# Functional heterogeneity of GEF-free initiation factor 2 purified from suckling and adult rat brain

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Received 8 July 1985

The functional behavior of initiation factor 2 was studied in purified preparations from the brains of suckling (4–12-day-old) and adult (60-day-old) rats. Adult eIF2 has lower GDP and GTP affinity than suckling eIF2, even in the presence of a large excess of GTP, whereas suckling eIF2 has a lower capacity to bind GTP. Since these two factors are free of guanine nucleotide exchange factor (GEF), and ribosomal fractions show an age-dependent difference in GEF activity, the observed functional heterogeneity may be due to a different ratio in eIF2 species (eIF2-GDP, eIF2(αP)).

*Developing brain* *Protein synthesis* *Initiation factor 2* *Guanine nucleotide exchange factor*

## 1. INTRODUCTION

Recent studies performed in our laboratory and elsewhere indicate that the decreased rate of protein synthesis initiation in the developing rat brain is accompanied by similar changes in the capacity of ribosomal salt-washed protein fractions to form the ternary complex eIF2–GTP–met-tRNA$_i$ [1,2]. This suggests that protein synthesis during development may be mainly determined by changes in eIF2 activity, as occurs in the reticulocyte system [3], where heme regulation of globin synthesis is modulated by phosphorylation of the $\alpha$-subunit of eIF2 [3,4]. This modification interferes with the action of the GDP/GTP exchange factor (GEF) which triggers the eIF2-GDP complex (release from the 40 S initiation complex) to bind first GTP and met-tRNA$_i$ [3–6], thus blocking recycling of eIF2($\alpha$P) [5]. A direct correlation has also been detected between the protein synthesis activity of reticulocyte lysate and its GEF activity [7] as well as between the presence of eIF2($\alpha$P) in fully inhibited lysates and a decrease in ribosomal GEF [8]. Since the degree of protein synthesis inhibition is related to differences in both ribosomal eIF($\alpha$P) and GEF activity levels, the decrease of protein synthesis initiation in developing rat brain is probably accompanied by such changes. The present study was performed to determine whether GEF has been lost during the purification procedure [9] and to analyze the functional behavior of purified eIF2 from both suckling (4–12 day old rats) and adult (60 day old rats) brain.

*Abbreviations:* eIF2, eukaryotic initiation factor 2; GEF, guanine nucleotide exchange factor (also referred to as eIF2-B [5] and RF [7,10]); met-tRNA$_i$, initiation methionine-tRNA

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## 2. MATERIALS AND METHODS

### 2.1. *Materials*

L-[*methyl*-$^3$H]Methionine (89 Ci/mmol) was purchased from the Radiochemical Centre, Amersham; [$^3$H]GDP (7 Ci/mmol) was from New England Nuclear, Dreieich; [$^3$H]met-tRNA$_i$ and ribosomal salt-washed protein (cIF fraction) were prepared as described in [9]. Unlabelled nucleotides, pyruvate kinase (PK) and phospho*enol*-

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00145793/85/$3.30 

pyruvate (PEP) were obtained from Sigma, St. Louis, MO.

### 2.2. *Preparation of rat brain eIF2*

eIF2 was isolated from the cIF fraction of both 4–12- and 60-day-old rat brains as in [9]. Purification steps included heparin-Sepharose, phosphocellulose and DEAE-cellulose chromatographies. The eIF2 obtained was 90 and 80% pure for suckling and adult preparations, respectively.

### 2.3. *Formation of ternary complexes*

Formation of eIF2–GTP–[$^3$H]met-tRNA$_i$ complexes was assayed as in [9]. Incubations (100 $\mu$l) contained 20 mM Hepes, pH 7.6, 100 mM KCl, 1 mM DTT, 0.5 mg/ml BSA, 1.5–3.0 pmol [$^3$H]met-tRNA$_i$ (30 Ci/mmol), and GTP and eIF2 at the concentrations specified in the figure legends.

