Characterization of the Aluminum and Beryllium Fluoride Species Which Activate Transducin

ANALYSIS OF THE BINDING AND DISSOCIATION KINETICS*

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Aluminofluoride and berylliofluoride complexes can activate the heterotrimeric G-proteins by binding next to GDP in the nucleotide site of their Go subunit and acting as analogs of the γ-phosphate of a GTP. However, the exact structures of the activatory complexes in solution as well as those of the bound complexes in the nucleotide site are still disputed. We have studied, by monitoring the activation-dependent tryptophan fluorescence of transducin Ta subunit, the pH ($\log[F^{-}]$) and pH dependencies of the kinetics of activation and deactivation of TaGDP in the presence of NaF and aluminum or beryllium salts. Comparisons were made with the calculated pH and dependencies of the distribution of the metallofluoride complexes, in order to identify the activating species. We observed that the contribution of a magnesium-dependent mechanism of activation by fluoride (Antonny, B., Bigay, J., and Chabre, M. (1990) FEBS Lett. 268, 277-280) and effects due to slow equilibration kinetics between various aluminofluoride complexes could give rise to puzzling kinetics that had caused misinterpretations of previous results. Once corrected for these effects, our results suggest that with aluminum AlF$_4^-$, the main activating species and that the bound form of the complex is tetracoordinated GDP-AlF$_3$. Deactivation kinetics depend on the free fluoride concentration in the medium, suggesting that the simple bimolecular scheme: TaGDP-AlF$_3$ ↔ TaGDP + AlF$_4^-$ (OH) does not fully describe the interaction. Fluorides in the bound complexes can also exchange with free F$^-$ ions in solution. With beryllium, two complexes are activatory: BeF$_4^-$H$_2$O and BeF$_3$(OH)$^-$H$_2$O. In the nucleotide site these give two tetracoordinated complexes, GDP·BeF$_3$ and GDP·BeF$_3$(OH), as shown by their different dissociation rates.

Following the demonstration by Sternweis and Gilman (1982) that aluminum or beryllium are necessary cofactors for the fluoride activation of G-proteins and that their ion could be the active form of the halide, Bigay et al. (1985) noticed that activation by fluoride and aluminum salts of transducin, the G-protein of retinal rod cells, required that the GDP had remained bound into the nucleotide site of the Ta

subunit. They proposed that the aluminofluoride complex binds into the nucleotide site, next to the GDP β-phosphate, and simulates the presence of a GTP γ-phosphate, thus promoting the switch of the Ta subunit to the active TaGTP conformation. Beryllium, which forms with fluoride tetrahedral complexes that are strictly isomorphous to a phosphate group, is as good a cofactor as aluminum for transducin activation (Bigay et al., 1987).

This phosphate-analog model has been extended to other guanine nucleotide binding proteins such as tubulin (Carlier et al., 1988) and even adenine nucleotide binding enzymes such as actin (Combeau and Carlier, 1988) and ATPases (Lunardi et al., 1988; Missiaen et al., 1988), in which fluoride effects also require aluminum or beryllium and depend on the presence of GDP or ADP in the nucleoside triphosphate binding site (Chabre, 1990). Chemical titration in F$_3$2 ATPase (Dupertis et al., 1989) and in tubulin (Combeau and Carlier, 1989) confirmed the binding of one metal ion per nucleoside site and of three to four fluoride anions per bound aluminum, or two to three fluorides per bound beryllium. $^{19}$F and $^{31}$P NMR spectroscopies have recently been used to characterize the bound complex in G-proteins (Higashijima et al., 1991) and suggest that three to five F$^-$ ions and a single aluminum bind to GaGDP. This binding causes an upfield shift of the fluoride as follows.

Thus the number of fluoride-bound fluorines might differ from the number of fluorines that were bound to the metal in the "activating complex" in solution. The metal-fluoride coordination in the protein nucleoside site and the exact structure of the activating species in solution are, however, still debated. Martin (1988) noticed that the calculations which had suggested AlF$_4^-$ and BeF$_3^-$ as the reactive species had not taken into account the hydroxyl groups that, in aqueous solution at neutral pH, also coordinate with aluminum or beryllium ions. A new calculation of the complexes equilibria predicted that at physiological pH, in millimolar fluoride, the ternary complex AlF$_4^-$(OH)$^-$ and neutral AlF$_3^-$ predominate over AlF$_4^-$.

Martin (1988) furthermore pointed out that AlF$_4^-$, as well as AlF$_3^-$ and all binary aluminum complexes, should be hexa-

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coordinated with water molecules occupying the free sites. By contrast \(\text{AlF}_3^-(\text{OH})\) could be tetrahedral, and thus appeared to be a better candidate for a phosphate analog.

