Studies on the Role of Eukaryotic Nucleotide Exchange Factor in Polypeptide Chain Initiation*

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Interactions of eukaryotic 5-dimethylaminonaphthalene-1-sulfonyl-initiation factor 2 (eIF-2) from rabbit reticulocytes and the guanine nucleotide exchange factor (GEF), Met-tRNAf, GTP, and GDP were monitored by changes in fluorescence anisotropy and radioactive filtration assays. At 1 mM Mg2+, radioactive filtration assays demonstrate that GEF is necessary for nucleotide exchange. We did not observe a GDP dependence in the association reaction of eIF-2-GEF for GDP concentrations from 0.01 to 20 μM. This is in disagreement with the model: eIF-2-GDP + GEF = eIF-2-GEF + GDP. The addition of GTP caused a decrease in fluorescence anisotropy which is interpreted as a dissociation of eIF-2-GEF. We propose an asymmetrical model of ternary complex (eIF-2-GTP • Met-tRNAf) formation where 1) GDP does not displace GEF or GTP (100 μM presumably as a binary (eIF-2.GDP) complex (6, 7). Bound complex transfers Met-tRNAf to an 80S reticulocyte eIF-2, phosphorylation of the α subunit of eIF-2, and 2) GTP replaces GEF and presumably GDP. For eIF-2 phosphorylation, the α subunit greatly inhibits protein synthesis. This inhibition derives neither from failure of GEF to bind to eIF-2(αP) nor from greatly enhanced binding of GEF. The inhibition results from the requirement of very high levels of GTP (100 μM) to dissociate the eIF-2(αP) • GEF complex.

Termination (eIF-2-GTP • Met-tRNAf) complex formation by eIF-2 is one of the first steps in protein synthesis (1-4). This complex transfers Met-tRNAf to an 80S initiation complex with the hydrolysis of GTP (5) and the release of eIF-2, presumably as a binary (eIF-2-GDP) complex (6, 7). Bound GDP must be exchanged for GTP in order to form a new ternary complex. In reticulocytes, GEF promotes this exchange (8-11). Phosphorylation of the α subunit of eIF-2, which occurs in hemin-deficient reticulocyte lysates, greatly inhibits the exchange of GTP for GDP under physiological conditions and inhibits protein synthesis.

Several mechanisms have been proposed for the nucleotide exchange reaction and the effects of phosphorylation of eIF-2 (8, 12-14). In this paper, we present data from membrane filter assays showing the overall reactions and a mechanism based on direct measurement by fluorescence anisotropy of the interaction of eIF-2 and eIF-2(αP) with GEF. These results are used to interpret the overall nucleotide exchange reactions and the effect of phosphorylation on the catalytic utilization of eIF-2 and GEF.

EXPERIMENTAL PROCEDURES

Materials—Dansyl chloride was obtained from Sigma. DEAE-cellulose type 52 and Sephadex G-25 (medium) were products of Whatman and Pharmacia Fine Chemicals, respectively. Reticulocyte lysate preparations were purchased from Green Hectares, Oregon, WI. Sources of other materials were previously described (15, 16). Buffer (A), used for fluorescence titrations, consisted of 0.1 M Tris-HCl, pH 7.5, 0.30 M KCl, 1 mM diithiothreitol, 1 mM MgCl2, and 10% (v/v) glycerol.

Preparations—Reticulocyte eIF-2 (15) and GEF (17) were purified from rabbit reticulocyte lysates. The eIF-2 was phosphorylated with the reticulocyte heme-controlled repressor and [γ-32P]ATP and subsequently isolated by chromatography on phosphocellulose (18).

Dansylation of eIF-2—Reticulocyte eIF-2 and eIF-2(αP) were labeled by the same procedures. A 100-fold molar excess of dansyl chloride in 5 μl of ethanol was added to 25 μl of eIF-2 or eIF-2(αP) containing 0.3-0.6 mg/ml of protein (1 μM = 0.127 mg/ml). The solution was incubated for 20 min at 4°C and chromatographed on a Sephadex G-25 column (0.4 × 15 cm) equilibrated with buffer A containing 0.05 mM EDTA and eluted with the same buffer. The first fluorescent band was collected (0.1-0.2 ml) and applied to a DE52 column (0.5 × 2 cm) equilibrated with the same buffer. Upon chromatography on the Sephadex G-25 column, the A280 and activity peaks eluted earlier than the fluorescence intensity maximum. The second column (DE52) removed additional noncovalently bound dye so that the A280, eIF-2 activity, and fluorescence intensity peaks corresponds and free fluorescent material was retained.

Activity Assays—Activity assays for labeled and unlabeled protein were carried out as previously described (15). The dansyl-labeled protein showed the same activity for binary and ternary complex formation as unlabeled protein.

