Interaction of Wheat Germ Protein Synthesis Initiation Factors eIF-3, eIF-(iso)4F, and eIF-4F with mRNA Analogues†

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Received February 12, 1991; Revised Manuscript Received May 1, 1991

ABSTRACT: The interaction of wheat germ eIF-3 with the wheat germ cap-binding proteins eIF-(iso)4F and eIF-4F as a function of pH and ionic strength is described. Direct fluorescence titration experiments are used to measure the equilibrium association constants (K_{eq}) for the binary protein/protein complexes as well as for the interaction of eIF-3 with methylated cap analogues and rabbit α-globin mRNA oligonucleotide analogues. The K_{eq} values for ternary eIF-3/eIF-(iso)4F/analogue and eIF-3/eIF-4F/analogue interactions were also measured. The equilibrium binding constants were used to calculate coupling free energies, which provide an estimate of the cooperativity for the interaction of the mRNA analogues, eIF-3, and either eIF-4F or eIF-(iso)4F. These data suggest a mechanism in which the binding of eIF-(iso)4F or eIF-4F to mRNA enhances the subsequent binding of eIF-3 to the message. This may lead to favorable positioning of the complex on the ribosome and thereby enhance translation.

Wheat germ eIF-3[1] is a large protein complex (660–770 kDa) consisting of approximately 11 polypeptides ranging in molecular mass from about 25 to 135 kDa (Checkley et al., 1981; Lax et al., 1986); eIF-3 isolated from mammalian cells has been found to contain 7–11 polypeptides (Safer et al., 1976; Benne & Hershey, 1976; Schreier et al., 1977; Thompson et al., 1977) ranging from 28 to 140 kDa in eIF-3 isolated from rabbit reticulocytes (Benne & Hershey, 1976). Various functions of eIF-3 have been described: mammalian eIF-3 has been shown to bind the 40S ribosomal subunit (Benne & Hershey, 1976; Trachsel & Staehelin, 1979; Behlke et al., 1986) and prevent association of the 40S and 60S ribosomal subunits (Trachsel & Staehelin, 1979; Goss et al., 1988) and to enhance the binding of Met-tRNA to the 40S subunit (Schreier & Staehelin, 1973; Trachsel et al., 1977; Benne & Hershey, 1978). eIF-3 is also essential for the binding of mRNA to the 40S subunit (Trachsel et al., 1977; Benne & Hershey, 1978; Peterson et al., 1979). Wheat germ eIF-3 however, did not prevent the association of wheat germ 40S and 60S ribosomal subunits or significantly enhance the dissociation of 80S subunits (Checkley et al., 1981).

eIF-3 has also been implicated in the interaction with the 5′-terminal cap of mRNA. Reticulocyte eIF-3 was found to associate with the 24-kDa polypeptide cap-binding protein (Sonenberg et al., 1978; Westerman & Nygard, 1984). Sonenberg et al. (1979) and Sonenberg and Shatkin (1977) have shown that the 28-, 93-, and 135-kDa subunits of wheat germ eIF-3 (and to a lesser extent the 16- and 50-kDa subunits) cross-link to the 5′-termini of oxidized viral mRNA, while Setyono et al. (1984) have shown that the 64-, 66-, 95-, and 110-kDa subunits of reticulocyte eIF-3 can be cross-linked to Semliki Forest virus mRNA in a complex consisting of eIF-3 and the mRNA alone.

In order to characterize the interaction of eIF-3 with mRNA and the cap-binding proteins, we have used direct fluoresence titration experiments to measure the equilibrium association constants (K_{eq}) for the binding of wheat germ eIF-3 to the wheat germ cap-binding proteins eIF-4F and eIF-(iso)4F as a function of pH and ionic strength. We have previously described the interaction of eIF-(iso)4F and eIF-4F with methylated cap analogues (Carberry et al., 1991a) and rabbit α-globin oligoribonucleotide analogues (Carberry & Goss, 1991); in this report, we describe the binary eIF-3/mRNA analogue interactions and the ternary eIF-3/eIF-(iso)4F/analogue and eIF-3/eIF-4F/analogue interactions as well. From these data, information on the stability and cooperativity of the formation of ternary complexes was obtained. These results suggest that eIF-3 binds mRNA in an eIF-4F/mRNA or eIF-(iso)4F/mRNA complex; this interaction may then assist in positioning the mRNA on the ribsome.

Materials and Methods

Wheat germ eIF-3 was prepared as described by Lax et al. (1986a); wheat germ eIF-4F and eIF-(iso)4F were isolated as described previously (Lax et al., 1986a,b; Carberry et al., 1991a). m^7GpppG was purchased from Pharmacia Molecular Biologicals (Milwaukee, WI), and $m^{2,7}$GpppG was kindly provided by E. Darzynkiewicz and was synthesized as described previously (Darzykiewicz et al., 1988). The rabbit α-globin mRNA oligoribonucleotides were prepared by cell-free transcription in a T7 RNA polymerase system according to the method of Milligan et al. (1987) and purified according to the method of Draper et al. (1988). Details of this protocol have been described elsewhere (Carberry et al., 1991b).