In this study, we further identify the active fluoride complexes by analyzing the pH and pF dependence, at fixed concentrations of aluminum or beryllium, of the kinetic rates of TaGDP activation. These rates should depend only on the concentration of the “active” complex in solution. The kinetics were followed by monitoring the intrinsic tryptophan fluorescence of transducin. Higashijima et al. (1987) have demonstrated that the activation of a G-protein correlates with a large change of tryptophan fluorescence. Philips and Cerione (1988) already studied by this technique the kinetics of transducin activation by fluorides, but they might not have taken into account two phenomena that could perturb those kinetics. First, the kinetics of formation and dissociation of some of the aluminumfluoride complexes are very slow and interfere with activation kinetics: equilibria between the various aluminumfluoride complexes may not be reached within a few minutes of the mixing of the fluoride and AlCl

solutions. Indeed, Paris and Pouysségur (1986) had earlier noticed that a lag occurred in the activation of a G-protein-dependent process, whenever they injected in their samples small amounts of concentrated stock mixtures of NaF + AlCl

. They suggested that this was due to the rate-limiting dissociation of highly fluorinated inactive complexes, such as \(\text{AlF}_3^2-\) and \(\text{AlF}_4^2-\), that form in the concentrated fluoride solutions and dissociate back only slowly toward the active species when the stock solutions were diluted in the sample for use. We suspected that some puzzling biphasic kinetics reported by Philips and Cerione (1988) for the activation of transducin by the AlCl

 + NaF mixture could be due to this phenomenon. Fluoride activation kinetics are also influenced by magnesium in the millimolar concentration range. We recently suggested that, in the absence of magnesium, when the NaF concentration is above 3 mM, fluoride ions bind to transducin into the sites that normally bind the oxygens of the \(\gamma\)-phosphate and can entrap a magnesium counterion that also mimicks the phosphorus of a GTP \(\gamma\)-phosphate, thus activating transducin (Antonny et al., 1990). The magnesium is only weakly bound, but millimolar concentrations of magnesium can compete with micromolar concentrations of aluminum or beryllium.

We indeed detected in our activation kinetics perturbations that are attributable to slow aluminiumfluoride complexation and to magnesium. After eliminating or correcting for these effects, the dependence on fluoride concentration of the rates of transducin activation by aluminum suggested that \(\text{AlF}_3^2-\) is the activating species, rather than \(\text{AlF}_4^2-\). With beryllium, \(\text{BeF}_3^2-\), \(\text{BeF}_4^2-\), and possibly \(\text{BeF}_2\) contribute to transducin activation, their relative contributions depending on the fluoride concentration and the pH of the solution.

Deactivation kinetics were also studied by diluting fully activated transducin samples in media devoid of fluoride complexes. Deactivation rate analyzing provided checks on the bimolecular character of the dissociation of the metalfluoride complex from TaGDP and on the possible multiplicity of bound species.

**MATERIALS AND METHODS**

**Transducin Purification**—The Ta subunit of transducin was extracted from cattle retinal rod outer segments and purified in its GDP-bound form as previously described (Detertre et al., 1984). Briefly, illuminated rod outer segment membranes were washed in a hypotonic buffer (5 mM Tris, pH 7.5) to remove soluble proteins, the pellet is resuspended in an isotonic buffer (120 mM KCl, 0.2 mM MgCl

, 5 mM Tris, pH 7.5) and TaGDP is preferentially eluted from the membranes upon the addition of 200 \(\mu\)M GTP (Kühn, 1980). After sedimentation of the membranes the excess GTP is removed from the supernatant by elution on a Sephadex G25 column. The solubilized Ta, which has returned to the GDP-bound form due to the intrinsic GTPase activity, is purified by anion-exchange chromatography on a Pharmacia polyamion SI column, with a linear 0 to 0.66 M NaSO

 gradient. A preparation from 40 retinas yields about 2 \(\mu\)g of 2-3 \(\mu\)M solution of pure TaGDP. Storage at \(-20^\circ\)C of aliquots with 40% glycerol prevents transducin from denaturation but the viscosity of glycerol is a limiting factor for fast injection and mixing when measuring fast kinetics. Therefore most of the measurements presented here have been performed on TaGDP solutions stored at 4 °C, without glycerol in the elution buffer of the polyamion column (100 mM NaSO

, 10 mM MgSO

, 20 mM Tris, pH 7.5, 5 mM \(\beta\)-mercaptoethanol, 0.1 mM phenylmethysulfonyl fluoride) and used within 3–4 days of the purification.

**Fluorescence Measurements**—Fluorescence measurements were performed with a Shimadzu RF-5000 fluorimeter. The excitation wavelength was set at 294 nm with a bandwidth of 1.5 nm and emission wavelength at 340 nm with a bandwidth of 30 nm. NaF. The sequence order of the injections is discussed with each experiment. Injection and mixing were completed within about 1 s.

For activation kinetics measurements, the concentrations of TaGDP were first activated at saturation by incubation with suitable concentrations of NaF and AlCl

 or BeCl

. Suitable amounts of AlCl

, BeCl

, and NaF were later injected from separate stock solutions of 1 mM AlCl

, 1 mM BeCl

, and 0.5 mM NaF. The sequence order of the injections is discussed with each experiment. Deactivation rate analyzing provided checks on the bimolecular character of the dissociation of the metalfluoride complex from TaGDP and on the possible multiplicity of bound species.

**RESULTS**

Fluorescence emission spectra of TaGDP before and after full activation by 1 mM NaF + 20 \(\mu\)M AlCl

 are shown on Fig. 1A. For a recently purified TaGDP preparation the fluorescence increase was of about 75%. After several days of storage of the protein at 4 °C, the amplitude of the fluoride-dependent effect could be slightly reduced, but the kinetics of variations were always preserved. This suggested that a fraction of the protein pool had denatured and lost its ability to be activated, while the other fraction had kept all its kinetic properties.

