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Interaction of protein synthesis initiation factor 2 from *Xenopus laevis* oocytes with GDP and GTP analogs

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The structural specificity of the purified protein synthesis initiation factor 2 (eIF-2) from *X. laevis* ovary towards analogs of GTP and GDP was studied. The relative affinity of the structural analogs was measured by their capacity to inhibit the formation of the [^{3}H]GDP·eIF-2 binary complex. The results obtained demonstrate that modifications in the ribose moiety are well tolerated by eIF-2 which binds dGTP, 2′,3′-dialdehyde GTP (oGTP) and 2′,3′-dialdehyde GDP (oGDP) and even the dinucleotide cytidylyl(5′-3′)guanosine 5′-triphosphate (pppGpC). Substitution in the polyphosphate chain by phosphorothioate groups in the β and γ positions (GDPβS or GTPγS) does not abolish the affinity for the nucleotides and the presence of an imido group between the β and γ phosphates in guanyl-5′-yl imidodiphosphate (GppNHp) still permits a weaker but significant binding. Guanine 5′-*O*-(2-fluorodiphosphate) (GDPβF) has an affinity considerably lower than GDPβS. Methylation of position 7 of the guanine (7-m GDP), however, completely eliminates the interaction of GDP with eIF-2. The analogs tested can be listed in the following order of descending affinities: GDP > GDPβS > oGDP $\geq$ GTPγS > GDPβF > pppGpC > GTP > GppNHp > oGTP $\gg$ 7-m GDP. Assays of the capacity of GTP analogs to form a ternary complex of the type met-tRNA$_i$·GTP·eIF-2 or of GDP analogs to inhibit the formation of this complex reflect, in general, the same order of relative affinities except for pppGpC, which is weaker in its capacity to form a ternary complex than GppNHp or oGTP, although it has a higher affinity than these compounds in the formation of a binary complex.

Protein synthesis initiation factor 2; G-protein; Guanine nucleotide analog; (Oocyte)

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Abbreviations: met-tRNA$_i$, initiation methionyl-tRNA; eIF-2, protein synthesis eucaryotic initiation factor 2; EF-1, protein synthesis eucaryotic elongation factor 1; EF-Tu, bacterial protein synthesis elongation factor Tu; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); oGDP, 2′,3′-dialdehyde of GDP; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); GDPβF, guanosine 5′-*O*-(2-fluorodiphosphate); pppGpC, cytidylyl(5′-3′)guanosine 5′-triphosphate; GppNHp, guanyl-5′-yl imidodiphosphate; oGTP, 2′,3′-dialdehyde of GTP; 7-m GDP, 7-methylguanosine diphosphate

1. INTRODUCTION

The binding of GDP and GTP by various protein factors (G-proteins) is a key step in regulating the macromolecular interactions that occur in important cellular processes such as protein synthesis, hormonal regulation of adenylate cyclase, light signal transduction in vision, microtubule assembly, olfaction, phosphatidylinositol turnover and possibly transformation by ras oncogenic proteins (reviews [1,2]). For this reason it has become important to compare the structural similarities of the guanine nucleotide binding proteins [3–5] and also to analyze the specificity that these proteins

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have towards the structure of the guanine nucleotides. Studies of this latter aspect have demonstrated some interesting similarities and differences in the affinities of some of these proteins toward GDP and GTP analogs [6–9]. Previous work from our laboratory has studied the nucleotide specificity of protein synthesis elongation factor 1 from eucaryotes and from procaryotes [10,11].

This communication summarizes the work carried out on the relative affinities for several guanine nucleotides of a pure preparation of eIF-2 obtained from *Xenopus laevis* oocytes. The results obtained demonstrate that the eIF-2 tolerates several modifications of the ribose moiety of GDP or GTP with only moderate changes in the relative affinity. Changes in the polyphosphate chain are also tolerated without complete loss of affinity. However, methylation of the guanine base in position 7 completely eliminates its interaction with eIF-2.

2. MATERIALS AND METHODS

2.1. *Animals*

Large *Xenopus laevis* females were purchased from the South African Snake Farm of Cape Province, RSA.

2.2. *Preparation of eIF-2 from* X. laevis *ovaries*

The purification and properties of eIF-2 from *X. laevis* ovaries will be published in detail elsewhere (Carvallo, P., García-Mateu, M., Sierra, J.M. and Allende, J.E., in preparation).