The interactions between eIF-3, eIF-4F [or eIF-(iso)4F], and the cap and globin mRNA analogues were determined by direct fluorescence titration. The fluorescence of eIF-3, eIF-4F, or eIF-(iso)4F is quenched by addition of cap or RNA analogue. The change in protein fluorescence was used to determine the amount of complex formed and hence the

† This work was supported by grants from the National Science Foundation (NSF 9007807) and the American Heart Association (AHA-NYC Established Investigatorship and Grant-in-Aid) and a PSC-CUNY faculty award (D.J.G.). S.E.C. is supported by an American Heart Association—NYC postdoctoral fellowship. This investigation was supported in part by a Research Centers in Minority Institutions award, RR-0307, from the Division of Research Resources, NIH, to Hunter College.
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[1] Abbreviations: m^7G, 7-methylguanosine; $m^{2,7}$G, 2,7-dimethylguanosine; eIF, eukaryotic initiation factor; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; kDa, kilodalton.

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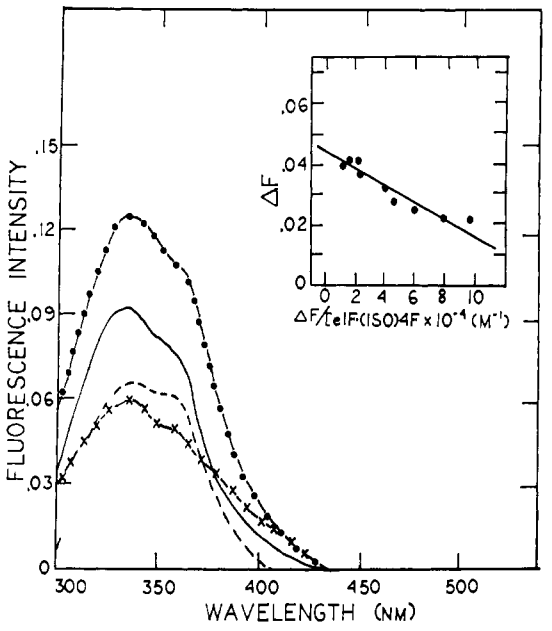


FIGURE 1: Fluorescence emission spectra of 0.4 μM wheat germ eIF-3 (—), 0.8 μM wheat germ eIF-(iso)4F (---), and a complex consisting of 0.4 μM eIF-3 and 0.8 μM eIF-(iso)4F (-●-). All measurements were made in buffer A, pH 7.6; a 258-nm excitation wavelength was used. The spectrum of the eIF-3/eIF-(iso)4F complex corrected for the free eIF-(iso)4F contribution is also shown (-×-). (Inset) Eadie–Hofstee plot of the corrected fluorescence data: a K_{eq} value of (21.6 ± 7.9) × 10^5 M^{-1} was obtained from the negative inverse of the slope.

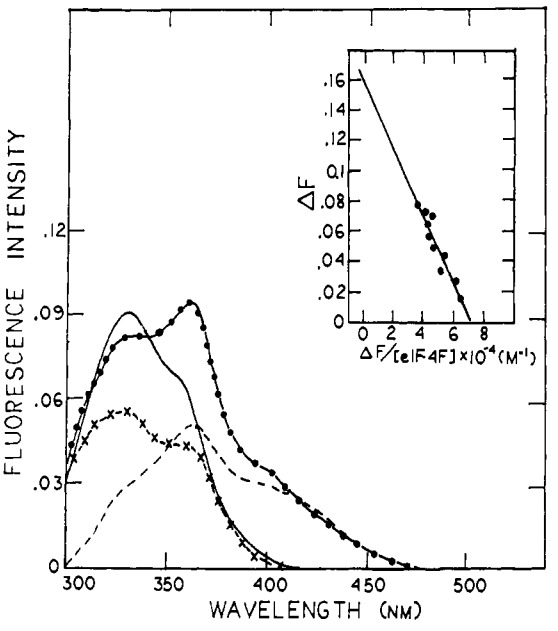


FIGURE 2: Fluorescence emission spectra of 0.4 μM wheat germ eIF-3 (—), 0.8 μM wheat germ eIF-4F (---), and a complex consisting of 0.4 μM eIF-3 and 0.8 μM eIF-4F (-●-) in buffer A, pH 8.0. Experimental conditions are the same as in Figure 1. The spectrum of the eIF-3/eIF-4F complex corrected for the free eIF-4F contribution is also shown (-×-). (Inset) Eadie–Hofstee plot of the corrected fluorescence data: a K_{eq} value of (4.54 ± 0.36) × 10^5 M^{-1} was obtained.

equilibrium constants. This protocol has been described in detail elsewhere (Carberry et al., 1989, 1990). All solutions for fluorescence measurement were prepared in buffer A, which consisted of 20 mM HEPES and 1 mM DTT, adjusted to the pH as indicated; all measurements were carried out at 23 ± 2 °C. All fluorescence spectra of the eIF-3/eIF-(iso)4F and eIF-3/eIF-4F complexes were corrected for the contribution of free eIF-(iso)4F or eIF-4F by subtracting this contribution, which was measured independently at the same time, from the fluorescence of the complex. The data were fit to account for free eIF-4F [or eIF-(iso)4F] and free eIF-3 forming complexes with the oligoribonucleotide. This fitting was accomplished with use of independently determined K_{eq} values for the binary protein/oligonucleotide interactions. The contribution of free cap or oligonucleotide analogue to the eIF-3 intensity at 330 nm was negligible. The equilibrium association constants (K_{eq}) were obtained from Eadie–Hofstee plots (Eadie, 1942).