### 2.4. *Assays for nucleotide exchange*

The formation of binary complexes was performed under the same conditions as described above, except that GTP and met-tRNA$_i$ were omitted and 1.4 $\mu$M GDP or [$^3$H]GDP (1 $\mu$Ci) was added.

The ability of eIF2 preparations to exchange eIF2-bound GDP with either GDP or GTP was monitored using 2 types of assays. (i) [$^3$H]GDP-eIF2 complexes were preformed for 10 min at 30°C, in a final volume of 250 $\mu$l. The samples were chilled on ice and the reaction was stopped by adding $MgCl_2$ (final concentration 1 mM). The exchange reaction was started by adding excess unlabelled GDP (1.4 mM final concentration). Following incubation at 30°C, 50-$\mu$l samples were taken at different times, immediately filtered through nitrocellulose membranes (HAWP from Millipore Ibérica, Spain), washed twice with ice-cold wash buffer (20 mM Tris-HCl, pH 7.6, 100 mM KCl, 10 mM $MgCl_2$, 2 mM DTT), and retained radioactivity was estimated by liquid scintillation counting. (ii) Binary complexes were preformed with unlabelled GDP in a final volume of 100 $\mu$l, for 10 min at 30°C, stopping the reaction by adding $MgCl_2$ (1 mM). GTP and [$^3$H]met-tRNA$_i$ were then added to form the ternary complex. PEP (3 mM) and PK (2 U/ml) were also present in the incubation mixture to avoid the inhibitory effect of unbound GDP. This exchange reaction was incubated for 15 min at 30°C. Samples preincubated without GDP were used as controls.

## 3. RESULTS AND DISCUSSION

When eIF2 was isolated from suckling rat brain, we observed that the purification of eIF2 activity did not correlate with the purity percentage, suggesting that GEF could be separated throughout the purification process [9]. Here, the absence of GEF factor in preparations from both suckling and adult animals was tested by measuring their capacitiy to exchange eIF2-bound GDP with GTP, in comparison with that of crude fractions. As shown in table 1, the GDP/GTP exchange capacity of isolated factors is much less compared with the crude ones. It has been established that in the presence of $Mg^{2+}$, eIF2 has a higher affinity for GDP than for GTP [11] and that the nucleotide exchange only occurs if GEF is present [3,4,6,7]. Therefore, the loss of nucleotide exchange capacity in our purified factors could be explained by the absence of GEF.

GEF was detected in the protein fraction eluted with 0.5 M KCl from the heparin-Sepharose column (HS fraction) after gradient elution of eIF2 activity. As shown in fig.1, both eIF2 preparations from suckling and adult brains have no capacity to exchange [$^3$H]GDP by free GDP. When the HS

Table 1

Ternary complex formation from preformed eIF2-GDP complexes

| Fraction | Bound met-tRNA$_i$ (pmol/mg protein) | |
|---|---|---|
| | Control | + GDP preincubation |
| Suckling cIF | 1.28 | 1.24 |
| Adult cIF | 0.85 | 0.45 |
| Suckling eIF2 | 24.51 | 4.53 |
| Adult eIF2 | 30.32 | 3.59 |

Binary eIF2-GDP complexes were preformed as described in the text, with 20–30 $\mu$g protein and 14 $\mu$M GDP for cIF fractions or 2.5 $\mu$g eIF2 and 1.4 $\mu$M GDP for purified preparations. GTP added was 1 mM or 80 $\mu$M for crude and purified eIF2, respectively. Values represent mean of 2 experiments