The preparation involves homogenization of 600 g wet wt ovary with a buffer containing 50 mM Hepes, pH 8.0, 30 mM benzamidine, 100 mM KCl, 7 mM β-mercaptoethanol, 0.1 mM EDTA and 10% glycerol. The homogenates are cleared by centrifugations at 5000 × *g* (10 min) and 27000 × *g* (20 min) and the supernatant fractions are centrifuged at 160000 × *g* for 2.5 h. The microsomal fraction is then extracted with a buffer containing 0.5 M KCl, 20 mM Hepes, pH 7.6, 0.1 mM EDTA, 7 mM β-mercaptoethanol and 10% glycerol and centrifuged again at 160000 × *g* for 2.5 h. This microsomal high salt wash fraction is then fractionated by $(NH_4)_2SO_4$ precipitation and by chromatography on carboxymethyl Sephadex, heparin Sepharose and DEAE-cellulose resins. This scheme yields an eIF-2 preparation that is more than 90% pure by the criterion of SDS gel electrophoresis and which has an apparent native molecular mass of approx. 160 kDa, and three different polypeptide subunits.

2.3. *Assay for the relative affinity of guanine nucleotides for eIF-2*

The relative affinity of guanine nucleotides for eIF-2 is measured by their capacity to inhibit the retention of the radioactive binary complex [^{3}H]GDP·eIF-2 on nitrocellulose filters. Approx. 1–2 μg purified factor were incubated with 1 μM [^{3}H]GDP (8000 cpm/pmol), 20 mM Hepes, pH 7.6, 150 mM KCl and the amount of the non-radioactive guanine nucleotide specified in each case in a total volume of 20 μl. Incubations were for 5 min at 30°C and were stopped by dilution with 2 ml of cold buffer A containing 20 mM Tris-HCl, pH 7.6, 100 mM KCl, 7 mM β-mercaptoethanol and 1 mM $Mg(CH_3COO)_2$. This mixture was filtered through nitrocellulose filters (0.45 μm pore diameter), the filters were washed with 3 × 5 ml of the same buffer, dried and counted.

2.4. *Assay for ternary complex formation*

The assay for the formation of [^{3}H]met-tRNA·eIF-2·GTP ternary complex was carried out as described by De Haro and Ochoa [12] with slight modifications. Approx. 1.5 μg eIF-2 were incubated with 1 pmol of [^{3}H]methionyl-tRNA (20000 cpm/pmol), 50 μM GTP, 20 mM Hepes, pH 7.6, 1 mM dithiothreitol and 150 mM KCl in 25 μl. The incubation is carried out for 5 min at 37°C and stopped by dilution with 2 ml of buffer A. The mixture is filtered through nitrocellulose membranes, washed with 15 ml of buffer A, and counted. Control values of [^{3}H]met-tRNA radioactivity retained in the absence of GTP are subtracted from the values obtained.

2.5. *Guanine nucleotides*

pppGpC was synthesized as described [13]. 7-m GDP was obtained from Dr A. Shatkin.

The 2′,3′-dialdehydes of GDP and GTP were prepared by periodate oxidation of these nucleotides, as published [14].

GDPβF was synthesized essentially as described by Haley and Yount [15] and by Eckstein et al. [7], and was a kind gift of Dr O. Monasterio.

GDPβS, GTPγS, GppNHp and all other nucleotides were purchased from Sigma.

[^{3}H]GTP, [^{3}H]GDP and [^{3}H]methionine were obtained from New England Nuclear.

2.6. *Other materials*

[^{3}H]met-tRNA was prepared as described [16], using tRNA from rat liver.

3. RESULTS

3.1. *Analogs as competitive inhibitors of [^{3}H]GDP·eIF-2 complex formation*

The relative affinities of different nucleotide analogs can be measured by their capacity to compete with GDP in the formation of the [^{3}H]GDP·eIF-2 binary complex which can be estimated by the retention of label on nitrocellulose membranes [6].

Fig.1 shows that GTP is approx. 50-fold less efficient than non-radioactive GDP in competing for the factor. The results coincide with our calculations of K_d for GDP, 7.2×10^{-8} M, and GTP, 3.8×10^{-6} M. These latter values were obtained by measuring direct binding of radioactive GDP and GTP and Scatchard plot analysis (not shown).