**Dependence of Fluoride Activation Kinetics on Aluminum or Beryllium**—A kinetic recording of the fluorescence increase correlated to the activation of TaGDP by NaF + AlCl

 is shown on Fig. 1B. NaF and AlCl

 were sequentially injected in the protein solution. With this low (1 mM) fluoride concentration, TaGDP activation appears strictly dependent on the subsequent injections of AlCl

 or BeCl

. Injection of 20 \(\mu\)M AlCl

 triggers a fluorescence increase that displays a typical exponential shape. A second addition of AlCl

 does not induce any further fluorescence enhancement, indicating that activation of the protein was completed by the first
plexes. The analysis of the $pF$ and $pH$ dependencies of with beryllium salts. This confirms that a single metal ion is to allow to visualize the initial fluorescence level of TaGDP, independently from fluoride as free A1$^{3+}$ or Be$^{2+}$ ions do not exist in significant amount in these solutions. These metal cations form metastable complexes of generic form MeF$J$OH)$,$ whose equilibrium constants (Martin, 1988) and depend on fluoride and hydroxyl concentration, that is on $pF$ and $pH$. For example at $pH$ 7.5 and without fluoride, aluminum is complexed mainly as Al(OH)$^3^-$. With increasing NaF concentrations the major complex shifts, successively, through AlF(OH)$^3$, AlF$_2$(OH)$^-$, AlF$^-$, and up to AlF$_6^{3-}$ (see Fig. 4B below). Analogous patterns are observed for beryllium salts (see Fig. 6B below). The mole fraction of a given complex does not depend on the metal concentration insofar as $F^-$ is in large excess over the metal and OH$^-$ is buffered. Under the experimental conditions of Fig. 1B, that is sequential injections of NaF and AlCl$_3$ in the protein solution, the apparent rate constant of activation ($k_{A1}$) limited by the rate of formation of the activating alumino-fluoride complex, as discussed later. To avoid this problem NaF and AlCl$_3$ (or BeCl$_2$) can be first mixed to the appropriate concentrations in the buffer and incubated in the fluorescence cell to allow equilibration between the metallofluoride complexes, and then the protein is injected from a concentrated stock solution (Fig. 2A). This technique has the drawback not to allow to visualize the initial fluorescence level of TaGDP, nor to check on the effect of low concentrations of NaF alone. But, using this method, with a NaF concentration of 1 mM a strict linearity of the apparent rate constant of activation with respect to aluminum concentration was observed, up to 15 $\mu$M AlCl$_3$ (data not shown). Similar data were observed with beryllium salts. This confirms that a single metal ion is involved in the activation and suggests a bimolecular mechanism between TaGDP and one or several AlF$_2$(OH)$^-$,AlF$^-$, complexes. The analysis of the $pF$ and $pH$ dependencies of TaGDP activation kinetics should then allow to identify the complexes that activate transducin.

**Activation by Magnesium at High NaF Concentrations in the Absence of Al$^{3+}$ or Be$^{2+}$**—We wanted to study the activation rates of TaGDP by fluoride in the concentration range where the presumed activating species, AlF$^-$, AlF$_3$, and AlF$_5$(OH)$^-$ (or BeF$^-$, BeF$_3$, BeF$_5$(OH)$^-$) predominate. Measurements at high fluoride concentrations were hence required. But when the NaF concentration reached 5 mM ($pF$ 2.3), the fluorescence started to increase already before the beryllium (or aluminum) salt were added (Fig. 2). The addition of beryllium (or aluminum not shown) still accelerated the rate of increase confirming that alumino- and berylliofluoride complexes were still efficient. This Al$^-$ and Be$^+$ dependent fluoride effect had already been observed by Higashijima et al. (1987), and we recently demonstrated (Antonny et al., 1990) that it is due to the presence of magnesium, at millimolar concentrations, in the buffer. But magnesium can hardly be suppressed from the buffer as it is required for the functional structure of the G-protein (Higashijima et al., 1987). The contribution of this magnesium-dependent mechanism must therefore be subtracted before one estimates the contributions of the alumino- and berylliofluoride complexes to the activation rates at high fluoride concentrations. The apparent rate constant $k_{MF}$ that corresponds to the Mg$^+$ - $F^-$ -mediated mechanism are obtained from the initial rate of TaGDP activation, at a given $pF$, in absence of aluminum or beryllium (Fig. 2). The initial rate in presence of aluminum or beryllium gives $k_{MF} + k_{A1}$ for Al and $k_{MF} + k_{Be}$ for Be where $k_{MF}$ and $k_{MF}$ are the
apparent kinetic constant for the specific binding of one fluorometallic complex (Fig. 2).

Interference of Slow Complexation Kinetics of Al$^{3+}$, F$^-$, and OH$^-$ with the Rate of Transducin Activation by the Complexes—To our knowledge, no accurate data were available on the rates of equilibration between aluminofluoride or beryllofluoride complexes. In the case of aluminum the hydrated Al$^{3+}$ ion is remarkable by the slow exchange of water molecules in its inner sphere ($\tau_0 \approx 10^9 \text{s}^{-1}$), and this feature could extend to many ligands (Martin, 1986). Thus two processes might interfere with our activation kinetics measurements: the slow formation of the active aluminofluoride complex upon the separate injections of AlCl$_3$ and NaF into the protein solution, or a slow dissociation toward the active form of highly fluorinated and inactive complexes that might have formed in a premixed concentrated solution of NaF + AlCl$_3$ before its dilution to the appropriate pF into the protein solution.