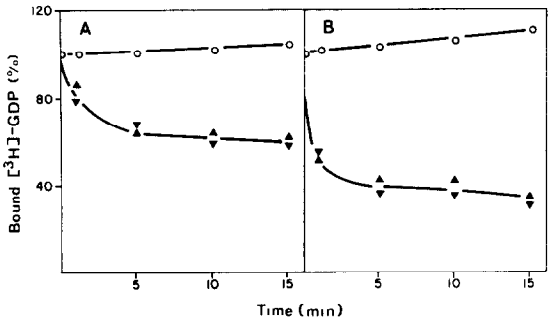


Fig.1. Kinetics of exchange of eIF2-bound [$^3$H]GDP with free GDP. Purified eIF2 (6.5 $\mu$g) from 4–12- (A) and 60-day-old (B) rat brain were assayed for their capacity to exchange [$^3$H]GDP with unlabelled GDP as described in section 2. Results (mean from 3 experiments) are expressed as a percentage of the initial level of complexes (immediately present before addition of unlabelled nucleotide). After preincubation with [$^3$H]-GDP, 20 $\mu$g HS protein fraction were added. (○) No addition, (▲) 4-day-old HS fraction, (▼) 60-day-old HS fraction.

fraction was added, this exchange was stimulated. The amount of exchange effected per mg protein in the 2 suckling and adult HS fractions was identical, indicating no differences in GEF specific activity. Nevertheless, the total protein content recovered was 20-times higher in the suckling HS fraction than in the adult one (not shown). This higher protein content, together with the fact that the GDP/GTP exchange capacity was greater in the suckling than in the adult cIF fractions (table 1), may be due to an increased GEF content in the younger cIF fraction. Since the protein synthesis initiation rate [1] is higher in the neonate brain, these results indicate a possible relationship between the GEF level in the ribosomal fraction and protein synthesis activity. A similar lower decrease in GEF level has been reported for heme-deficient reticulocyte vs fully active lysates [7,8].

Heterogeneity of these GEF-free eIF2 purified from suckling and adult brain was demonstrated by studying their capacity to bind GTP and their affinities for both GTP and GDP. In the ternary complex formation, the binding of 1 mol met-tRNA$_i$ occurs only if 1 mol GTP has been bound to 1 mol eIF2 [3,4,12,13]. The measure of met-tRNA$_i$ binding as a function of GTP concentration can thus be considered an indirect measure of eIF2 affinity for GTP. Although the eIF2 affinity for

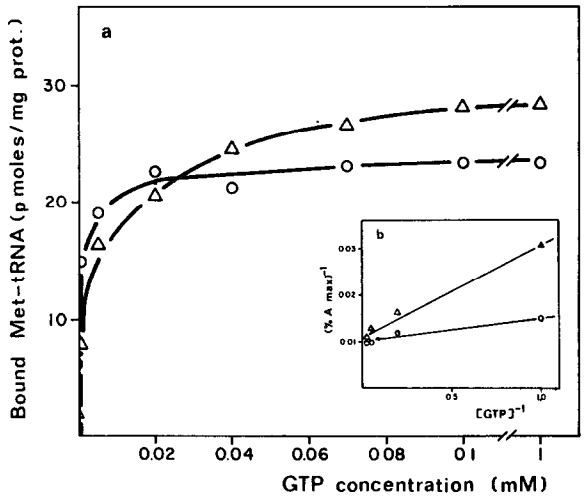


Fig.2. Ternary complex formation as a function of GTP concentration. 5 $\mu$g purified eIF2 from 4–12- (○) and 60-day-old (△) rat brain were assayed for its capacity to form ternary complexes at various GTP concentrations. (a) Saturation curves, (b) double-reciprocal plot of the saturation curves. $A_{max}$ was considered as the amount of met-tRNA$_i$ binding at 1 mM GTP and the other values were referred to this. The values represent mean of 3 experiments. $r$ values: 0.997 (○), 0.995 (△).