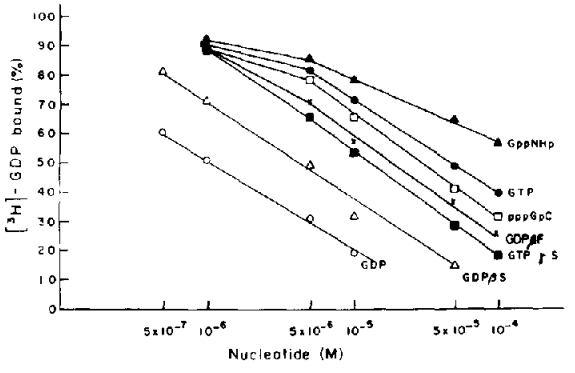

Fig.1. Relative affinities of guanine nucleotides for eIF-2 measured by their capacity to inhibit [^{3}H]GDP·eIF-2 complex formation. The formation of the [^{3}H]GDP·eIF-2 binary complex was assayed as described in section 2, using 0.6 μg eIF-2 and 1 μM [^{3}H]GDP (8000 cpm/pmol) and the non-radioactive nucleotides at the concentrations specified in the abscissa, and 1 mM $MgCl_2$. (○) GDP, (△) GDPβS, (■) GTPγS, (×) GDPβF, (□) pppGpC, (▲) GppNHp.

Comparing, in the same fig.1, the relative affinities of two GDP analogs, it can be seen that GDPβS and GDPβF have affinities that are respectively 5- and 20-fold lower than GDP. Among the GTP analogs, it is interesting to note that GTPγS and the dinucleotide pppGpC have higher affinities than GTP, while GppNHp has a considerably lower affinity than the naturally occurring triphosphate.

Using a similar method, several other analogs of GDP and GTP were tested at a single concentration (table 1). It is noteworthy that methylation in position 7 of the guanine base completely eliminates the interaction with eIF-2 as observed with 7-m GDP. On the other hand, the 2′,3′-dialdehyde of GDP or of GTP retains appreciable capacity to bind the factor. UDP and ADP have no detectable affinity while dGTP is as efficient as GTP.

3.2. *Effect of analogs on ternary complex (met-tRNA$_i$·eIF-2·GTP) formation*

The interaction of eIF-2 with GTP changes the conformation of this protein allowing it to bind selectively to the initiator methionyl-tRNA forming a ternary complex (met-tRNA$_i$·eIF-2·GTP) that subsequently interacts with the 40 S ribosomal subunit to initiate protein synthesis [17]. GDP is a

Table 1

Inhibition of [^{3}H]GDP·eIF-2 formation by different GDP and GTP analogs

Nucleotide added	[^{3}H]GDP·eIF-2 bound (pmol)	% of control
–	0.80	100
GDP (10 μM)	0.15	18
7-m GDP (10 μM)	0.73	92
oGDP (10 μM)	0.46	57
UDP (50 μM)	0.75	94
ADP (50 μM)	0.76	95
GTP (100 μM)	0.28	35
dGTP (100 μM)	0.26	33
oGTP (100 μM)	0.60	76

The assay for binary complex formation was carried out as described in section 2 using 0.6 μg of eIF-2 with 1 μM [^{3}H]GDP (8000 cpm/pmol) and the nucleotide analog specified. The radioactivity retained on the nitrocellulose filters was measured

potent inhibitor of ternary complex formation because eIF-2·GDP cannot recognize met-tRNA$_i$.

The results presented in fig.2 demonstrate the inhibitory capacity of GDP and 3 analogs in the formation of the ternary complex with the oocyte eIF-2. It can be seen that again GDPβS is somewhat less efficient than GDP, while the oGDP is a much weaker inhibitor. The 7-m GDP appears to be completely inactive also in this assay.

In all these cases, the inhibition caused by GDP and its analogs is much less than expected from their relative affinities as compared to that of GTP, as determined with the [^{3}H]GDP·eIF-2 complex assay.

On the other hand, GTP analogs were tested for their capacity to replace GTP in the formation of the ternary complex, as shown in fig.3. The capacity of GTPγS, dGTP and GppNHp seems to be very similar to that of GTP. However, in this assay oGTP and the dinucleotide pppGpC are much less effective in promoting the interaction of eIF-2 with met-tRNA$_i$.