We have looked for the possible interference of complexation kinetics by comparing the transducin activation rates observed when adding separately NaF and AlCl$_3$ to the protein solution, to that observed when injecting a small amount of concentrated protein into a solution into which both NaF and AlCl$_3$ had been diluted to the same final concentrations of 1 mM NaF and 40 $\mu$M AlCl$_3$, and had been allowed to equilibrate for 24 h at the final concentration. This time was presumed sufficient to allow full equilibration between all complexes. No kinetic difference was detectable between the two types of experiments. Thus the rate of formation of the active complex, starting from NaF and AlCl$_3$ solutions, does not kinetically limit transducin activation.

By contrast, significant slowing down of the transducin activation kinetics was observed when the activating concentrations of 1 mM fluoride and 40 $\mu$M aluminum were obtained by adding in the transducin solution a small amount of a premixed concentrated solution of 250 mM NaF + 10 mM AlCl$_3$ (Fig. 3A). The effect clearly depended on the "age" of the concentrated mixture, that is the time during which the aluminum salt had been in contact with a high concentration of fluoride. Not apparent when the contact time was in the range of seconds, the slowdown of transducin activation kinetics became noticeable if the aluminum had been in contact with the high fluoride concentration for 0.5 h before the mixture was diluted down in the protein solution. After a 24-h incubation of the concentrated AlCl$_3$ + NaF mixture, the transducin activation kinetics became extremely slow. A similar experiment was performed with a 250 mM NaF + 0.5 mM AlCl$_3$ mixture that was diluted down to 5 mM NaF and 10 $\mu$M AlCl$_3$ in the TaGDP solution (Fig. 3B). For short incubation times, the activation kinetics looked similar to that observed previously. But after 24 h of preincubation a fast but partial component subsisted in the kinetics, giving a clearly biphasic pattern. This was due to the final concentration of 5 mM fluoride which was sufficient to allow some magnesium-dependent activation: this fast but partial component of activation is not affected by the incubation of the concentrated mixture of NaF + AlCl$_3$ since magnesium does not form long-lived complexes with fluoride. The incubation time effect is hence specific to aluminofluoride complexes and only affects the aluminum-dependent part of the activation kinetics.

These experiments demonstrate that in concentrated fluoride solution highly fluorinated and inactive species form, that lose slowly their excess fluoride to get back to the less fluorinated "active" complexes when fluoride concentration is reduced down to the millimolar range. The influence on the activation kinetics of the duration of incubation of AlCl$_3$ with NaF at high concentration, before the mixture is diluted down into the protein solution, demonstrates that the rate of formation of the highly fluorinated inactive species is in the range of hours.

In another series of experiments we studied the dependence of the recovery of fast transducin activation kinetics on the duration of a delay introduced between the dilution of the concentrated salts mixture and its contact with the protein. An "aged" (more than 24 h old) concentrated AlCl$_3$ + NaF mixture was diluted down to 5 mM NaF concentration for various lengths of time before the protein was added, from a small volume of concentrated solution (Fig. 3C). When the dilution preceded the protein injection by less than 1 min, the activation kinetics was as slow as that observed by injecting directly the required small amount of the concentrated salts mixture in the diluted protein solution. Even with a 30-min incubation between the dilution of the salt mixture and the protein injection, the kinetics remained significantly slowed down. Incubation times of the order of 24 h were needed to recover the fast activation kinetics that correspond to the full re-equilibration of the aluminofluoride complexes. This suggests that the highly fluorinated, inactive complexes that form slowly in the concentrated fluoride solutions reverse also slowly toward the less fluorinated, active species that predominate at equilibrium in 5 mM fluoride.

Similar experiments were performed with concentrated solutions of BeCl$_2$ and NaF, but no effect of incubation on TaGDP activation kinetics were observed. This suggests that berylliofluoride complexes are in fast equilibrium and that
their formation and dissociation cannot rate-limit TaGDP activation.

Identification of AlF$_4$OH$^{-}$ as the Complex Activating TaGDP—From the experiments presented above, it was obvious that an identification of the activating aluminofluoride complex through the analysis of the pH dependence of the rate of activation by AlCl$_3$ requires (i) solutions with equilibrated aluminofluoride complexes, (ii) substraction of the contribution of the Mg$^{2+}$/F$^{-}$ mechanism.

Appropriate amounts of NaF and 10 μM Al were added to TKM buffer from two separate solutions of NaF and AlCl$_3$ (0.5 M and 1 mM, respectively). To allow equilibration of the fluoride complexes, these Al$^{3+}$/F$^{-}$ solutions were prepared 24 h before use. The activation rates were deduced from the initial slopes of the fluorescence increase observed after injection of 50 nM TaGDP in these equilibrated solutions, which gives the sum of the two rate constants $k_{sp} + k_{sr}$. Activation kinetics measured in the absence of aluminum (Fig. 2) give $k_{SR}$ which is subtracted and $k_{sp}$ is then plotted as a function of pH (Fig. 4A). With 10 μM AlCl$_3$, activation became detectable for NaF concentrations above 0.3 mM (pH 3.5), it peaked for 5 mM NaF (pH 2.25) and then decreased down to zero for NaF concentrations above 15 mM, if the contribution of magnesium was properly subtracted. This dose-response curve coincides with the calculated distribution curves of the two trifluorinated species: neutral AlF$_3$ and AlF$_3$(OH)$^{-}$, rather than with the curve calculated for AlF$_4$-, which peaks at a lower pH. This was the first indication that AlF$_4$- might not be the main activating complex.

