Equilibrium and Kinetic Study of the Conformational Transition toward the Active State of p21Ha-ras, Induced by the Binding of BeF$_3^-$ to the GDP-bound State, in the Absence of GTPase-activating Proteins*

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Hitherto ras-related GTP-binding proteins have been considered not to bind phosphate analogs (Kahn, R. A. (1991) J. Biol. Chem. 266, 15595–15597), at least in the absence of activating proteins (Mittal, R., Reza, M., Goody, R., and Wittinghofer, A. (1996) Science 273, 115–117). In this work, we have used a fluorescent active mutant (Y32W) of p21Ha-ras to demonstrate that BeF$_3^-$ binds to the GDP-p21Ha-ras complex in the absence of activating proteins. It induces a conformational change leading to a state with fluorescence properties similar to those of the active state. The binding has a low affinity ($K_d$ at 25 °C = 8.1 ± 0.3 mM) and is endothermic ($\Delta H = 22.3 \pm 1.6$ kJ mol$^{-1}$). The similarity between the GTP-bound form and the GDP-BeF$_3^-$-bound form has been confirmed using lifetime analysis of the tryptophan fluorescence. The kinetic analysis of the process indicates that the binding can be divided into a first bimolecular step, which accounts for the association of the anion with its binding site, and a second step, which corresponds to an internal conformational transition of the GDP-BeF$_3^-$-p21Ha-ras complex to its final state. Both steps are endothermic ($\Delta H_1 = 15 \pm 2$ kJ mol$^{-1}$ and $\Delta H_2 = 8 \pm 2$ kJ mol$^{-1}$). The kinetically determined enthalpy change of $23 \pm 4$ kJ mol$^{-1}$ is in excellent agreement with the equilibrium analysis.

Fluoride ions at relatively high (millimolar) concentrations are poisonous. This effect has been known and is due to their influence on the activity of enzymatic systems via the activation of adenylate cyclase (1). Later it was found that fluoride acts on the GTP-binding proteins (G-proteins) that couple hormone-sensitive membrane receptors to adenylate cyclase (2). Fluoride acts on the G$_\alpha$ subunit that carries the nucleotide-binding site (3). Further insight came when Sternweis and Gilman (4) proved that fluoride activation of adenylate cyclase requires the presence of trace amounts of aluminum. This fact had not been noticed up to then since, at the concentration used, the fluoride solution etched aluminum out of the glassware. These authors also found that the only metal that can replace aluminum is beryllium. Based on the fact that the activation required also GDP or a suitable analog in the nucleotide site, a mechanism of activation was proposed by Bigay et al. (5). These authors suggested that the aluminum fluoride complex ion acts as an analog of the phosphate anion and is able to bind next to the $\beta$-phosphate of the GDP molecule, mimicking the $\gamma$-phosphate and hence inposing on the protein its active conformation.

Among the group of G-proteins, the products of the ras oncogenes are the most studied proteins. They were originally found as the transforming (i.e. tumor-causing) oncogene in animal tumor viruses. Indeed, ~30% of the human tumors were found to contain an activated allele of the ras gene. The ras-related superfAMILY of GTP-binding proteins is a family of small 20–25-kDa proteins that bind guanine nucleotides very tightly and cycle between an inactive GDP-bound state and an active GTP-bound state. These proteins are involved in the signal transduction pathway (6).

As with the other G-proteins, GTP is bound in the active state, and to switch to the inactive state, the $\gamma$-phosphate of the nucleotide has to be hydrolyzed and released, while the GDP remains bound to the protein. Both states show differences in certain areas of the molecule (7, 8) that lead to two different conformational states: the GTP-bound form and the GDP-bound form. In the active conformation, these proteins interact with an effector molecule. The lifetime of the interaction is controlled by the GTPase activity of the protein (9–11). This GTPase activity on its own can be influenced by several effectors such as GTPase-activating proteins (GAPs)$^1$ (12). Since it is reasonable to assume that all G-proteins have a similar mechanism of GTP hydrolysis (on the basis of structural and other similarities), it was surprising to find that ras-related proteins did not become activated by AlF$_4^-$ (13).