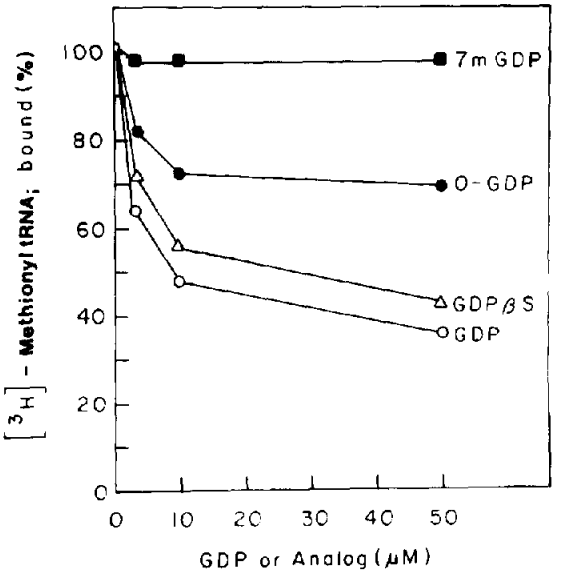

Fig.2. Inhibition of [^{3}H]met-tRNA$_i$·eIF-2·GTP ternary complex formation by GDP analogs. The formation of [^{3}H]met-tRNA$_i$·eIF-2·GTP was assayed as described in section 2, using 1.4 μg eIF-2, 1 pmol [^{3}H]met-tRNA$_i$ (20000 cpm/pmol), 50 μM GTP, and the guanine nucleotides as indicated. The amount of [^{3}H]met-tRNA$_i$ retained on the filter in the presence of only GTP (100%) was 4558 cpm.

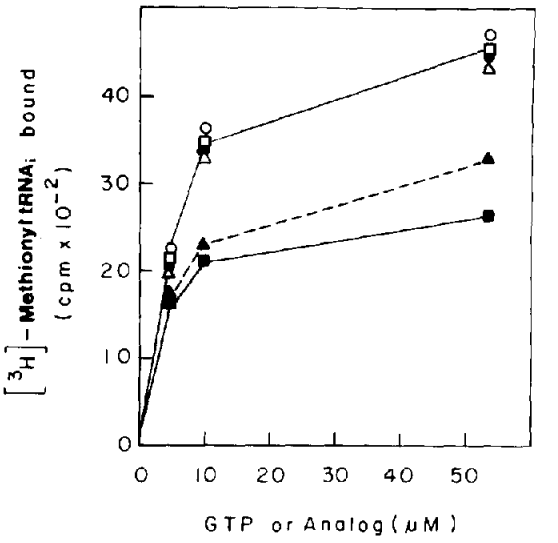

Fig.3. The capacity of different GTP analogs to form a ternary complex with eIF-2 and [^{3}H]met-tRNA$_i$. The capacity of different analogs of GTP to form a ternary complex with [^{3}H]met-tRNA$_i$ and eIF-2 was assayed as described in section 2, using 1.4 μg eIF-2 and 1 pmol [^{3}H]met-tRNA (20000 cpm/pmol) and the concentrations of the nucleotides specified in the abscissa. (●) GTP, (○) GTPγS, (□) dGTP, (△) GppNHp, (▲) oGTP, (■) pppGpC.

4. DISCUSSION

The results obtained provide some useful information about the structural specificity of oocyte eIF-2 towards guanine nucleotides.

In the first place it seems clear that the factor tolerates several modifications in the ribose ring of GDP or GTP. The affinity of dGTP is practically the same as that of GTP. Even the substitution in the 3′-hydroxyl of the ribose moiety by a nucleotide (pppGpC) does not greatly reduce the interaction with eIF-2. In this respect the initiation factor is very similar to EF-1 from wheat germ and different from *E. coli* EF-Tu [10]. It is interesting, however, that the dinucleotide which is better than the oxidized dialdehyde GTP or GppNHp in forming a binary complex with the factor is not as efficient as these compounds in promoting the formation of ternary complex. It may be that the extra nucleotide bound at the GTP site may repel, to some degree, the interaction with the met-RNA$_i$.

The fact that oGDP and oGTP still retain appreciable interaction with eIF-2 may be useful because these compounds are able to form Schiff-base adducts to neighboring ϵ-amino groups of lysines in the protein [14]. Since these bonds can be stabilized by borohydride reduction, it may be possible to affinity label the guanine nucleotide binding site of eIF-2 using these oxidized derivatives. The results with analogs having modifications in the 5'-polyphosphate chain demonstrate that the β or γ phosphorothioate derivatives have great affinity for eIF-2. The introduction of the sulfur atom in place of oxygen in the terminal phosphate is known to cause an acidification of the pK values of that phosphate [18]. In the case of transducin, this change increases the binding of GTPγS as compared to GTP [9]. A similar observation is obtained with oocyte eIF-2, however, the GDPβS is less efficient than GDP in binding. In both cases, the replacement of an imido group for the phosphodiester bond between the β and γ phosphates considerably decreases the affinity of the factors. This change is known to increase the pK of the terminal phosphate [19]. GDPβF is a poorer competitive inhibitor of [^{3}H]GDP·eIF-2 formation than GDPβS. This might be due to the loss of a negative charge of the β phosphate in the case of GDPβF, a loss that does not occur with GDPβS. It may be relevant to recall that GTPγF was practically inactive in the binding of transducin [9].