As we knew that the very slow kinetics of formation of the highly fluorinated species could distort the activation kinetics, we wanted to check whether such effects could affect the identification of the activating species. We thus studied the apparent pH dependence of TaGDP activation by deliberately nonequilibrated NaF + AlCl$_3$ mixtures. The AlCl$_3$ concentration was still fixed at 10 μM, but this salt was now added to the NaF-containing buffer solution 10 s before the protein.

The results are shown on Fig. 4A (closed circles). Below 10 mM NaF (pH > 2) the kinetics of activation are comparable to that obtained in the experiments were the aluminum and fluoride ions had been in contact for 24 h before the addition of transducin. This confirmed that the formation of the trifluorinated complex from NaF + AlCl$_3$ is nearly instantaneous and does not substantially rate limit the activation process. By contrast, for pH < 2, the rate observed with the nonequilibrated solutions increases instead of decreasing, with an apparent maximum at pH 1.7 which coincides with the maximum for AlF$_4$- in the calculated distribution curves. According to the equilibrium constants AlF$_3^{-}$ and AlF$_3$(OH)$^{-}$ are the majority species for pH < 2. But if these complexes form slowly while the lower fluorinated species form rapidly, then AlF$_3$ and AlF$_3$(OH)$^{-}$ will first accumulate and may momentarily become predominant over AlF$_3$ and AlF$_3$(OH)$^{-}$ in the pH range where these high fluorinated complexes will prevail when equilibrium will be reached. This explains the shift of the peak of TaGDP activation toward the high fluoride concentrations when one uses nonequilibrated NaF/AlCl$_3$ solutions. We tried to estimate the time required for full equilibration between the complexes when the fluoride concentration exceeds 10 mM. AlCl$_3$ (10 μM final) and NaF (17.5 mM final, pH 1.75) were diluted in TKM buffer from separate solutions of NaF (0.5 M) and AlCl$_3$ (1 mM). Transducin was added at various times after the mixing and its activation was monitored. No significant change in the fast activation rate were detectable for delays up to 1 h. After a 3-h delay the activation rate became significantly lower, and after a 24-h delay it was nearly similar to that obtained in the absence of AlCl$_3$, which is due entirely to the contribution of the Mg$^{2+}$/F$^{-}$ mechanism. Thus the temporary accumulation process last for hours and may well lead to misidentification of the activating species.

Calculations of the equilibria between the various AlF$_3$(OH)$_2$ complexes, using the constants given by Martin (1988), predict that at pH 3 (1 mM NaF) the possible activating species AlF$_4$OH$^{-}$, AlF$_3$ and AlF$_4$- should differ considerably in their sensitivity to pH (Fig. 4C). The dependence on pH of the transducin activation rate in 1 mM NaF and 10 μM AlCl$_3$
show a maximum at pH 7.2 and resembles the pH-dependent pattern calculated for AlF(OH). One cannot exclude that an effect of pH on the protein itself might reduce the binding and activation by the aluminofluoride species that are favored at the lower pH, but the bimodal pattern clearly favors AlF(OH) over AlF and AlF for the activator of TaGDP.

Identification of the Activating Berylliofluoride Complexes from the Activation Kinetics—We identified the active berylliofluoride complexes using the same method as for the aluminofluoride complexes, that is through the analyses of the pF and pH dependencies of the rate of transducin activation by BeCl. By contrast with the case of aluminofluoride complexes, no effect related to slow kinetics of beryllofluoride complexes formation of dissociation were ever detected.

At the lower fluoride concentrations (pF > 2.5), the magnesium-dependent activation is negligible. If BeF was the only beryllium species that activated TaGDP, the kinetics should follow a simple exponential as for an apparent first order mechanism. Surprisingly, the fluorescence enhancement was biphasic (Fig. 5). A fast phase of limited amplitude was followed by a slower enhancement which goes to saturation. This suggested the presence of two activating species in solution. One species would bind rapidly to TaGDP either because of its high kinetic binding constant (k) or because of its high mole fraction in this pF range, but the nontotal activation by this fast process suggests also a high dissociation constant (k). The second species would have a much lower dissociation constant and could saturate the protein despite its slow rate of binding in this pF range. Then the first phase of fluorescence enhancement would correspond to the concurrent binding of the two complexes, and the second phase would correspond to slower but higher affinity binding of the second complex. The kinetics were analyzed with this two-component model (Fig. 5), and the apparent kinetic constants (k) of each phase were estimated and plotted as a function of pF (Fig. 6A).

At the higher fluoride concentrations (pF < 2.5), the apparent activation rates had to be corrected for the contribution of the magnesium-dependent activation, measured at the same pF on aliquots deprived of BeCl. Once this correction was made, the kinetics looked monophasic up to saturation. The activation rates promoted by 10 μM BeCl and corrected for the magnesium-dependent activation are shown in Fig. 6A. The rate peaks around pF 2.25, where the calculated distribution curve for BeF reaches its maximum. This suggested that BeF was the main beryllium complex activating TaGDP.

