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Probing the Transducin Nucleotide Binding Site with GDP Analogues

Stéphane P. Vincent,[a] Sonya Grenier,[b] Charles Mioskowski,[a] Christian Salesse[b] and Luc Lebeau[a,*]

[a]*Laboratoire de Synthèse Bioorganique associé au CNRS, Université Louis Pasteur, 74, route du Rhin, BP 24, 67401 Illkirch cedex, France*
[b]*GREIB, Département de Chimie-Biologie, Université du Québec à Trois-Rivières, Trois-Rivières, Québec, Canada G9A 5H7*

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Abstract—An affinity study between the G protein of the visual photoreceptor, transducin, and eight different non-hydrolyzable GDP analogues is described. Imidodiphosphate derivatives have been shown to exhibit good affinities to transducin. This very important heterotrimeric G protein is shown to be highly restrictive with regard to structural modifications of the nucleotide at the pyrophosphate moiety, at the 3′ position on ribose, as well as at the N^1 position of the purine. © 2001 Elsevier Science Ltd. All rights reserved.

In 1971, Martin Rodbell and his group proved the existence of G proteins for the first time by using the non-hydrolyzable GTP analogues G-PPNP and G-PPCP.[1,2] Several years later, Alfred Gilman and co-workers isolated and characterized the first G protein, opening the way to the discovery of one of the most important signal transduction pathways.[3] In a general manner, these proteins bind GDP in their inactive state, and GTP when activated. They belong to the GTPase family since they possess the intrinsic ability to hydrolyze GTP into GDP during their inactivation process. Transducin, the G protein of the visual photoreceptors, is one of the most studied heterotrimeric G proteins, and is often considered a model for other G proteins.[4] As a matter of fact, transducin was the first heterotrimeric G protein whose structure was obtained at high resolution by X-ray crystallography.[5–7] Since Rodbell's discovery, stable GTP analogues have been extensively used to study G proteins in different physiological systems.[8–13] Therefore, the interactions between G proteins and analogues of GTP modified on the γ-phosphate or at the bridging position between the β- and the γ-phosphorus atoms have been investigated to a great extent. Surprisingly, little is known about the consequences of structural modifications of GDP at the α-phosphorus as well as at the ribose or the purine moieties, and whether they are compatible or not with a tight binding to G proteins. To the best of our knowledge, only the phosphorothioate analogues GDP-βS and GDP-αS have been assayed with transducin.[13] Even though these compounds are usually considered to be useful non-hydrolyzable GDP analogues, they are not totally inert towards enzymatic hydrolysis.[14,15] Furthermore, they have been shown to be potent reducing agents at physiological pH causing possible discrepancies during biological tests involving G proteins.[16] Since finding good pyrophosphate mimics is still a challenging problem with interesting applications in fundamental biochemistry and drug design, we embarked on a program aiming to synthesize new GDP analogues and to evaluate their biological properties with respect to transducin.

Thus, we developed a new convergent approach for the preparation of stable GDP analogues based on a key Mitsunobu coupling reaction.[17,18] This strategy allowed us to synthesize compounds **1**–**5** (Fig. 1). To ensure their stability towards chemical and enzymatic hydrolysis, compounds **1**–**4** bear a methylene group instead of the central oxygen atom in the natural pyrophosphate moiety, while in compound **5** it is substituted for an imido group. The α-phosphoryl groups in **1** and **2**, and the β-phosphoryl group in **1** and **3** have been replaced by a

*Corresponding author. Tel.: +33-388-67-6857; fax: +33-388-67-8891; e-mail: lebeau@aspirine.u-strasbg.fr

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carbonyl group. The affinity for transducin of these five non-hydrolyzable GDP analogues has been measured following a procedure[19] derived from the competition experiment previously established by Yamanaka et al.[13,20,21] The IC_{50} values obtained for analogues **1**–**5** are reported in Table 1 and representative curves are shown in Figure 2.