RESULTS

Characterization of eIF-3/eIF-4F and eIF-3/eIF-(iso)4F Complexes. Fluorescence titration experiments were utilized to characterize the interaction of wheat germ eIF-3 with eIF-4F and eIF-(iso)4F. The fluorescence emission spectra of eIF-3 and eIF-(iso)4F are shown in Figure 1: eIF-3 has a maximum at 330 nm and a shoulder at 360 nm (solid line). Addition of eIF-(iso)4F to eIF-3 causes an apparent *increase* in the eIF-3 intensity at 330 nm; however, when the contribution of free eIF-(iso)4F (dashed curve) is accounted for, a quenching of the initial fluorescence results (crossed curve). When this correction is made for each eIF-(iso)4F concentration on the titration curve, an Eadie–Hofstee plot for the eIF-3/eIF-(iso)4F interaction can be constructed by utilizing the corrected fluorescence values (such a plot is shown in the inset of Figure 1) and a K_{eq} value of (21.6 ± 7.9) × 10^5 M^{-1} is obtained. In the presence of salt, this value is found to be

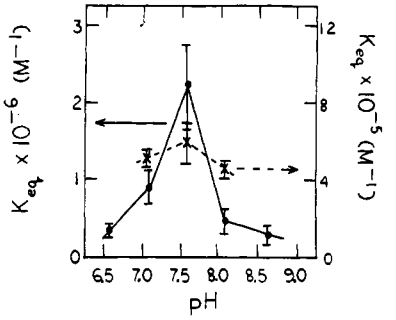


FIGURE 3: pH dependence of binding of eIF-(iso)4F (●) and eIF-4F (×) to eIF-3. All solutions were in buffer A, adjusted to the pH indicated.

(7.76 ± 2.3) × 10^5 M^{-1}. The large error in this K_{eq} value is due to the significant fluorescence correction required for the eIF-(iso)4F concentrations employed (0.2–2.0 μM).

When eIF-3 is titrated with eIF-4F, there is an apparent *decrease* in the fluorescence intensity at 330 nm (Figure 2). When the correction for free eIF-4F (dashed curve) is made, a further quenching is observed (crossed curve). The Eadie–Hofstee plot constructed from the corrected data is shown in the inset of Figure 2; a K_{eq} value of (4.54 ± 0.36) × 10^5 M^{-1} results. This value is 4.8-fold *lower* than for the eIF-3/eIF-(iso)4F interaction. In the presence of salt, pH 7.6, a K_{eq} value of (7.82 ± 0.25) × 10^5 M^{-1} is obtained for the eIF-3/eIF-4F interaction; this value is 1.7-fold greater than the value obtained in the absence of salt at pH 8.0.

The pH dependence of binding of eIF-(iso)4F and eIF-4F to eIF-3 is also different (Figure 3). At pH 8.0, the K_{eq} value for the eIF-3/eIF-4F interaction is (4.54 ± 0.36) × 10^5 M^{-1}, while at pH 7.6, the value is (6.04 ± 0.83) × 10^5; this is only a 1.3-fold increase in affinity at pH 7.6 relative to pH 8.0. However, the binding of eIF-(iso)4F to eIF-3 shows a significant pH dependence: the interaction is 5.4-fold stronger at pH 7.6 than at 8.0.

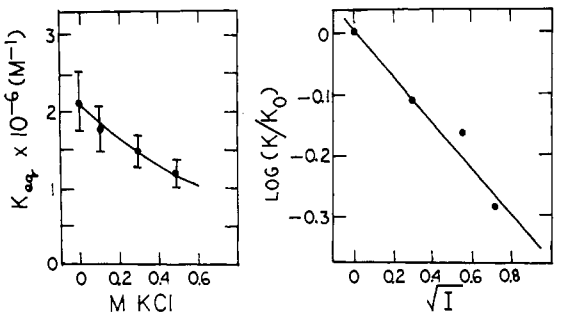
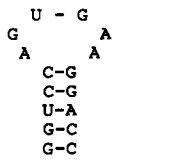


FIGURE 4: (A) KCl dependence of binding of eIF-(iso)4F to eIF-3. All solutions were in buffer A, pH 8.0. (B) Debye–Huckel plot of the date in (A).

OLIGORIBONUCLEOTIDE STRUCTURE



I m^7GpppGGCGCUCU-ACCAUGGUGC

II m^7GpppGGCGCUCUACCAUG

FIGURE 5: Rabbit α-globin analogues employed in this study.

In order to determine whether or not ionic interactions were involved in the eIF-3/eIF-(iso)4F interaction, the binding was measured as a function of KCl concentration (Figure 4). The K_{eq} value in the presence of 500 mM KCl is approximately 2-fold lower than in the absence of KCl (Figure 4A). Similar results were obtained with eIF-4F. When these data are analyzed according to Debye–Huckel theory as previously described in detail (Carberry et al., 1989), a $z_A z_B$ value of −0.38 is obtained (Figure 4B); a $z_A z_B$ value of −1.0 is predicted for the interaction of a single positive and negative charge. Therefore, electrostatic interactions are not dominant in the eIF-3/eIF-(iso)4F interaction.