Fig. 5. Fluorescence evolution of TaGDP upon its dilution (50 nM final) in a fluorescence cuvette containing TKM buffer with NaF 0.56 mM (pF 3.25) and BeCl 10 μM. The biphasic shape of the fluorescence enhancement suggests the binding of two berylliofluoride species. The apparent activation constant of the high affinity species: k (pF) = 1/τ1 - 1/τ2. As we could not resolve the contributions of each species, the two constants were set equal with error bars extending to their extreme possible values. At other pF the error bars correspond to the variations observed over two series of experiments. B, theoretical distribution of the main berylliofluoride complexes at pH 7.5. C, the kinetics of TaGDP activation by BeCl, 10 μM and NaF 0.56 mM (pF 3.25), was measured in a buffer containing 120 mM KCl, 2 mM MgCl, 1 mM DTT, and 20 mM HEPES and adjusted at various pHs. The two apparent activation constants k (pF) and k (pF) are plotted as a function of pH and compared to the theoretical distributions of BeF, BeF(OH), and BeF.

The apparent rate constant of the fast and partial phase peaks around pF 3, which suggests that it is due to another "active" beryllium complex. The calculated distribution curves for BeF(OH) and for neutral BeF both peak in this pF range. As for the aluminofluoride complexes, the pH dependence of the equilibria between the various BeF complexes was used to discriminate between the possible activating complexes; in a beryllium salt solution at pF 3.25, when going from pH 7 to 8, the calculated proportions of BeF and BeF complexes both diminish about 3-fold, while by contrast the proportion of BeF increases 3-fold (Fig. 6C). We analyzed the variations of the two components of activation by BeCl at pF 3.25 as a function of pH. This confirmed the assignment to BeF of the second component and suggested that the first component is due to BeF(OH) rather than to BeF (Fig. 6C).

Fig. 6. Dependencies on pF (A and B) and pH (C) of transducin activation by 10 μM BeCl. A, the same method was used as for AlF(OH) identification (see Fig. 4A). At low NaF concentrations (pF > 3) where a biphasic kinetics is observed (see Fig. 5) the two apparent activation constants are plotted: k (pF) (○) and k (pF) (□). C, At pH 2.75 the kinetics looks monophasic and hence gives only one apparent activation constant (18 × 10 s). As we could not resolve the contributions of each species, the two constants were set equal with error bars extending to their extreme possible values. At other pF the error bars correspond to the variations observed over two series of experiments. B, theoretical distribution of the main berylliofluoride complexes at pH 7.5. C, the kinetics of TaGDP activation by BeCl, 10 μM and NaF 0.56 mM (pF 3.25), was measured in a buffer containing 120 mM KCl, 2 mM MgCl, 1 mM DTT, and 20 mM HEPES and adjusted at various pHs. The two apparent activation constants k (pF) (○) and k (pF) (□) are plotted as a function of pH and compared to the theoretical distributions of BeF, BeF(OH), and BeF.
Further Identification of the Activating Complexes from Deactivation Kinetics—Activation kinetics suggested that two different beryllium-fluoride species can activate TaGDP: BeF$_3^-$ and BeF$_2$(OH)$^-$. If the bound forms of these two complexes in the activated protein are also different, one expects the deactivation kinetics to be bimodal. We measured the apparent deactivation rate of concentrated TaGDP samples that had been previously activated at saturation with 40 $\mu$m BeCl$_2$ and various concentrations of NaF, and were diluted in a buffer devoid of NaF. The residual NaF concentration after the dilution was enough low to allow complete deactivation. Two deactivation rates were observed. The fast process was predominant when the protein had been activated at high pH, and the slow one when it had been activated at low pH. After an activation achieved with BeCl$_2$ at pH 3.25, pH 7.5, the fast deactivation phase was not complete but followed by the slow one (Fig. 7). This implied that two forms of bound complex can exist in the activated protein, in agreement with our above suggestion that two different forms of free beryllium-fluoride complex can activate transducin. At low pH, activation is promoted mainly by the binding of BeF$_2$ which has a low $k_{off}$ rate ($2.5 \times 10^{-3}$ s$^{-1}$) and at high pH this binding competes with that of BeF$_2$(OH)$^-$, which has a much higher $k_{off}$ rates ($8 \times 10^{-3}$ s$^{-1}$), hence its lower affinity.

For transducin that had been activated by aluminofluoride (10 $\mu$m AlCl$_3$ and 3 mM NaF), and then diluted in a NaF-free TKM buffer, the deactivation kinetic constant was about $10^{-3}$ s$^{-1}$ (Antonny et al., 1990). A similar value has been measured for Gs, (Higashijima et al., 1987) under similar conditions. The deactivation rate did not depend on pH during activation, confirming the suggestion that only one aluminofluoride species activates TaGDP.

Dependence of Deactivation Kinetics of Aluminofluoride-activated Transducin on Free Fluoride Concentration—We had assumed up to now that deactivation was a simple monomolecular mechanism and corresponded to the exit of the whole complex from the $\gamma$-phosphate site, as if the complexes would be undissociable. But we know that in solution, the metal-bound fluorides of the complexes continuously exchange with free fluorides: that is the way the complex form and equilibrate depending on pH. The fluorides of a protein-bound complex, which are hydrogen-bonded to the protein and ionically bonded to the metal, might still be exchangeable with external fluorides. Deactivation might then also be induced by the dissociation of the bound complex in the protein site.

