side of tryptophan 5 (Glu-105) which could participate in the binding proposed by Ueda et al. (1991). Their data, however, fail to account for the pH dependence of binding.

The small subunit, p28, has cap binding properties very similar to native eIF-(iso)4F. This observation is similar to the relative affinities of mammalian eIF-4E and eIF-4F binding to globin mRNA (eIF-4E: \( K_{eq} = (20.9 \pm 1.0) \times 10^6 \text{ M}^{-1}; \) eIF-4F: \( K_{eq} = (18.6 \pm 1.1) \times 10^6 \text{ M}^{-1}; \) Goss et al., 1990). In the mammalian system eIF-4E has about the same affinity for mRNA as the larger eIF-4F complex. These data suggest that the essential role of the large subunit is involvement in other interactions such as protein-protein interactions and helicase activity.

The p86 subunit, which also binds to m\( ^7 \)GTP, although less tightly, is unlikely to be the specific cap binding subunit in eIF-(iso)4F for the following two reasons: p86 shows no significant difference between capped RNA, and uncapped RNA binding and p86 does not distinguish \( m^7 \)GTP from GTP. The fact that p86 has a relatively high binding affinity for RNA suggests the native protein may have an RNA site involving both subunits and that p86 stabilizes the contact with RNA during the course of protein synthesis. In addition, photoaffinity labeling with m\( ^7 \)GTP analogues label only the small subunit.\(^3\)

The stopped-flow kinetic data showed that the small subunit, p28, changed conformation approximately 10 times faster than eIF-(iso)4F (p28: \( k_2 = 123.5 \pm 8.6 \text{ s}^{-1}; \) eIF-(iso)4F: \( k_2 = 12.2 \pm 0.5 \text{ s}^{-1}. \) The faster conformational change rate of the p28-cap may be partially caused by an agile movement of its small mass (p28, 28 kDa compared with eIF-(iso)4F, 110 kDa). These results are surprising, since one would expect p86 to increase the rate of complex formation and hence increase the rate of interaction. However, if the initial equilibrium between the cap and protein is approximately the same for p28 and eIF-(iso)4F, then the off rate for eIF-(iso)4F would also be much slower than the off rate for p28. This would allow for interaction of other initiation factors or ribosomes and favor formation of an active initiation complex.

A working model is that p28 contains the binding site for the cap and locates the complex at or near the 5’ end of the mRNA. While p86 does not affect the equilibrium affinity for cap, it does affect the rate of complex formation. In order for the eIF-(iso)4F complex to perform helicase activity (which requires both p28 and p86), the complex must process down the RNA. This would in all probability require release of the cap during the course of protein synthesis. In addition, photoaffinity labeling with m\( ^7 \)GTP analogues label only the small subunit.\(^3\)

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\(^3\) D. E. Friedland, M. Shoemaker, C. Hagedorn, and D. J. Goss, unpublished results.
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