A recent work by Mittal et al. (14) showed that AlF$_4^-$ binding is possible if GAPs are present in the solution. The question arises whether GAP induces the binding of AlF$_4^-$ by providing an additional interaction site for AlF$_4^-$ or by inducing a conformational change. We decided to study the system using BeF$_3^-$ instead of AlF$_4^-$ for solubility reasons. If BeF$_3^-$ binds, AlF$_4^-$ should bind even more strongly since it is a transition state analog, and enzymes are supposed to bind transition state analogs with higher affinity.

To investigate the binding of beryllium fluoride using a spectroscopic technique, we have used a fluorescent mutant of p21 (Y32W); the tryptophan fluorescence yield of this mutant is significantly smaller (by ~45%) when GTP is bound as compared with the GDP-bound state (15). The structure of the mutant does not show any disturbance in comparison with the wild type (15).

Since the ratio between the affinities of GTP and GDP for

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§ The abbreviation used is: GAPs, GTPase-activating proteins.
p21H\textsuperscript{ras} is very low, i.e. of the order of 2 (15), it is logical to suppose that the affinity of BeF\textsubscript{3} for the GDP-bound state of p21H\textsuperscript{ras} will be low (even if the analog has higher affinity for the site than the phosphate ion (16)). In any case, the binding constant should be far away from the micromolar range used in previous studies (13, 14). We have used the complex formation constants given by Mesmer and Baes (17) to calculate the optimal concentrations of Be\textsuperscript{2+} and F\textsuperscript{-} ions to obtain millimolar concentrations of BeF\textsubscript{3} and to obtain an exact value of the concentration of the complex to have a rigorous thermodynamic characterization of the binding.

Time-resolved fluorescence spectroscopy is widely used in biological science to probe the structure and dynamics of matter at the molecular level. The fluorescence response (the decay of the fluorescence after an infinitely short pulse of light) of tryptophan in a protein is multiexponential (18), showing the microheterogeneity of the environment of the fluorophore (19). Given the fact that the Y32W mutant contains a single tryptophan, we have use the method as a tool to compare the different conformational states of the protein.

In addition, the binding of BeF\textsubscript{3} to the GDP-bound state of the Y32W mutant of p21H\textsuperscript{ras} produces a change in the intensity and in the decay parameters of the fluorescence similar to the one that can be seen in the presence of GDP. We can use this change in fluorescence to study the kinetics of binding of BeF\textsubscript{3} to the GDP-bound state of p21H\textsuperscript{ras} as well as the kinetics of the thus induced conformational transition toward the active state.

**EXPERIMENTAL PROCEDURES**

The Y32W mutant of p21 was produced and purified as described (20) using a *E. coli* expression system kindly donated by Prof. Dr. Alfred Wittinghofer (Max-Planck-Institut für Molekulare Physiologie, Dortmund, Germany). Its functionality was checked by the nucleotide binding assay (20).

All experiments were performed in buffer containing 64 mM Tris, 50 mM HCl, 1 mM Na\textsubscript{2}SO\textsubscript{4}, 1 mM dithioerythritol, 0.6 mM EDTA, 10 µM nucleotide (GDP or GTP depending on the nucleotide binding), and 1 mM MgCl\textsubscript{2}, pH 7.2, with different amounts of KCl, and BeSO\textsubscript{4} calculated using the complexing constants for Be\textsuperscript{2+} and F\textsuperscript{-} given by Mesmer and Baes (17) to obtain free concentrations of BeF\textsubscript{3} ranging from 0 to 87.7 mM.

GDP-bound p21H\textsuperscript{ras} was prepared by chromatography in a cold Sephadex G-25 column equilibrated in the GDP-containing buffer. GDP-bound p21H\textsuperscript{ras} was prepared using a two-step interchange procedure similar to the one described previously (21), which takes advantage of the higher dissociation rates of the nucleotides in the absence of Mg\textsuperscript{2+} (22). The concentrations of the samples were measured using bovine serum albumin as standard (23).

The steady-state fluorescence measurements were performed on a Spex spectrophotofluorometer (Fluorolog 1891) with excitation and emission slit widths of 7.2 and 3.6 nm, respectively. Fluorescence lifetimes were measured using multichannel phase fluorometry between 1.6 MHz and 1 GHz as described previously (24, 25). An excitation wavelength of 295 nm was used to measure exclusively the tryptophan fluorescence, while the emission wavelength was set at 340 nm using an Oriel filter (340FS10–25). N-Acetyctryptophanamide in water (lifetime at 4.8 °C = 4.129 ns)\textsuperscript{2} was used as reference.