Interaction of Cap and Globin mRNA Analogues with eIF-3, eIF-(iso)4F, and eIF-4F. Having demonstrated that eIF-3 forms binary complexes with eIF-4F and eIF-(iso)4F, we next wanted to investigate the role eIF-3 plays in mRNA binding and how this interaction affected the eIF-4F/mRNA and eIF-(iso)4F/mRNA interactions. We have previously shown that eIF-(iso)4F has a 2-fold higher affinity for $m^{2,7}$GpppG than m^7GpppG and preferred linear rabbit α-globin mRNA oligoribonucleotide analogues, whereas eIF-4F preferred m^7G cap analogues and hairpin-containing oligoribonucleotide analogues (Carberry et al., 1991a; Carberry & Goss, 1991). Therefore, we chose the cap analogues $m^{2,7}$GpppG and m^7GpppG and the rabbit α-globin mRNA oligoribonucleotide analogues I and II, shown in Figure 5, to investigate the eIF-3/analogue, eIF-3/eIF-4F/analogue, and eIF-3/eIF-(iso)4F/analogue interactions. For these measurements, the pH optima for the eIF-4F/analogue and eIF-(iso)4F/analogue interactions (pH 8.0 and 7.6, respectively; Carberry et al., 1991a) were used, since we wanted to compare the effect of eIF-3 binding on the message/cap-binding protein interaction. Measurements were also made under physiological conditions (100 mM KCl, 2 mM $MgCl_2$, pH 7.6) for comparison. The experimentally determined K_{eq} values for these interactions are summarized in Table I.

Table I: K_{eq} Values for the Interaction of Wheat Germ Initiation Factors eIF-3, eIF-(iso)4F, and eIF-4F with $m^{2,7}$GpppG, m^7GpppG, and Oligoribonucleotides I and II

interactions with eIF-(iso)4F	K_{eq} × 10^{-5} (M^{-1}): 0 mM KCl, 0 mM $MgCl_2$, pH 7.6	100 mM KCl, 2 mM $MgCl_2$, pH 7.6
eIF-3/eIF-(iso)4F	21.6 ± 7.9	7.76 ± 2.3
eIF-3/$m^{2,7}$GpppG	1.05 ± 0.04	
eIF-(iso)4F/$m^{2,7}$GpppG[a]	2.24 ± 0.07	
eIF-3/eIF-(iso)4F/$m^{2,7}$GpppG	0.82 ± 0.04	
eIF-3/m^7GpppG	0.88 ± 0.11	
eIF-(iso)4F/m^7GpppG[a]	1.21 ± 0.10	
eIF-3/eIF-(iso)4F/m^7GpppG	1.82 ± 0.18	
eIF-3/oligo I	3.38 ± 0.21	2.46 ± 0.25
eIF-(iso)4F/oligo I	4.19 ± 0.17[b]	2.68 ± 0.45
eIF-3/eIF-(iso)4F/oligo I	7.08 ± 0.31	3.91 ± 0.08
eIF-3/oligo II	2.25 ± 0.39	
eIF-(iso)4F/oligo II[b]	5.95 ± 0.66	
eIF-3/eIF-(iso)4F/oligo II	8.20 ± 1.30	

interactions with eIF-4F	K_{eq} × 10^{-5} (M^{-1}): 0 mM KCl, 0 mM $MgCl_2$, pH 8.0	100 mM KCl, 2 mM $MgCl_2$, pH 7.6
eIF-3/eIF-4F	4.54 ± 0.36	7.82 ± 0.25
eIF-3/m^7GpppG	1.05 ± 0.10	
eIF-4F/m^7GpppG[a]	2.32 ± 0.10	
eIF-3/eIF-4F/m^7GpppG	0.98 ± 0.24	
eIF-3/oligo I	10.2 ± 0.61	2.46 ± 0.25
eIF-4F/oligo I	8.69 ± 0.17[b]	4.44 ± 0.58
eIF-3/eIF-4F/oligo I	6.15 ± 0.14	7.16 ± 0.85
eIF-3/oligo II	4.10 ± 0.21	
eIF-4F/oligo II[b]	2.14 ± 0.66	
eIF-3/eIF-4F/oligo II	4.77 ± 0.29	

[a] Data taken from Carberry et al. (1991a). [b] Data taken from Carberry and Goss (1991).

A comparison of the K_{eq} values for the interaction of cap analogues with eIF-3 and eIF-(iso)4F in the absence of salt indicates that eIF-3 has 2.1- and 1.4-fold lower affinity for the cap analogues $m^{2,7}$GpppG and m^7GpppG, respectively, than eIF-(iso)4F. In the case of the oligoribonucleotide analogues, eIF-3 has 1.3-fold lower affinity for the hairpin-containing oligoribonucleotide I than eIF-4F and 2.6-fold lower affinity for the linear oligoribonucleotide II than eIF-(iso)4F. eIF-3 has 2.2-fold lower affinity for m^7GpppG than eIF-4F and 1.2–1.9-fold greater affinity for oligoribonucleotides I and II than eIF-4F. The hairpin-containing oligoribonucleotide I, however, shows a pH-dependent binding to eIF-3 (Table I). The binding of this oligoribonucleotide to eIF-3 at pH 8.0 is 3-fold greater than at pH 7.6; in contrast, oligoribonucleotide II and m^7GpppG have only marginally (1.8- and 1.2-fold, respectively) greater affinity for eIF-3 at pH 8.0 relative to 7.6.

