Guanine nucleotides and magnesium dependence of the association states of the subunits of transducin

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When $GTP_\gamma S$ is bound to transducin (T), the two subunits $T_\alpha \cdot GTP_\gamma S$ and $T_{\beta\gamma}$ dissociate, independently of the ionic environment. When GDP is bound, these subunits are associated as a monomeric $T_\alpha \cdot GDP–T_{\beta\gamma}$ complex of 75 kDa when the ionic environment is comparable to that of the cytoplasm, but they dissociate in the presence of 10–100 mM Mg^{2+} or Ca^{2+}. Using this property, the subunits could be separated and purified by a rapid one-step procedure on an ion-exchange column (FPLC), and their molecular masses were verified by neutron small angle scattering. The physiological relevances of the dissociating effect of Mg^{2+} are discussed.

Transducin *GTP-binding protein* *Magnesium* *Retinal rod*

1. INTRODUCTION

The visual transduction process in retinal rods involves a light triggered cascade of reactions leading to the hydrolysis of about 10^5 cGMP per s and per captured photon [1]. Photoactivated rhodopsin activates the cGMP phosphodiesterase (PDE) through the mediation of a GTP-binding protein called G protein, GTPase or transducin [2–4]. Transducin is composed of 3 subunits, $T\alpha$ (38 kDa) which contains the binding site for guanine nucleotide, $T\beta$ (35 kDa) and $T\gamma$ (6 kDa). In the resting state $T\alpha$ binds a GDP [5] and the 3 subunits form a monomeric complex $T\alpha \cdot GDP–T\beta T\gamma$ [6]. Photoexcited rhodopsin (R*) catalyses a GTP/GDP exchange on $T\alpha$ [5] and this causes the dissociation of the R*–transducin complex and the separation of $T\alpha \cdot GTP$ from the two other subunits which remain bound as a $T\beta\gamma$ unit [7]. The end of the activation cycle is achieved by the spontaneous hydrolysis of GTP to GDP on the $T\alpha$ subunit, which then reassociates to $T\beta\gamma$.

Structural and functional similarities exist between transducin and the GTP-dependent protein (G/F or Gs, and Gi) involved in the hormone sensitive adenylate cyclase system [8]. In all cases the activation by GTP or non-hydrolysable GTP analogs is accompanied by the dissociation of the two main subunits which are bound in the resting state. However, some dissociation in the GDP state has been observed under non-physiological conditions, such as alkaline pH [9] or upon elution from a long anion exchange chromatography column [4].

We report here that, in the GDP state, the binding between the two main subunits is also affected by the ionic environment, especially the presence of divalent cations (Mg^{2+} and Ca^{2+}). This led to a rapid procedure of separation and purification of transducin subunits. It also permits new speculations about the significance of the subunit dissociation upon activation, and suggests a plausible explanation of the effect of Mg^{2+} on hormone-dependent cyclase systems which are known to rely on GTP-binding proteins analogous to transducin.

Abbreviations: T, transducin; ROS, rod outer segments; $GTP\gamma S$, guanosine 5′-*O*-(3-thiotriphosphate); FPLC, fast protein liquid chromatography

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2. MATERIALS AND METHODS

Transducin from bovine ROS [6] was solubilized in the presence of either GTPγS (10 μM) or GTP (1 mM) and equilibrated with buffer A (20 mM Hepes, pH 7.0, 1 mM DTT). Ion-exchange chromatography of transducin samples was carried out with a Pharmacia FPLC system, using a Polyanion SI column (diameter 5 mm, length 5 cm). The different transducin samples were eluted from the column with a linear gradient (13.3 mM/min) from buffer A to buffer B (20 mM Hepes, pH 7.0, 1 mM DTT, 1 M NaCl or other salt at the same ionic strength, see below) at a flow rate of 0.5 ml/min. The fractions were analyzed by SDS-Laemmli gels, and protein concentrations determined by the Coomassie blue binding method [10].

Neutron small angle scattering measurements were performed on the D11 apparatus at the Laüe-Langevin Institute. Molecular mass determination was based on Guinier analysis [11].

3. RESULTS

Transducin in the 'permanently activated' state with bound GTPγS or in the 'resting' GDP state (Tα·GDP–Tβγ) was eluted by gradients of NaCl, Na_2SO_4, $MgCl_2$ or $MgSO_4$. The separation of the protein subunits under these various conditions is sketched in fig.1.