Fluorescence Measurements—Fluorescence anisotropy was measured as previously described (19, 20). Titrations were carried out in buffer A. Concentrations of eIF-2 and eIF-2(αP) ranged from 3 to 17 nM.

Data Fitting—Equilibrium constants for eIF-2 and eIF-2(αP) binding to GEF were obtained by fitting fluorescence anisotropy titrations to equations described previously for prokaryotic ribosomal protein S1 binding to 70 S ribosomes (20). The eIF-2-GEF equilibrium constant is: K0 = ([eIF-2(GEF)]/[eIF-2 • GEF]). The fluorescence anisotropy at any point in the titration of dansyl-labeled eIF-2 with GEF is given as robs = (a - b + b2 - 4ac)/2(a) where a, b, and c are analogous to the prokaryotic S1 ribosome (S1-Rb) case: [eIF-2-GEF] ~ [S1 • RB], [eIF-2] ~ [S1], and [GEF] ~ [Rb] in the previous equations (20).

The fluorescence intensity was unchanged when GEF was added to eIF-2 or eIF-2(αP). The total eIF-2 and GEF concentrations were known, and therefore the equilibrium constant in principle could be determined from any point on the anisotropy curve (29). The values for robs and robs were measured and known. The value for robs was easily measured before addition of GEF, and the value for robs was determined from the end point of the anisotropy titration. The values for eIF-2 • GEF data were fit with a Fletcher-Powell sum of squares minimization using only one variable (K0) for the entire titration curve (20).
RESULTS

Binary (eIF-2-GDP) and ternary (eIF-2-GTP-Met-tRNAf) complex formation with phosphorylated and nonphosphorylated eIF-2 is shown in Table I. In the absence of Mg\(^{2+}\), reticulocyte eIF-2(\(\alpha\P\)) formed binary and ternary complexes as efficiently as the nonphosphorylated form. However, the addition of Mg\(^{2+}\) reduced these reactions 70–86%. In the presence of a factor, GEF, isolated from either the postribosomal supernatant or the high salt wash of ribosomes, [\(^{3}H\)]GDP binding or [\(^{35}S\)]Met-tRNA\(_f\) binding occurred to the same extent in the presence or absence of 1 mM Mg\(^{2+}\). When the \(\alpha\) subunit of reticulocyte eIF-2 was phosphorylated, addition of GEF in the absence or presence of Mg\(^{2+}\) did not promote significant binding of [\(^{3}H\)]GDP or [\(^{35}S\)]Met-tRNA\(_f\).

Labeling of eIF-2 with dansyl chloride permitted direct measurement of the interaction of eIF-2 and eIF-2(\(\alpha\P\)) with GEF. Dansyl-eIF-2 showed a large change in fluorescence anisotropy when GEF was added to the solution (Fig. 1). The apparent equilibrium constant (Table II) for eIF-2.GDP + GEF did not change significantly with GDP concentration (0.01–20 \(\mu\)M). The eIF-2 bound GEF

### Table I

**Binary (eIF-2-GDP) and ternary (eIF-2-GTP-Met-tRNAf) complex formation with phosphorylated and nonphosphorylated eIF-2**

| Additions         | [\(^{3}H\)]GDP bound | [\(^{35}S\)]Met-tRNA\(_f\) bound |
|-------------------|-----------------------|----------------------------------|
|                   | \(-\text{Mg}^{2+}\)  | \(+1\text{ mm}\text{Mg}^{2+}\) | \(-\text{Mg}^{2+}\)  | \(+1\text{ mm}\text{Mg}^{2+}\) |
| eIF-2             | 11,200                | 3,260                            | 8,730                | 1,700                          |
| eIF-2 + GEF       | 10,300                | 11,000                           | 9,890                | 1,700                          |
| eIF-2(\(\alpha\P\)) | 9,490                | 1,300                            | 6,800                | 1,600                          |
| eIF-2(\(\alpha\P\)) + GEF | 8,960              | 1,380                            | 8,100                | 1,600                          |

Additions Experimental conditions
|                    | \(K_d\) \(nM\) |
|--------------------|---------------|
| eIF-2 + GEF        | A             | 0.05 (0.02, 0.06) |
| eIF-2-GDP + GEF    | B             | 0.20 (0.1, 0.25)  |
| eIF-2 + GEF + GDP  | C             | 0.15 (0.1, 0.17)  |
| eIF-2(\(\alpha\P\)) + GEF | A          | 0.53 (0.4, 0.6)   |
| eIF-2(\(\alpha\P\)) + GEF + GDP | C        | 0.31 (0.21, 0.35) |

*The numbers in parentheses following \(K_d\) are the values of \(K_d\) for the 95% confidence region. \(K_d\) is the equilibrium constant for the dissociation of the eIF-2.GEF or eIF-2(\(\alpha\P\)).GEF complex. We do not specify whether GDP is bound to the complex. See text for details.