GTP is higher at 4–12 than at 60 days (fig.2b), at saturating GTP concentrations, the capacity of younger eIF2 to bind GTP is less than that of adult one (fig.2a), a surprising result, since the suckling crude fraction had higher eIF2 activity [1]. Up to 50% of eIF2 in active reticulocyte lysates reportedly consists of eIF2-GDP [14]. Our eIF2 preparation, isolated from the more active ribosomal fraction (suckling animals), should contain a higher eIF2-GDP/eIF2-eIF2($\alpha$P) ratio than the adult one. This different ratio can explain the differences observed on maximal GTP binding since eIF2-GDP binding is very tight [6] and only free eIF2 or eIF2($\alpha$P) would be able to form ternary complexes in the absence of GEF. In addition, the presence in suckling preparation of a higher eIF2-GDP/eIF2-eIF2($\alpha$P) ratio can also explain the lower [$^3$H]GDP/GDP exchange rate observed with exogenous GEF (HS fraction) (fig.1), since free GDP would exchange not only with eIF2-bound [$^3$H]GDP which is what we measure, but also with the endogenous eIF2-bound GDP.

In the presence of $Mg^{2+}$, the eIF2 affinity for GDP is 100-times higher than that for GTP [11] and eIF2 sensitivity to $Mg^{2+}$ inhibition may be a

Table 2

Ternary complex formation: effect of $Mg^{2+}$ and a GTP-regenerating system

| Addition | Bound met-$tRNA_i$ (pmol/mg protein) | |
|---|---|---|
| | Suckling eIF2 | Adult eIF2 |
| None | 24.5 | 30.3 |
| $+Mg^{2+}$(0.5 mM) | 5.3 (25) | 9.7 (35) |
| $+Mg^{2+}$(2.0 mM) | 2.4 (10) | 7.6 (25) |
| $+Mg^{2+}$(0.5 mM) +PEP/PK | 16.1 (66) | 14.8 (49) |

Ternary complex formation assays were performed as described in section 2, with 2.5 $\mu$g eIF2 and 80 $\mu$M GTP. PEP (3 mM) and PK (2 U/ml) were added when indicated. Values are expressed as mean of 3–5 experiments. In parentheses: percentage with respect to control values

suitable indirect measure of eIF2 affinity for GDP. The presence of a GTP-regenerating system eliminates all the GDP present and maintains a large excess of GTP (which can even displace bound GDP), and $Mg^{2+}$ inhibition can be abolished [15,17], unless the eIF2 is $\alpha$-phosphorylated [15]. As shown in table 2, the inhibition produced by $Mg^{2+}$ on ternary complex formation is more pronounced for suckling than for adult eIF2, which retains 25% of its activity even at 2 mM $Mg^{2+}$. The GTP-regenerating system produced 3- and 0.5-fold stimulation in suckling and adult eIF2, respectively. Our results show that adult eIF2 has a diminished affinity for GTP (fig.2b) and for GDP (table 2), even when a great excess of GTP is present in the reaction mixture. This behavior is reportedly characteristic of eIF2($\alpha$P) [15,16]. We may conclude, therefore, that the ratio eIF2($\alpha$P)/eIF2 is higher in 60-day-old than in suckling eIF2 preparation.

These findings indicate that rat brain protein synthesis activity is closely correlated with GEF activity in ribosomal fractions. A high ribosomal GEF content in suckling animals leads to high eIF2-GDP content in purified eIF2 preparations from these animals and the presence of a higher eIF2($\alpha$P) level is detected in partially inhibited preparations from adults, containing a lower ribosomal GEF activity. The described model, involving the study of rat brain at 2 differentiated developmental stages, which reflects 2 physiological situations but no induced modifications, should contribute to a better knowledge of the mechanisms which control protein synthesis alterations occurring in eukaryotic cells during development and/or differentiation.

## ACKNOWLEDGEMENTS

The authors thank Caroline S. Delgado for her editorial help. This work has been supported by grants from the Comisión Asesora y Técnica (CAYCYT no.1397/82), Ministerio de Educación y Ciencia, and Fondo de Investigaciones Sanitarias, Ministerio de Sanidad y Consumo, Spain.

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