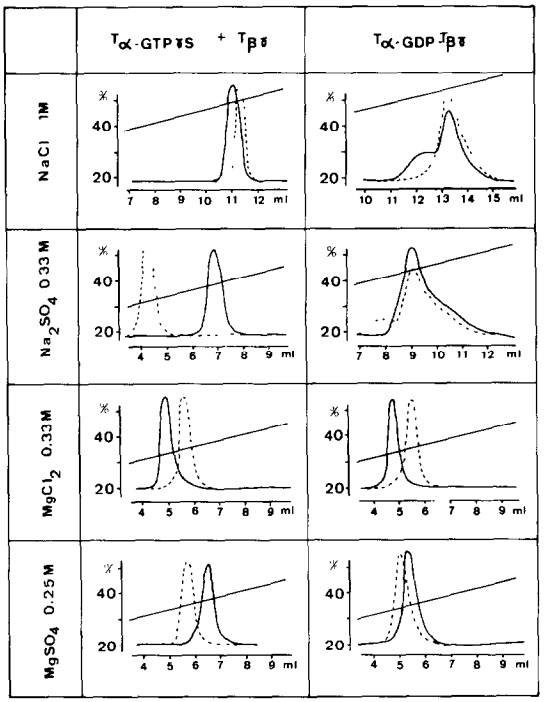

Fig.1. FPLC elution profiles of transducin as a function of the state of transducin and the nature of the elution salt. Transducin extracted either with GTPγS (left column), or with GTP, i.e., with GDP bound (right column). The elution gradient (straight line) is given as a percentage of buffer B in buffer A. The salts in buffer B are indicated on the left. The concentrations (arbitrary units) of Tα (– – –) and Tβγ (——) in the various fractions were determined by densitometry of Coomassie blue stained gels.

3.1. *Dissociation of transducin subunits in the GTPγS activated state*

When extracted from illuminated ROS in the presence of GTPγS, irrespective of the salt in buffer B, transducin always eluted in two well separated peaks corresponding to Tα·GTPγS and Tβγ. This is in accordance with the idea that, in the permanently activated state, Tα and Tβγ are predominantly dissociated.

With Cl^- as the anion, e.g., in $MgCl_2$ (fig.2a), Tβγ is eluted first, but with SO_4^{2-}, e.g., in Na_2SO_4 (fig.2b), Tα·GTPγS is eluted before Tβγ. In both cases the proteins in the first peak are quasi-pure. This property has been used to purify the subunits in two successive chromatograms. For example, with material extracted from 100 retinas, a fraction of pure Tα·GTPγS with a concentration of up to 3 mg/ml was obtained in one elution by an Na_2SO_4 gradient. The fractions corresponding to the second peak were pooled, diluted 50-fold with buffer A and loaded onto an FPLC column. Pure Tβγ at 1–2 mg/ml was eluted by an $MgCl_2$ gradient. Neutron small angle scattering measurement on the purified subunits at ~1 mg/ml in their respective elution media led to a similar molecular mass estimation of 35 ± 5 kDa for both Tα·GTPγS and Tβγ, and to radii of gyration of 24 ± and 22 ± 2 Å, respectively. This indicates that under these conditions the eluted subunits are monomeric, in agreement with [4,6], but in contrast to [9].

The ability of the purified Tα·GTPγS to activate PDE was comparable to that observed in [2].

3.2. *Dissociation of the transducin subunits in the GDP binding state*

When extracted from illuminated ROS in the

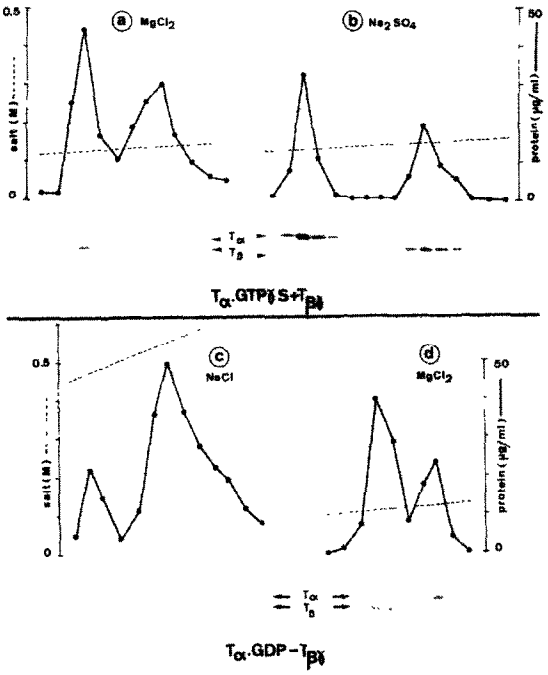

Fig.2. Analysis of the elution patterns of transducin in the GTPγS binding state (a,b) and in the GDP binding state (c,d). (——) Protein concentration in each fraction. (– – –) Salt concentration in the elution gradient. The fraction volume was 0.25 ml for a, b, d and 0.5 ml for c. Gel electrophoresis patterns in the 35 kDa region are shown below each elution profile.