The relative capacity of the GTP analogs to support ternary complex formation or of GDP analogs to inhibit the formation of such a complex runs in parallel to their capacity to bind eIF-2 with the exception of the pppGpC already commented. However, the capacity of GDP and its analogs to inhibit ternary complex formation is much less than one would expect by merely considering their relative affinities vis-à-vis GTP in binary complex formation. It is obvious that the presence of met-tRNA$_i$ which interacts selectively with the eIF-2·GTP binary complex and not with eIF-2·GDP should drive the reaction in favor of the triphosphate and diminish the inhibition observed with GDP. This observation is interesting in considering the problem of how the cellular ratio of GTP/GDP might affect protein synthesis initiation.

Finally, it is interesting that methylation in position 7 of the guanine essentially abolishes the interaction with eIF-2 as it does with EF-1 [9]. This modification, of course, occurs in the 'cap' structure of most eucaryotic mRNAs. It has been demonstrated that initiation factor 4F and some of its subunits specifically recognize 7-m GTP and 7-m GDP and this capacity indicates that this factor plays a role in the correct initiation of translation at the AUG closest to the cap structure [20]. It is probably relevant that eIF-2 has no affinity for such analogs and therefore cannot interfere with eIF-4F function.

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REFERENCES

[1] Allende, J.E. (1982) Arch. Biol. Med. Exp. 15, 347–355.
[2] Hughes, S.M. (1983) FEBS Lett. 164, 1–8.
[3] Halliday, K.R. (1983) J. Cyclic Nucleotide Protein Phosphorylation Res. 9, 435–448.
[4] LaCour, T.F.M., Nyborg, J., Thirup, S. and Clark, B.F.C. (1985) EMBO J. 4, 2385–2388.
[5] Jurnak, F. (1985) Science 230, 32–36.
[6] Walton, G.M. and Gill, G.N. (1975) Biochim. Biophys. Acta 390, 231–245.
[7] Eckstein, F., Bruns, W. and Parmeggiani, A. (1975) Biochemistry 14, 5225–5232.
[8] Northup, J.K., Smigel, M.D. and Gilman, A.G. (1982) J. Biol. Chem. 257, 11416–11423.
[9] Yamanaka, G., Eckstein, F. and Stryer, L. (1986) Biochemistry 25, 6149–6153.
[10] Allende, J.E., Allende, C.C., Simoncsits, A. and Tomasz, J. (1975) J. Biol. Chem. 250, 2056–2061.
[11] Allende, J.E., Tarragó, A. and Shatkin, A.J. (1978) Arch. Biochem. Biophys. 187, 335–338.
[12] De Haro, C. and Ochoa, S. (1979) Proc. Natl. Acad. Sci. USA 76, 1741–1745.
[13] Simoncsits, A., Tomasz, J. and Allende, J.E. (1975) Nucleic Acids Res. 2, 257–263.
[14] Olate, J., Anker, R. and Allende, J.E. (1985) FEBS Lett. 185, 170–176.
[15] Haley, B. and Yount, R.G. (1972) Biochemistry 11, 2863–2869.

[16] Stringer, E.A., Chaudhuri, A. and Maitra, U. (1979) J. Biol. Chem. 254, 6845–6848.
[17] Moldave, K. (1985) Annu. Rev. Biochem. 54, 1109–1149.
[18] Eckstein, F. (1985) Annu. Rev. Biochem. 54, 367–402.
[19] Reynolds, M.A., Gerlt, J.A., Demou, P.C., Oppenheimer, N.J. and Kenyon, G.L. (1983) J. Am. Chem. Soc. 105, 6475–6481.
[20] Grifo, J.A., Tahara, S.M., Morgan, M.A., Shatkin, A.J. and Merrick, W.C. (1983) J. Biol. Chem. 258, 5804–5810.