Having determined the binary interactions, we examined the ternary interactions. A summary of the directly measured binary and ternary complexes in the presence and absence of salt is given in Table I. A schematic representation for the interaction of eIF-3 with eIF-4F or eIF-(iso)4F and oligoribonucleotide I in the presence of salt is shown in Figure 6. The equilibrium association constants K_1, K_2, K_4, and K_5 were directly measured by fluorescence titration experiments in the presence of salt as follows. The titration of eIF-(iso)4F (or eIF-4F) with oligoribonucleotide I is represented by K_1; the titration of eIF-3 with oligoribonucleotide I is given by K_5, and the titration of eIF-3 with either eIF-4F or eIF-(iso)4F is represented by K_2. K_4 was determined by titrating an eIF-

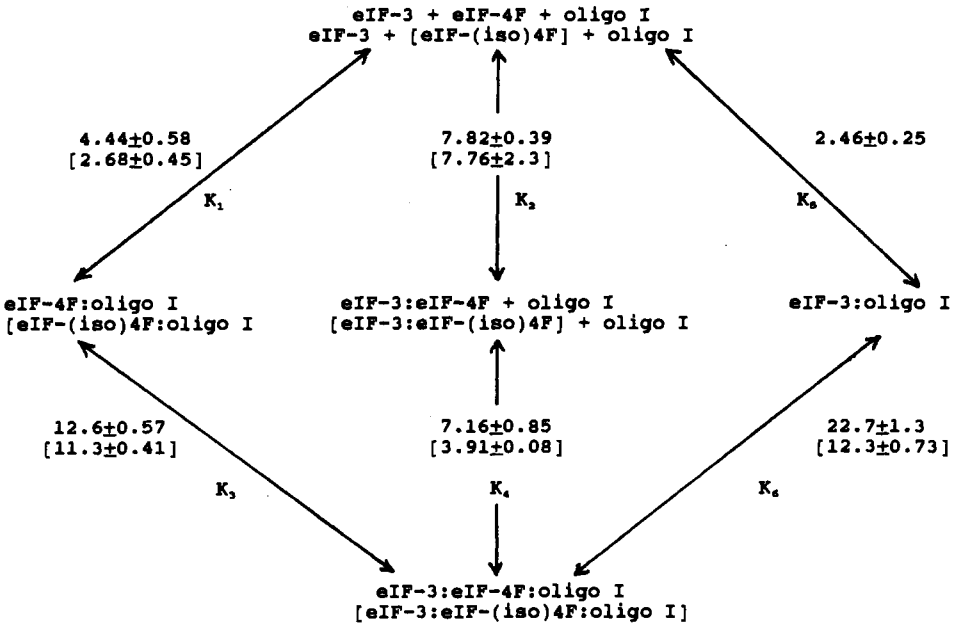


FIGURE 6: Schematic representation of the interaction of eIF-3, oligoribonucleotide I, and either eIF-(iso)4F or eIF-4F in the presence of 100 mM KCl/2 mM $MgCl_2$, pH 7.6. K_1, K_2, K_4, and K_5 were determined experimentally; K_3 and K_6 were calculated as described in the text. The values in brackets are for interactions involving eIF-(iso)4F. All values cited are 10^5 M^{-1}.

3/eIF-(iso)4F complex (in which 40–62% of the eIF-3 and 16–25% of the eIF-(iso)4F were bound) or an eIF-3/eIF-4F complex (in which 45–58% of the eIF-3 and 10–12% of the eIF-4F were bound) with oligoribonucleotide I. The interaction of the free factors with oligoribonucleotide was taken into account from the previously determined binary equilibrium constants. In this scheme, K_3 and K_6 were chosen as the thermodynamically dependent equilibrium constants and were calculated from the relationships

$$K_3 = (K_2K_4)/K_1 \quad (1a)$$

$$K_6 = (K_2K_4)/K_5 \quad (1b)$$

A comparison of the cross-terms in Figure 6 shows that the binding of the oligoribonucleotide to eIF-4F [or eIF-(iso)4F] enhances the binding of eIF-3 (comparing K_1 and K_4); similarly, the binding of the oligoribonucleotide to eIF-3 enhances the binding of eIF-4F [or eIF-(iso)4F; comparing K_5 and K_4].

In order to quantitate these interactions, coupling energies were calculated according to the method of Weber (1975) and as previously described in detail (Goss et al., 1984, 1990). The coupling energies reflect the overestimation or underestimation of the free energy of binding for the formation of the ternary eIF-3/eIF-4F/mRNA [or eIF-3/eIF-(iso)4F/mRNA] complex ($\Delta G°_{M3F}$) calculated from the addition of the component binary binding energies for the interaction of cap or oligoribonucleotide analogue with eIF-4F [or eIF-(iso)4F] ($\Delta G°_{MF}$) or with eIF-3 ($\Delta G°_{M3}$) of and eIF-4F [or eIF-(iso)4F] with eIF-3 ($\Delta G°_{3F}$). These coupling energies therefore represent different binding perspectives and are defined by

$$\Delta G°_{M,3F} = \Delta G°_{M3F} - \Delta G°_{M3} - \Delta G°_{MF} \quad (2a)$$

$$\Delta G°_{F,M3} = \Delta G°_{M,3F} + \Delta G°_{M3} - \Delta G°_{3F} \quad (2b)$$

$$\Delta G°_{3,MF} = \Delta G°_{M,3F} + \Delta G°_{MF} - \Delta G°_{3F} \quad (2c)$$