The kinetics of the binding were measured in a stopped-flow instrument, specially designed for fluorescence measurements and built in the laboratory. A Hamamatsu mercury-xenon 200-watt arc lamp (L2482) was used. The monochromator was set at 280 nm. The optical path length was 2 mm and perpendicular to the flow direction. The light beam was 8 mm wide in the direction of the flow. Emission was collected over a wide angle so that a large part of the front surface fluorescence was collected as well. A filter with a cutoff of 300 nm was used in the emission pathway. The dead time of the instrument was determined using the reaction of N-bromosuccinimide with N-acetyltryptophanamide (26) and was found to be ~1.5 ms.

The fitting of the curves to a sum of two exponentials was done using a nonlinear least-squares program based on the Marquardt algorithm (27). Fitting with a sum of two exponentials was accepted on the basis of a substantial decrease in χ² (at least 2-fold) and a better random distribution of the residual (see Fig. 4, inset). The direct fitting of a complete set of curves at a given temperature to a set of kinetic constants was done using the KINSIM and FTFITSIM packages (28) for analysis of kinetic data running on a Silicon Graphics Indigo 2 workstation equipped with a MIPS R10000 processor.

**RESULTS**

**Binding of BeF\textsubscript{3} to p21H\textsuperscript{ras}**—Fig. 1A shows the fluorescence emission spectra of the GDP-bound form of this mutant before and after the addition of millimolar amounts of BeF\textsubscript{3}. It can be seen clearly that there is a reduction in the fluorescence intensity of ~47% after the addition of the analog. The addition of similar amounts of BeF\textsubscript{3} to a GTP-bound p21H\textsuperscript{ras} sample (Fig. 1B) produced only a very small effect on the fluorescence that can be assigned to the small amount of GDP bound to the site. This is not surprising since the affinities of p21H\textsuperscript{ras} for GDP and GDP are very similar and the commercial GTP preparations always contain small amounts of GDP. Moreover, some GDP is formed due to the small GTPase activity. A similar problem in obtaining a fully GTP-bound GTPase protein has been previously reported (21). The addition of neither fluoride nor beryllium alone is able to produce the effect, which is maximum when the concentrations of Be\textsuperscript{2+} and F\textsuperscript{-} are optimal, for obtaining the BeF\textsubscript{3} ions according to Mesmer and Baes (17). These results demonstrate that BeF\textsubscript{3} is able to bind to the GDP-bound form of p21H\textsuperscript{ras} and to induce the conformational transition from the GDP-bound form to a GTP-like conformation. However, the concentrations necessary to fully turn the conformational transition are on the order of 0.1 mM BeF\textsubscript{3}, which is between 3 and 4 orders of magnitude higher than the ones used by Mittal *et al.* (14).

The equilibrium was thermodynamically fully characterized at different temperatures. The reaction is very fast, and equilibrium is reached within the mixing time in the cuvette of the fluorometer. The reaction is endothermic with a ΔH of 22.3 ± 1.6 kJ mol\textsuperscript{-1}, as can be deduced from the van't Hoff plot (Fig. 2). The overall K\textsubscript{eq} at 25 °C is 8.1 ± 0.3 mM, which represents an overall affinity between 3 and 4 orders of magnitude lower than the one observed in the presence of GTTPase-activating proteins (14).

**Phase Fluorometry**—To obtain additional evidence of the similarity between the GTP-bound state and the state induced by the binding of the anion to the GDP-bound state, fluorescence lifetime studies were performed to determine the trypto-
phan fluorescence characteristics in the different conformational states (Fig. 3). To prevent hydrolysis of GTP during the measurements, fluorescence lifetimes were measured at 4.8 °C, at which hydrolysis of the nucleotide is slow in comparison with the time necessary to measure the lifetimes.

The time dependence of the tryptophan fluorescence emission can be described by a sum of three exponentials, whose values and amplitudes are shown in Table I. The fluorescence lifetimes of tryptophan in the three states (GDP, GDP-BeF$_3^-$, and GTP) are approximately the same; the only differences arise from the amplitude fractions of the middle and longer lifetime. After hydrolysis of the γ-phosphate, the amplitude fraction of the longer lifetime increased 2.5 times, whereas the amplitude fraction of the middle lifetime decreased 4 times.