The imidodiphosphate derivative **5** proved to be the only compound exhibiting an affinity comparable to that of the natural ligand (GDP, $K_d = 10\,\mu m$).[22,23] The replacement of any of the two phosphoryl groups in GDP by a carbonyl moiety thus prevents a tight binding of the analogues to transducin. This can be rationalized considering that the nucleotide is complexed with Mg^{2+} in the ligand binding site, which probably greatly contributes to the nucleotide–protein binding strength.

More surprising was the huge loss of affinity displayed by compound **4** compared to GDP as the affinity decreases by three orders of magnitude. Indeed, the IC_{50} value is shifted from the micromolar to the millimolar range. In that case, binding to divalent metal cations cannot be invoked since bisphosphonates are known to be good ligands for these cations.[24–28] On the other hand, the IC_{50} value for compound **5** is much closer to the GDP association constant with transducin. Such a significant difference between the affinity of **4** and **5** for transducin can be explained considering both the geometry of the molecules and their ionization state. Structurally, imidodiphosphates are much closer to pyrophosphates than are bisphosphonates, as evidenced by X-ray crystallography studies.[29] The averaged values for P–X–P dihedral angles are 129°, 127°, and 117° for P–O–P, P–N–P, and P–C–P derivatives, respectively. Consequently, the distance between Pα and Pβ is 3.05, 3.01, and 2.94 Å for pyrophosphates, imidodiphosphates, and methylene bisphosphonates, respectively. Moreover, pK_a values of the three first acidic protons of the imidodiphosphate moiety are close to those of pyrophosphates, whereas the presence of a methylene bridge between the two phosphorus atoms induces a significant increase in the pK_a values.[30–32] For all these reasons, the bisphosphonate **4** happens to be a poor GDP analogue for transducin, compared to **5**, despite their small structural differences.

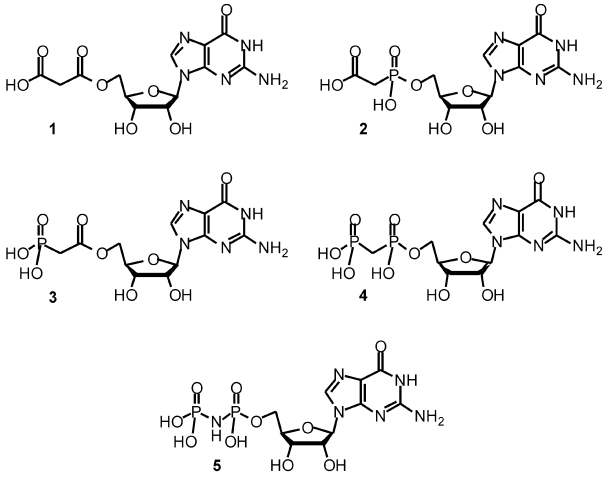

Figure 1. Non-hydrolyzable GDP analogues.

Table 1. IC_{50} values of compounds **1**–**8** for transducin

Compound	IC_{50} (μM)[a]	Compound	IC_{50} (μM)[a]
1	> 1000	**5**	12
2	> 1000	**6**	> 1000
3	> 1000	**7**	> 1000
4	> 1000	**8**	500

[a]GDP's K_d value with transducin = 10 μM.

With the imidodiphosphate **5** in hand, we could push our investigation further by probing the transducin binding site. As part of our research program, we are also interested in developing new tools for the two-dimensional (2-D) crystallization of proteins.[33] Though the structures of several G proteins have been recently determined at high resolution, the way these proteins interact with their partners during the transduction process is still intriguing. This problem could be clarified by obtaining structural information on the complexes formed between the G proteins and the receptors or the effectors involved in the transduction process. Such multimeric protein complexes have a high molecular weight and will probably remain outside the reach of current X-ray crystallography and solution NMR techniques. In order to carry out 2-D crystallization experiments with the transducin–rhodopsin complex, lipids functionalized with a stable analogue of GDP are required. However, before coupling the GDP analogue to any lipid moiety, the adequate anchoring site on the nucleotide has to be determined. Consequently, compound **5** has been functionalized at different positions and the interaction of the resulting derivatives with transducin has been investigated.