presence of GTP, Tα·GTP hydrolyses its GTP, Tα·GDP reassociates with Tβγ and transducin is recovered in the GDP binding state as a holoenzyme unable to reexchange its GDP in the absence of photoexcited rhodopsin [5]. We confirmed by neutron scattering measurements that our preparation contained the monomeric holoenzyme of ~70 kDa. This again is in agreement with [4,6] but at variance with [9]. This holoenzyme has a radius of gyration of ~34 Å.

3.2.1. Elution by a gradient of Na^+ salts

When eluted by a gradient of Na^+ salt (NaCl or Na_2SO_4), the protein emerges mainly in one major peak containing both Tα·GDP and Tβγ subunits. This peak is preceded by a minor peak of pure Tβγ (if the anion in the eluting salt is Cl^-, e.g., NaCl of fig.2c), or pure Tα·GDP (with SO_4^{2-}, e.g., Na_2SO_4, see fig.1). About 10% of Tβγ (or Tα·GDP) is thus separated from the holoenzyme. We checked that this partial separation was not related to the presence of a contamination by Tα·GTP due to an incomplete hydrolysis of GTP after the extraction. The pooled fractions from the major peak, containing Tα·GDP–Tβγ, were diluted in buffer A, loaded again on the same column and eluted a second time with the same salt gradient: the same proportion of separated subunits was then observed as in the first elution. The separation thus results from an equilibrium dissociation Tα·GDP–Tβγ $\rightleftharpoons$ Tα·GDP + Tβγ. One notices that the anion dependence of the sequence of elution for the partially dissociated subunits Tα·GDP and Tβγ reproduces that observed for the dissociated subunits Tα·GTPγS and Tβγ.

3.2.2. Elution by a gradient of Mg^{2+} or Ca^{2+}

With divalent cations the elution patterns resemble that observed for transducin extracted with GTPγS. Three salts were tested: $MgCl_2$, $CaCl_2$, $MgSO_4$. As seen in fig.2d for $MgCl_2$, there are two protein peaks corresponding to the two separated subunits: there is here no secondary maximum of Tβγ associated with the maximum of Tα. However, the peaks are not as well separated as for Tα·GTPγS and Tβγ with the same salt (fig.1). With $CaCl_2$ (not shown) the same type of pattern was observed as for $MgCl_2$. With $MgSO_4$, as expected, the pattern was similar, but reversed, Tα·GDP eluting first (fig.1).

These data indicate that, at the concentration required to elute the proteins from the column, Mg^{2+} (and Ca^{2+}) induces a dissociation of the subunits. To differentiate the ionic strength effect which releases the proteins from the column from the divalent cation effect which dissociates the subunits, elutions were performed with NaCl gradients superimposed on constant levels of $MgCl_2$. It was observed (not shown) that 10 mM $MgCl_2$ is sufficient to enhance significantly the dissociation observed in NaCl, but that 100 mM was necessary to obtain complete separation.

Two other different approaches have been attempted to observe this dissociation:

(i) To check whether Tα·GDP–Tβγ dissociation by Mg^{2+} could be observed in situ, extraction experiments [6,12] were carried out on ROS fragments in the dark, using buffers containing