![Fig. 1. Titration of eIF-2 with the nucleotide exchange factor GEF. The y axis is the normalized anisotropy change (\(r_{set} - r_{set-g} - r_{set-f}\)). The triangles represent the data points for eIF-2-GDP (protein \(=8\) nm, GDP total \(=0.1\) \(\mu\)M)–binding GEF. The anisotropy range was 0.13–0.20. The range varied slightly with different preparations, probably due to the difficulty in removing free dye. Circles are the observations for 8 nm eIF-2 and 20 \(\mu\)M GDP titrated with GEF. In this case, the anisotropy range was 0.16–0.22. The solid and dotted lines represent the calculated equilibrium curves for \(K_{eq} = 0.15\) and 0.20 nm, respectively. The temperature was 20 °C.](image1)

![Fig. 2. Kinetics of [\(^{3}H\)]GDP release from eIF-2. [\(^{3}H\)]GDP or eIF-2(\(\alpha\P\)).[\(^{3}H\)]GDP. The assay mixture in 75 \(\mu\)l contained 100 mM KCl, 20 mM Tris-HCl, pH 7.8, 1 mM Mg\(^{2+}\), 0.2 mM GTP as indicated, 42 ng (approximately 0.14 pmol) of GEF, and 0.6 pmol of reticulocyte eIF-2(\(\alpha\P\)).[\(^{3}H\)]GDP, and eIF-2(\(\alpha\P\)).[\(^{3}H\)]GDP. In the absence of GTP, no release of [\(^{3}H\)]GDP occurred in the 10-min assay period when amounts of GEF up to 1 \(\mu\)g were added.](image2)

about three to four times more tightly than the binary (eIF-2-GDP) complex.

The effects of GTP on this system were as follows. For nonphosphorylated reticulocyte eIF-2, the addition of GEF caused a fluorescence anisotropy change from about 0.11 to 0.19–0.22. At low levels of GDP (stoichiometric, 0.005–0.015 \(\mu\)M) to about 0.5 \(\mu\)M, the addition of GTP (10–15 \(\mu\)M) caused a decrease in anisotropy to about 0.08–0.09, presumably from dissociation of the eIF-2.GEF complex. The phosphorylated eIF-2 behaved quite differently. The GTP level was increased approximately 10-fold to 100 \(\mu\)M before dissociation took place.

Fig. 2 shows the kinetics of [\(^{3}H\)]GDP release from eIF-2 or eIF-2(\(\alpha\P\)). GDP was released from eIF-2 but not from...
eIF-2(aP) by the addition of 125 μM GTP and GEF. In the absence of GTP, the addition of a 5-fold excess of GEF over eIF-2·[3H]GDP did not cause the release of [3H]GDP within the 10-min assay.

**DISCUSSION**

For reticulocyte eIF-2, the addition of GEF was necessary to promote significant exchange of GTP for GDP in the presence of Mg$^{2+}$. A symmetrical model was proposed to represent the GEF catalytic cycle of ternary complex formation (8, 14). The model can be represented as follows.

$$\begin{align*}
&\text{GDP} \quad \text{GTP} \\
&\text{eIF-2-GDP} \quad \text{eIF-2-GTP} \\
&\text{eIF-2-GEF} \quad \text{Met-tRNA}_f
\end{align*}$$

In this model, GEF serves to displace GDP and is itself displaced by GTP. The eIF-2-GTP can then form a ternary complex with Met-tRNAf.

This model predicts that the observed dissociation of eIF-2·GEF for the three-component system (eIF-2, GEF, and GDP) would depend on GDP concentration. The reaction can be written as: eIF-2·GDP + GEF ⇌ eIF-2·GEF + GDP, where $K_0 = ([\text{eIF-2·GEF}][\text{GDP}])/([\text{eIF-2·GDP}][\text{GEF}])$. Here, we assume there is negligible free eIF-2, since $K_p$ for eIF-2·GDP is ~10^{-8} M (13), eIF-2 concentration was 0.007-0.02 μM, and [GDP] > 0.1 μM. If this model were correct, then the observed anisotropy change upon addition of GEF to eIF-2 would be due to formation of eIF-2·GEF rather than eIF-2·GDP·GEF. The equilibrium expression for the symmetrical model may be written as: $\log K_0 = \log \rho + \log[GDP]$, where $\rho = ([\text{eIF-2·GEF}])/([\text{eIF-2·GDP}][\text{GEF}])$, where $\rho$ is readily calculated from the anisotropy data, and total concentration of GEF and eIF-2. If this model were correct, a plot of $\log \rho$ versus $\log [\text{GDP}]$ would have a slope of -1 (Fig. 3 solid line).