To test this hypothesis we have compared TaGDP-AlF$_3^-$ deactivation kinetics upon two conditions under which AlF$_3^-(OH)^-$ concentration was reduced equivalently in the milieu, but residual free fluoride concentration differed. One way was to chelate aluminum, which suppressed essentially all aluminofluoride complexes while free fluoride concentration was maintained in the millimolar range; the other way was to dilute fluoride, which also suppresses the aluminofluoride complexes that convert to Al(OH)$_3$. EDTA was used as a chelant because of its extremely high affinity for aluminum ($K_c = 10^{30-38}$ M$^{-1}$ at pH 7.5). However, in order to maintain the free Mg$^{2+}$ concentration the same amount of MgCl$_2$ must be added to the buffer. We have checked that EDTA chelation removed within seconds all the activating aluminofluoride complexes from the solution: if 10 mM EDTA-MgCl$_2$ had been added to a standard activating solution (1 mM NaF and 10 $\mu$m AlCl$_3$ in TKM buffer) 10 s before the injection of TaGDP, no activation occurred. That this quenching was only due to the removal of the aluminofluoride complexes could be checked by the further addition of 10 $\mu$m BeCl$_2$, which has a low affinity for EDTA, and activated transducin (data not shown). Fig. 8 shows the evolutions of two aliquots of a TaGDP solution previously activated with 40 $\mu$m AlCl$_3$ and 3 mM NaF, upon their dilution (50 times) in TKM buffer containing either 1 mM NaF and 10 mM EDTA-Mg or 10 $\mu$m AlCl$_3$ but no NaF. The deactivation kinetics upon the dilution of fluoride in the presence of AlCl$_3$ is faster than that observed when aluminum is chelated and fluoride is maintained. The apparent kinetic constants are 8.10$^{-4}$ s$^{-1}$ and 3.10$^{-4}$ s$^{-1}$, respectively.

**DISCUSSION**

We have analyzed the kinetics of activation and deactivation of the G-protein transducin by fluoride complexes of aluminum or beryllium, in order to identify the activating complexes and characterize their bound form in the nucleotide site of transducin. But the complexes that form spontaneously upon mixing NaF and AlCl$_3$ or BeCl$_2$ are not stable entities in an aqueous solution. Our data first indicated that slow exchange of F$^-$ or OH$^-$ ions between the various possible AlF$_3$(OH)$_2$ complexes could defer equilibration over long times, depending on the previous "history" of the mixed solutions of NaF+ AlCl$_3$. This may lead to wrong estimates of the actual distribution of the various complexes at the time of the activity measurement, hence to misidentification of the activating form of the complex. Activation kinetics are also perturbed by the presence in the buffer of magnesium, since this metal ion, although it does not form complexes with fluoride anions in solution, associates to bound fluoride in the
For the aluminofluoride complexes, once the effects of these two "parasitic" phenomena have been eliminated or corrected for, the dependence on fluoride concentration and on pH of the transducin activation rates in the presence of AlCl₃, when compared to the calculations of equilibria between the multiple forms of AlF₄(OH)₃ complex (Martin, 1988), suggested that AlF₄(OH)⁻ is, rather than AlF₃⁺, the active species in solution. Deactivation kinetics seemed independent of the pH and pF at which activation had taken place. This suggested that there is a single form of bound complex but did not necessarily imply that there was a single form of free activating complex: the bound form of the complex might be -P-O-AlF₄⁻ for an activation by AlF₄(OH)⁻ as well as for an activation by AlF₃⁺. The original model proposed by Bigay et al. (1985) for AlF₃⁺ may still be preserved by suggesting now that a condensation reaction occurs between the β-phosphate of GDP and AlF₄(OH)⁻ with the elimination of a water molecule, rather than with AlF₃⁺ that would lose one fluoride. Why should AlF₄(OH)⁻ be more reactive than AlF₃⁺? This may be due to its tetrahedral geometry in solution, which makes it a better candidate phosphate analog than AlF₃⁺ now presumed to be octahedral in aqueous solution (Martin, 1988). One may also conjecture that the hydroxyl in AlF₄(OH)⁻ exchanges faster than any of the fluorides in AlF₃⁺. In any case one must notice that the rate constant for the activation by AlF₄(OH)⁻ remains very low: from the apparent activation constant and the molar concentration of AlF₄(OH)⁻ at pH 7.2 (Fig. 4) one gets kₐ = 6 × 10⁷ M⁻¹ s⁻¹, which is 10⁸ to 10⁹ slower than for a diffusion-controlled process. This must be related to the fact that the reaction is not a simple binding of the "active" complex, but involves a condensation between the hydroxyl of the incoming AlF₄(OH)⁻ and the terminal oxygen of the GDP β-phosphate. This reaction may require the occurrence of a highly improbable transient state of the tetrahedral "active" AlF₄(OH)⁻ species, like tetrahedral AlF₄⁻.

A bimolecular reaction scheme: TaGDP + AlF₄(OH)⁻ » TaGDP-AlF₄⁻ + H₂O, in which no individual F⁻ ion would bind or be released, infers that the deactivation kinetics should depend only on the concentration of free aluminofluoride complexes, not on the free fluoride concentration. Our observation that the suppression of free fluoride from a solution depleted of aluminofluoride complexes further increases the speed of transducin deactivation sets a limit on the validity of this simple scheme and suggests that fluorides from the bound complex in the activated protein exchange at a significant rate with the free fluoride anions in solution. When the solution is depleted of NaF, the released F⁻ cannot be replaced; this induces a faster dissociation of the bound complex and deactivation of the protein.