where 3, F, and M denote eIF-3, eIF-4F [or eIF-(iso)4F], and cap or oligoribonucleotide analogue, respectively. $\Delta G°_{MF}$, $\Delta G°_{3F}$, and $\Delta G°_{3M}$ are determined from K_1, K_2, and K_5 in Figure 6, respectively, in the presence of salt. The respective K_{eq} values in the absence of salt are given in Table I. $\Delta G°_{M3F}$ is determined from the addition of the $\Delta G°$ values calculated from K_2 and K_4. These interaction energies indicate how the binding of one component to its site affects the binding of a second component to its site; thus, each component (eIF-3, eIF-4F or eIF-(iso)4F, and analogue) is treated as if it possesses two binding sites. For example, $\Delta G°_{F,3M}$ shows how the binding of eIF-3 to one site on eIF-4F affects the affinity of the analogue for its binding site on eIF-4F. The coupling energies may be positive, negative, or zero, depending on whether the interactions are anticooperative, cooperative, or noncooperative due to the binding of the second component, respectively.

Table II. Summary of the Interaction Energies for the Wheat Germ Factors and Oligoribonucleotide Analogues[a]

	eIF-(iso)4F		eIF-4F	
	0 mM KCl, 0 mM $MgCl_2$, pH 7.6	100 mM KCl, 2 mM $MgCl_2$, pH 7.6	0 mM KCl, 0 mM $MgCl_2$, pH 8.0	100 mM KCl, 2 mM $MgCl_2$, pH 7.6
$m^{2,7}$GpppG				
$\Delta G°_{M,3F}$	−1.19			
$\Delta G°_{F,3M}$	+0.60			
$\Delta G°_{3,MF}$	+0.15			
m^7GpppG				
$\Delta G°_{M,3F}$	−2.13		−0.36	
$\Delta G°_{F,3M}$	−0.25		+0.51	
$\Delta G°_{3,MF}$	−0.42		+0.04	
oligo I				
$\Delta G°_{M,3F}$	−1.41	−0.90	+0.69	−0.97
$\Delta G°_{F,3M}$	−0.31	−0.22	+0.30	−0.28
$\Delta G°_{3,FM}$	−0.44	−0.27	+0.21	−0.63
oligo II				
$\Delta G°_{M,3F}$	−1.53		+0.07	
$\Delta G°_{F,3M}$	−0.19		+0.13	
$\Delta G°_{3,MF}$	−0.76		−0.09	

[a] All $\Delta G°$ values are reported in kilocalories per mole and were obtained as described in the text. The notation used is 3, eIF-3; eIF-(iso)4F or eIF-4F as indicated; M, cap or oligoribonucleotide analogue as indicated.

The coupling energies calculated in this manner are given in Table II. In the absence of salt, the coupling energies for the interaction of eIF-4F, eIF-3, and either m^7GpppG or oligoribonucleotide I or II are generally small and positive, indicative of anticooperative interactions. In contrast, the coupling energies for the interaction of eIF-(iso)4F, eIF-3, and m^7GpppG or oligoribonucleotide I or II are negative, indicative of cooperative interactions. However, the interactions involving

$m^{2,7}$GpppG are varied: the binding of eIF-3 and eIF-(iso)4F to the analogue is cooperative and similar in magnitude to the interactions involving the other analogues ($\Delta G°_{M,3F}$), whereas the binding of the analogue to either eIF-3 or eIF-(iso)4F in the eIF-3/eIF-(iso)4F complex is anticooperative ($\Delta G°_{F,3M}$ or $\Delta G°_{3,MF}$).

In the presence of salt, the coupling energies for the interaction of oligoribonucleotide I with eIF-3 and either eIF-4F or eIF-(iso)4F are cooperative (Table II). In the case of eIF-(iso)4F, the coupling energy values reflect slightly less cooperativity in binding in the presence of salt than in the absence of salt, whereas for eIF-4F, the interactions that were anticooperative in the absence of salt have become cooperative upon addition of salt. This difference may be due to the presence of the salt itself, which stabilizes mRNA structure and therefore may alter the interactions, and/or the difference in pH (Tables I and II).

Discussion

The coupling free energies calculated from the experimentally determined K_{eq} values may be used to determine the interactions of various species leading up to the formation of the eIF-3/eIF-4F/mRNA [or eIF-(iso)4F/mRNA] complex. In the absence of salt, the binding of either eIF-(iso)4F or eIF-3 to cap analogue or oligoribonucleotide enhances the subsequent binding of the second factor to the analogue ($\Delta G°_{M,3F}$). The smaller values of $\Delta G°_{F,3M}$ and $\Delta G°_{3,MF}$ for the interaction of m^7GpppG or oligoribonucleotide I and II with eIF-3 and eIF-(iso)4F suggest that (i) the formation of the eIF-3/eIF-(iso)4F complex only slightly enhances the affinity of eIF-(iso)4F for the analogue and (ii) that the binding of the analogue to eIF-(iso)4F only slightly enhances the interaction of eIF-(iso)4F with eIF-3. In the case of $m^{2,7}$GpppG, however, $\Delta G°_{F,3M}$ and $\Delta G°_{3,FM}$ are anticooperative, suggesting that the bulkier hypermethylated cap analogue may be unable to fit in its cap-binding site on eIF-(iso)4F due to (partial) overlap of this site by the bound eIF-3. These coupling energies are therefore consistent with a mechanism in which the formation of a protein/mRNA complex [presumably an eIF-4F/mRNA or an eIF-(iso)4F/mRNA complex] is favored over the formation of an eIF-3/eIF-4F [or eIF-3/eIF-(iso)4F] complex prior to mRNA binding.