Exploiting the synthetic strategy mentioned above, we prepared several G-PNP derivatives bearing a short spacer at different positions on the nucleotide.

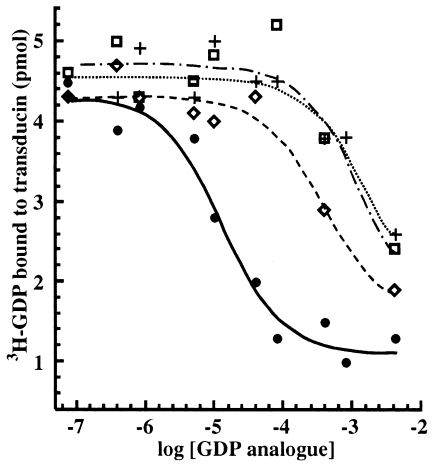

Figure 2. Representative competition experiments between GDP analogues and [^{3}H]-GDP: **5** (–●–), **4** (–·–□–·–), **8** (- -◇- -), **7** (··· + ···). To improve clarity, the curves corresponding to compounds **1**, **2**, **3**, and **6** have been omitted.

Compounds **6** and **7** are functionalized on the purine at the N^1 and O^6 positions, while compound **8** is modified at the 3′ position on the ribose moiety (Fig. 3). To our surprise, compounds **6** and **7** displayed IC_{50} values beyond the millimolar range which suggests that, in each case, the presence of the spacer prevents a crucial interaction between transducin and the imidodiphosphate derivative. Taking into account that G proteins have to discriminate, in vivo, between GDP and other nucleoside diphosphates, especially inosine and adenosine diphosphate, a tight and very specific interaction involving the N^1 position or its vicinity in the guanine moiety can be expected. The affinity measurements with compounds **6** and **7** indeed acknowledge that. It is worth noting however that Osterman et al. have described fluorescent GTP derivatives functionalized at the N^2 position that are still recognized by the small GTPases H-*Ras* and cdc 42.[34]

More surprising is the loss of affinity of compound **8** for transducin since a 3′-*O*-anthranoyl GTP derivative has been described as a potent fluorescent probe for the G protein p21 (in its activated form), another member of the *Ras* G protein subfamily.[35] However, these data cannot be directly compared to ours since activated G proteins generally bind GTP (with K_d in the nanomolar range), whereas when inactivated, they preferentially bind GDP (in the micromolar range). Nevertheless, these results likely indicate that transducin binds GDP in a different way than small G proteins. This means that, even if all G proteins share the same general biochemical characteristics regarding GDP and GTP binding properties, as well as GTPase activity, the structure of their nucleotide binding site can be significantly different. However, the 3′-hydroxyl group of the GDP ribose moiety appears to be the best position for the attachment of a lipid in view of 2-D-crystallization experiments of transducin.

In conclusion, an affinity study between transducin and eight different non-hydrolyzable GDP analogues shows that this very important heterotrimeric G protein is highly restrictive with regard to GDP structural modifications at the pyrophosphate moiety, at the 3′ position of the ribose, as well as at the N^1 position of the purine.

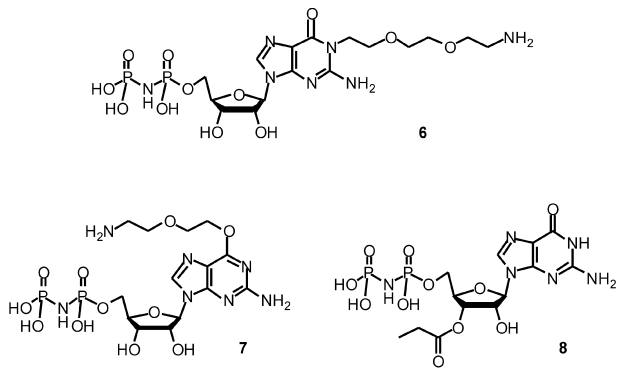

Figure 3. Functionalized non-hydrolyzable GDP analogues derived from **5**.

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