With beryllium, no effect due to slow equilibration between different complexes in aqueous fluoride solution were detected. The pH and pF dependacies of activation kinetics suggest that two different forms of free complex, BeF₂⁻ and BeF₂(OH)⁻ are both activatory, the former being predominant at low pF, the later at higher pF. The activation rates are of the same order of magnitude as for the aluminofluoride complex: kₐ = 4.10⁹ M⁻¹ s⁻¹ for BeF₂⁻ and kₐ = 10⁹ M⁻¹ s⁻¹ for BeF₂(OH)⁻. The duality of deactivation kinetics further indicates that the bound form of the two complexes are also different. All beryllium complexes keep a tetrahedral coordination geometry, whatever the number of coordinants. In aqueous solution, the fourth coordination of BeF₂⁻ is occupied by a polarized water molecule which is most probably released and replaced by a direct bonding to the β-phosphate oxygen of the GDP when the complex binds into the nucleotide site of transducin. BeF₂(OH)⁻ fits in the γ-phosphate site with the same bonding geometry as for BeF₂⁻, but with the hydroxyl group replacing one of the three bonding fluorines: this might be the cause of the weaker bonding strength, implied by the high deactivation rate of transducin activated at high pF, where BeF₂(OH)⁻ predominates.

Our conclusions for the bound forms of beryllium complexes in transducin agree with that of Combeau and Carlier (1989) for F-actin and tubulin, and by Dupuis et al. (1989) for mitochondrial F₁-ATPase. In the nucleotide sites of these proteins the berylliofluoride complexes bind quasi irreversibly, and the bound fluoride and beryllium were measured by extraction and chemical assay. Stoichiometries of two to three bound fluorides per bound beryllium were obtained. The higher values were observed at low pF and low pH, in full agreement with our proposal that this favors the binding of BeF₂⁻ whereas high pF and high pH favor the binding of BeF₂(OH)⁻. Our data seem to exclude that the biferuorinated complex would be neutral BeF₂. Thus all berylliofluoride complexes appear to be tetracoordinated into the protein γ-phosphate site, either through three fluorides or through two fluorides and one hydroxyl, the fourth coordination being always with the β-phosphate oxygen of the GDP (Fig. 9).

As for the bound aluminofluoride complexes, a stoichiometry of four bound fluorines per bound aluminum has been measured in F-actin and tubulin (Combeau and Carlier 1989) and in mitochondrial F₁-ATPase (Dupuis et al., 1989). To accommodate four fluorines, they must assume either a pentacoordinated state of the aluminum, equivalent to the bipyramidal "transit state" of a γ-phosphate undergoing hydrolysis, or keep a tetracoordinated aluminum, that is therefore not bonded to the nucleotide β-phosphate and is equivalent to a "bound phosphate" already dissociated from the bound GDP or ADP. Both hypothesis are plausible since aluminofluoride complexes inhibit and block these proteins in states which are not fully characterized. This is, however, at odds with our suggestion that for transducin the main form of activating complex is AlF₄(OH)⁻. But in transducin, and other G-proteins, the active state reached with aluminofluoride complexes appears to be octahedral in aqueous solution (Martin, 1988).

\[
\begin{align*}
\text{GDP} + \text{AlF}_3(OH)^- & \rightarrow \text{GDP-AlF}_3^{-} + \text{H}_2\text{O} \\
\text{BeF}_2^{-} + \text{H}_2\text{O} & \rightarrow \text{BeF}_2(\text{OH})^{-} + 2\text{H}^+
\end{align*}
\]

**Fig. 9.** The activating aluminio- and berylliofluoride species in solution and their presumed coordination state in the nucleotide site of activated transducin. Our kinetic evidences favor AlF₄(OH)⁻ as the activating species -Pβ-O-AlF₄⁻ as the bound form, but the neutral charge of bound AlF₃ contrasts with the negative charge of the other bound complexes. The aluminofluoride complexes bind mainly by a bimolecular mechanism, but the sensitivity of deactivation kinetics to the free fluoride concentration demonstrates that the fluoride of the bound complexes is still exchangeable with free fluorides with a faster rate than for the release of the bound complex. With beryllium, BeF₂⁻, H₂O and BeF₂(OH)⁻ - H₂O are activatory, and two types of bound complexes can be formed. Their kₐ rates have only been measured in the absence of external free fluoride and thus include a possible contribution of the release of fluoride from the bound complexes.
ide complexes and GDP is equivalent to the permanently active state obtained with GTPγS: this is most probably not a bipyramidal transition state and certainly not a “GDP + bound P” state. We therefore maintain the hypothesis that in transducin the main activatory aluminofluoride complex is AlF₃(OH)⁻ and the bound form is P₇-O-AlF₃. We are, however, conscious of the problem that the electrical charge of bound complexes such as P₇-O-BeF₃ or P₇-O-BeF₂(OH) are different of that of P₇-O-AlF₃. A fourth F⁻ in the nucleotide site might be needed to compensate for the excess charge of the aluminium ion over that of a beryllium. By fluorine NMR, Higashijima et al. (1991) could only obtain an estimate of three to five bound fluorines per transducin, which does not allow to discriminate between the various models.

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