We did observe a GDP dependence in the dissociation reaction of eIF-2·GEF for GDP concentrations from 0.01 to 20 μM. If binding of GEF to eIF-2 were a displacement reaction, high levels of GDP should reverse the binding and promote dissociation of the complex. The observed data are clearly not in accord with this model.

An alternative model of the GEF catalytic cycle of ternary complex formation is (13) as follows.

$$\begin{align*}
&\text{GTP} \quad \text{Met-tRNA}_f \\
&\text{eIF-2-GTP} \\
&\text{eIF-2·GEF} \quad \text{eIF-2·GTP-Met-tRNA}_f
\end{align*}$$

In this model, eIF-2·GEF forms a stable complex first with GDP and then with GTP during the exchange reaction. Our data are not in conflict with the first part of the model, formation of a stable eIF-2·GDP·GEF. We cannot measure directly release of GDP and therefore cannot distinguish, from anisotropy measurements, between an eIF-2·GEF complex and eIF-2·GDP·GEF. The proposal of a stable eIF-2·GEF·GTP complex is not in accord with our data. The addition of GTP caused a decrease in anisotropy, which is most reasonably interpreted as a dissociation of eIF-2·GEF since the end point corresponded to the anisotropy of labeled eIF-2-GTP (see Fig. 4).

In this model, eIF-2·GEF forms a stable complex first with GDP and then with GTP during the exchange reaction. The effects of increasing GTP concentrations on the anisotropy of eIF-2·GEF and eIF-2(aP)·GEF. The y axis is the normalized anisotropy ($r_0/r_c$), where $r_c$ is the observed anisotropy for eIF-2·GEF or eIF-2(aP)·GEF with no added GTP. The x axis is the total GTP concentration. The circles are the data points for eIF-2·GEF, and the triangles are the data points for eIF-2(aP)·GEF. The temperature was 20 °C.

Our data are consistent with what might be termed an asymmetrical model of ternary complex formation.

$$\begin{align*}
&\text{eIF-2-GFP} \quad \text{GTP} \\
&\text{eIF-2-GDP} \quad \text{GEF} + \text{GDP} \\
&\text{eIF-2-GTP-Met-tRNA}_f
\end{align*}$$

In this model, GEF binding does not displace GDP. This conclusion is supported by the fact that the association equilibrium for eIF-2·GEF does not depend on GDP concentration in the range 0.010-20 μM, a >10-fold excess of GDP over eIF-2 and GEF.

The binding of GTP releases GEF and presumably GDP, so that a stable eIF-2·GTP·GEF is not formed. In terms of nucleotide binding, the model presented is asymmetrical: the first reaction, GEF associating with eIF-2·GDP, does not require release of nucleotide, and the second reaction, GTP·GDP exchange, causes release of GEF. This model incorporates the first part of the model of Safer et al. (13) and the second part of the symmetrical model (8, 14). Phosphorylation

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1 We represent the reactions of eIF-2, GEF, and GDP by a single equilibrium given by $K$ (above) since, according to the model being tested, the ternary complex (eIF-2·GDP·GEF) is not allowed and GDP and GEF are present in large enough amounts to ensure that we may neglect unassociated eIF-2·GDP. Calculation shows that for the datum in Fig. 3 at 0.01 μM GDP, unassociated eIF-2·GDP was <12% of the total; for the other points, the percentage was <2%.
of the reticulocyte eIF-2 inhibits the second part of the reaction.

Two opposed explanations were provided for the inhibition of protein synthesis in rabbit reticulocyte lysates. 1) Phosphorylated eIF-2 binds GEF much more tightly than nonphosphorylated eIF-2 and sequesters it from the active pool (17). This explanation is supported by the observation that even low levels of phosphorylation (10-20%) will inhibit protein synthesis as much as 90-95%. 2) Phosphorylated eIF-2 fail to interact with GEF, and therefore nucleotide exchange does not take place, and consequently a ternary complex cannot be formed (9).

Our equilibrium data (Table II) show that the second interpretation (non-interaction) is not correct. We have measured directly the interaction of GEF and eIF-2(αP) and find tighter binding and a 10-fold larger equilibrium constant must be smaller. This observation is in accord with data (17) that show inhibition of protein synthesis as much as 90-95%.

For eIF-2(αP), higher levels of GTP are required for the same bound. Addition of GTP favors dissociation of the complex. The anisotropy, however, is a measure of the GEF bound. Addition of GTP favors dissociation of the complex. For eIF-2(αP), higher levels of GTP are required for the same degree of dissociation, and in terms of this mechanism the equilibrium constant must be smaller. This observation is in accord with data (17) that show inhibition of protein synthesis can be overcome by increasing the GTP/GDP ratio.

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