The binding of either eIF-3 or eIF-4F to m^7GpppG slightly enhances the binding of the second factor in the absence of salt ($\Delta G°_{M,3F}$). However, when the oligoribonucleotide analogues are used, this interaction becomes anticooperative, especially for hairpin-containing oligoribonucleotide I. This result suggests that the eIF-4F/eIF-3 interaction may prevent eIF-4F from interacting with other structural features of the mRNA (e.g., the hairpin), since it is known that eIF-4F prefers interacting with hairpin-containing oligoribonucleotides (Carberry & Goss, 1991). This interpretation is also consistent with the finding that the linear oligoribonucleotide II shows less of an anticooperative effect upon interacting with eIF-3 and eIF-4F (Table II).

In the presence of salt, the interaction energies for eIF-4F and eIF-(iso)4F with oligoribonucleotide I and eIF-3 are similar (Table II): the binding of either eIF-3 or eIF-4F [or eIF-(iso)4F] to oligoribonucleotide I enhances the binding of the second factor to the message ($\Delta G°_{M,3F}$). In the case of eIF-4F, this result is in contrast to that obtained in the absence of salt, suggesting that increased stability of the oligoribonucleotide in the presence of salt enhances the binding of the protein factors. This is not surprising, since eIF-4F binds more strongly to oligoribonucleotides with secondary structure (see Figure 5 and Table I). The binding of the oligoribonucleotide to eIF-4F [or eIF-(iso)4F] in the eIF-3/eIF-4F [or eIF-3/eIF-(iso)4F] complex shows only slight cooperativity ($\Delta G°_{F,3M}$); from the perspective of eIF-4F [or eIF-(iso)4F], this indicates nearly independent binding of eIF-3 and mRNA. Therefore, the protein/protein interactions are not significantly increased by the mRNA binding, perhaps due to an induced conformational change in eIF-4F [or eIF-(iso)4F]. In contrast to the cooperative effects observed here in the presence of salt, the interaction energies for the formation of a mammalian eIF-4E/eIF-4A/globin mRNA complex were found to be anticooperative (Goss et al., 1990); those data had suggested that the formation of an eIF-4E/eIF-4A complex may precede the binding of the mRNA to eIF-4E.

From these data, a mechanism can be proposed for the sequence of events leading to the formation of an initiation complex. eIF-4F [or eIF-(iso)4F] binds to the mRNA cap first; in the case of eIF-(iso)4F, hypermethylated caps are preferred (see Table I; Carberry & Goss, 1991). The formation of this binary complex enhances the subsequent binding of eIF-3. The interaction of eIF-3 with the mRNA cap region may act as a bridge to aid in correctly positioning the ribosome and thereby enhance translation.

References

Behlke, J., Bommer, U.-A., Lutsch, G., Henske, A., & Bielka, H. (1986) *Eur. J. Biochem. 157*, 523–530.

Benne, R., & Hershey, J. W. B. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3005–3009.

Benne, R., & Hershey, J. W. B. (1978) *J. Biol. Chem. 253*, 3078–3087.

Carberry, S. E., & Goss, D. J. (1991) *Biochemistry 30*, 4542–4545.

Carberry, S. E., Rhoads, R. E., & Goss, D. J. (1989) *Biochemistry 28*, 8078–8083.

Carberry, S. E., Darzynkiewicz, E., Stepinski, J., Tahara, S. M., Rhoads, R. E., & Goss, D. J. (1990) *Biochemistry 29*, 3337–3341.

Carberry, S. E., Drazynkiewicz, E., & Goss, D. J. (1991a) *Biochemistry 30*, 1624–1627.

Carberry, S. E., Kapsis, E., Rhoads, R. E., & Goss, D. J. (1991b) *Biochemistry* (submitted for publication).

Checkley, J. W., Cooley, L., & Ravel, J. M. (1981) *J. Biol. Chem. 256*, 1582–1586.

Darzynkiewicz, E., Stepinski, J., Ekiel, I., Jin, Y., Haber, D., Sijuwade, T., & Tahara, S. M. (1988) *Nucleic Acids Res. 16*, 8953–8962.

Draper, D. E., White, S. A., & Kean, J. M. (1988) *Methods Enzymol. 164*, 221–237.

Eadie, G. S. (1942) *J. Biol. Chem. 146*, 85.

Goss, D. J., Parkhurst, L. J., Mehta, A. M., & Wahba, A. J. (1984) *Biochemistry 23*, 6522–6529.

Goss, D. J., Rounds, D., Harrigan, T., Woodley, C. L., & Wahba, A. J. (1988) *Biochemistry 27*, 1489–1494.

Goss, D. J., Carberry, S. E., Dever, T. E., Merrick, W. C., & Rhoads, R. E. (1990) *Biochemistry 29*, 5008–5012.

Lax, S. R., Lauer, S. J., Browning, K. S., & Ravel, J. M. (1986a) *Methods Enzymol. 118*, 109–128.