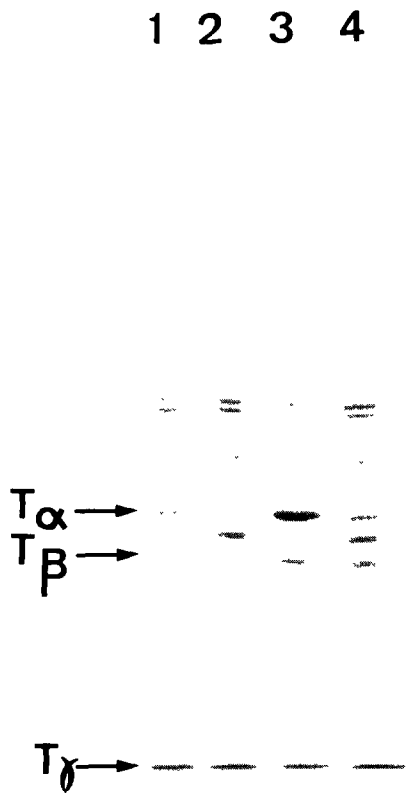

Fig.3. Partial release of Tα from ROS membranes in the dark, in the presence of high magnesium concentrations. Gel electrophoresis of supernatants of dark-adapted ROS membranes suspensions (3.5 mg/ml rhodopsin) in a medium containing 5 mM Hepes, 1 mM DTT, pH 7.0, and the following salts: lane 1, 50 mM $MgCl_2$; lane 2, 150 mM NaCl; lane 3, 150 mM $MgCl_2$; lane 4, 300 mM NaCl. Much more Tα is released with 100 mM $MgCl_2$ (lane 3) than in the other cases.

various concentrations of $MgCl_2$ or NaCl at the same ionic strength. In 150 mM NaCl (physiological ionic strength) or in 50 mM $MgCl_2$, a minimal amount of transducin is eluted from the membranes (fig.3, lanes 1,2). But with 100 mM $MgCl_2$ in the medium (fig.3, lane 3), a significant excess of Tα·GDP is extracted, as compared to the aliquot extracted with the NaCl medium at the same ionic strength (fig.3, lane 4). No variation is seen on Tβγ which remains preferentially bound to the membrane. This further confirms that in the dark, Tα is bound to the membrane through its binding to Tβγ, as shown in [7].

The dissociation, however, appears far from complete in this experiment even with 100 mM $MgCl_2$. This is not contradictory with the near total dissociation observed by elution from the anion exchange column: one cannot compare quantitatively both experiments, neither of them measuring an equilibrium dissociation. When transducin subunits progress on the column or when they are extracted by centrifugation, their physical separation from the holoenzyme pulls strongly the equilibrium towards the dissociation.

(ii) Small angle neutron scattering measurements on Tα·GDP–Tβγ at 0.3 mg/ml (~4 μM) in the presence of $MgCl_2$ confirmed that the dissociation by Mg^{2+} remains very partial at equilibrium. The addition of up to 100 mM $MgCl_2$ was found not to affect notably the apparent molecular mass (70 kDa) of the particle in solution, indicating that less than 30% of the holoenzyme was dissociated at equilibrium in this medium. One would probably have to use submicromolecular concentrations to observe a significant dissociation at equilibrium, but such low concentrations are not measurable by neutron small angle scattering.

4. DISCUSSION

A partial dissociation of transducin in the GDP binding state is observed upon the elution from an ion exchange column by a monovalent ion concentration which is higher than that of the cytoplasm. Moreover, the physiological concentration of transducin in the rods amounts to about 500 μM, certainly many orders of magnitude higher than the dissociation constant, and the enzyme in its GDP 'resting state' is therefore, in physiological conditions, the Tα·GDP–Tβγ monomeric complex.

In the presence of high concentration (10^{-2}–10^{-1} M) of divalent ions, the transducin dissociation is significantly enhanced. This may be conveniently used to separate and purify the subunits. But this effect should not be confused with the requirement of Mg^{2+} in the submillimolar range (10^{-6}–10^{-4} M) for the activation of PDE by R*, described in [1] and [13]. Using the infrared light scattering technique [14], Kühn (personal communication) has observed that in ROS membranes at moderate ionic strength (110 mM NaCl)

but in the total absence of Mg^{2+} (i.e., with EDTA), $T\alpha \cdot GDP–T\beta\gamma$ binds to R* but that a concentration of $\sim 10^{-4}$ Mg^{2+} is required for the GDP/GTP exchange and ensuing dissociation of transducin from R* to take place. Mg^{2+}-GTP would be the required substrate for the activation of $T\alpha$.

As the physiological activation of transducin, and other equivalent GTP binding proteins, always involves their dissociation, it is tempting to suggest that the artificial dissociation by high divalent ion concentrations could lead to an artificial activation. For example, artificially dissociated $T\alpha \cdot GDP$ might partially activate the PDE in the absence of GTP, or the artificial dissociation might induce or facilitate a spontaneous GDP/GTP exchange on $T\alpha$ without requiring the catalysis by R*. Similarly, an artificial dissociation of the GTP-binding protein in the hormonally controlled adenylate cyclase system could occur explaining, for example, the various effects of high magnesium concentrations observed in several tissues [15–17].

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