Lax, S. R., Browning, K. S., Maia, D. M., & Ravel, J. M. (1986b) *J. Biol. Chem. 261*, 15632–15636.

Milligan, J. F., Groebe, D. R., Witherell, G. W., & Uhlenbeck, O. C. (1987) *Nucleic Acids Res. 15*, 8783–8798.

Peterson, D. T., Merrick, W. C., & Safer, B. (1979) *J. Biol. Chem. 254*, 2509–2516.

Safer, B., Adams, S. L., Kemper, W. M., Berry, K. W., Lloyd, M., & Merrick, W. C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2584–2588.

Schreier, M. H., & Staehelin, T. (1973) *Nature New Biol. 242*, 35-38.
Schreier, M. H., Erni, B., & Staehelin, T. (1977) *J. Mol. Biol. 116*, 727-753.
Setyono, B., van Steeg, H., & Voorma, H. O. (1984) *Biochim. Biophys. Acta 782*, 242-246.
Sonenberg, N., & Shatkin, A. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4288-4292.
Sonenberg, N., Morgan, M. A., Merrick, W. C., & Shatkin, A. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75* 4843-4847.
Sonenberg, N., Morgan, M. A., Testa, D., Colonno, R. J., & Shatkin, A. J. (1979) *Nucleic Acids Res. 7*, 15-29.
Thompson, H. A., Sadnik, I., Scheinbuks, J., & Moldave, K. (1977) *Biochemistry 16*, 2221-2230.
Trachsel, H., & Staehelin, T. (1979) *Biochim. Biophys. Acta 565*, 305-314.
Trachsel, H., Erni, B., Schreier, M. H., & Staehellin, T. (1977) *J. Mol. Biol. 116*, 755-767.
Weber, G. (1975) *Adv. Protein Chem. 29*, 2-78.
Westerman, P., & Nygard, O. (1984) *Nucleic Acids Res. 12*, 8887-8897.

Overexpression of the cAMP Receptor 1 in Growing *Dictyostelium* Cells†

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Received February 14, 1991; Revised Manuscript Received April 19, 1991

ABSTRACT: cAR1, the cAMP receptor expressed normally during the early aggregation stage of the *Dictyostelium* developmental program, has been expressed during the growth stage, when only low amounts of endogenous receptors are present. Transformants expressing cAR1 have 7-40 times over growth stage and 3-5-fold over aggregation stage levels of endogenous receptors. The high amounts of cAR1 protein expressed constitutively throughout early development did not drastically disrupt the developmental program; the onset of aggregation was delayed by 1-3 h, and then subsequent stages proceeded normally. The affinity of the expressed cAR1 was similar to that of the endogenous receptors in aggregation stage cells when measured either in phosphate buffer (two affinity states with K_d's of approximately 30 and 300 nM) or in 3 M ammonium sulfate (one affinity state with a K_d of 2-3 nM). When expressed during growth, cAR1 did not appear to couple to its normal effectors since these cells failed to carry out chemotaxis or to elevate cGMP or cAMP levels when stimulated with cAMP. However, cAMP stimulated phosphorylation, and loss of ligand binding of cAR1 did occur. Like aggregation stage control cells, the cAR1 protein shifted in apparent molecular mass from 40 to 43 kDa and became highly phosphorylated when exposed to cAMP. In addition, the number of surface cAMP binding sites in cAR1 cells was reduced by over 80% during prolonged cAMP stimulation. These results define a useful system to express altered cAR1 proteins and examine their regulatory functions.

Dictyostelium discoideum normally live as freely growing amoebae, but when deprived of nutrients, cell division and growth cease, and the cells enter a developmental program that results in the formation of a multicellular structure. During early development, organizing centers arise which secrete cAMP[1] every 6 min. The released cAMP stimulates neighboring cells, which relay the chemical signal outward in the form of concentric or spiral waves (Tomchik & Devreotes, 1981). The propagated waves of cAMP act as chemoattractant gradients which coordinate the migration of cells toward the aggregation center (Devreotes, 1982).

Early aggregation is coordinated by a G-protein-linked signal transduction system. Extracellular cAMP binds to a cell surface receptor, coupled to a G-protein, which leads to activation of adenylyl cyclase. The newly synthesized intracellular cAMP is then secreted from the cell. Ligand binding also causes adaptation which uncouples the receptor from its effectors within minutes. The rapid removal of extracellular cAMP by cell surface phosphodiesterases allows the receptors to resensitize, and the cycle is reinitiated (Klein et al., 1985; Janssens & Van Haastert, 1987; Gundersen et al., 1989).

A cAMP receptor (denoted cAR1) has been cloned and its primary sequence determined (Klein et al., 1988). Characteristic of other G-protein-coupled receptors, such as rhodopsin (Hargrave, 1986) and the adrenergic receptors (Dohlman et al., 1987), its predicted sequence encodes a protein consisting of seven transmembrane domains followed by a hydrophilic C-terminal region. This cytoplasmic region contains 18 serines,

† This work was supported by National Institutes of Health Grant GM-34933.
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[1] Abbreviations: cAR, cAMP receptor; cAMP, adenosine cyclic 3′,5′-monophosphate; cGMP, guanosine cyclic 3′,5′-monophosphate; LLB, loss of ligand binding; DB, developmental buffer; HBS, Hepes-buffered saline; PB, phosphate buffer; AS, ammonium sulfate; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

0006-2960/91/0430-6982$02.50/0