It is possible to follow the hydrolysis of the γ-phosphate using phase fluorometry. When samples of GTP-bound p21$^{Ha-ras}$ were measured after different incubation times at 25 °C, a slow progressive shifting of the fluorescence decay parameters toward the GDP parameters could be easily seen (data not shown).

Comparison of the fluorescence decay parameters of the conformational state induced by the binding of BeF$_3^-$ to GDP with those of the GDP- and GTP-like conformations shows that the parameters of the GDP-BeF$_3^-$-bound state are very similar to those of the GTP-bound state (Table I). This indicates that the tryptophan in the GDP-BeF$_3^-$-bound state is practically in the same conformation as in the GTP-bound form.

**Kinetics of Binding of BeF$_3^-$ to p21$^{Ha-ras}$—**Since the binding of BeF$_3^-$ to the GDP-bound form of a mutated p21$^{Ha-ras}$ containing tryptophan instead of tyrosine at residue 32 (15) produces a large decrease in the quantum yield of the tryptophan very similar to the one induced by the binding of GTP, the reaction can therefore be followed by the decrease in the fluorescence observed at 340 nm. Since the reaction is very fast, we studied

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**TABLE I**

| State          | $a_1$ | $\tau_1$ | $a_2$ | $\tau_2$ | $a_3$ | $\tau_3$ | $\chi^2$ |
|----------------|-------|----------|-------|----------|-------|----------|-----------|
| GDP            | 0.12 ± 0.01 | 0.38 ± 0.13 | 0.15 ± 0.01 | 2.28 ± 0.35 | 0.73 ± 0.01 | 6.08 ± 0.15 | 1.2 |
| GDP + BeF$_3^-$ (25,65 mM) | 0.19 ± 0.02 | 0.28 ± 0.14 | 0.53 ± 0.02 | 2.26 ± 0.10 | 0.28 ± 0.03 | 6.12 ± 0.20 | 3.1 |
| GDP + BeF$_3^-$ (43.35 mM) | 0.19 ± 0.02 | 0.25 ± 0.09 | 0.58 ± 0.02 | 2.19 ± 0.07 | 0.23 ± 0.02 | 6.07 ± 0.16 | 2.5 |
| GTP            | 0.15 ± 0.02 | 0.32 ± 0.02 | 0.56 ± 0.02 | 2.14 ± 0.10 | 0.29 ± 0.02 | 6.09 ± 0.16 | 2.6 |

*a_1*, amplitude fraction of lifetime $i$ at 340 nm.
It is straightforward to assign the first step explained by a scheme of coupled reactions with comparable rate constants (29). It is possible to determine the four individual kinetic constants of the reaction (29). Nevertheless, the errors are much smaller in the latter case. This method has allowed us to obtain a very precise determination of the overall equilibrium binding constant that can be compared with the one obtained by direct binding measurements, and it has allowed us to assign the fluorescence change to the first reaction step.

The best values of the numerical fit at all temperatures were obtained when the fluorescence change was supposed to occur completely in the first step of the reaction, i.e., binding of BeF$_3$ to the GDP-bound form. This approach has allowed us to assign the fluorescence change to the first step of the reaction (29). When the two observed rate constants depend measurably on the concentration (Fig. 5), it is possible to determine the four individual kinetic constants of the reaction (29). Nevertheless, to take advantage of the large set of stopped-flow data obtained at each temperature, we decided to apply a global analysis by direct fitting of the experimental data using a numerical approach (28) to solve the differential equations derived from the mechanism identified by the analytical method. The differences between the values obtained using the direct fitting approach and the values obtained by global analysis are very small, but the errors are much smaller in the latter case. This method has allowed us to obtain a very precise determination of the kinetic constants and an independent determination of the overall equilibrium binding constant that can be compared with the one obtained by direct binding measurements, and it has allowed us to assign the fluorescence change to the first reaction step.

The best values of the numerical fit at all temperatures were indeed obtained when the fluorescence change was supposed to occur completely in the first step of the reaction, i.e., binding of BeF$_3$ to the GDP-bound form. The complete set of kinetic parameters determined at different temperatures is summarized in Table II.

**DISCUSSION**

**Binding of BeF$_3$ to p21$^{Ha-ras}$—**The results presented here support the hypothesis of Mittal et al. (14) about the contribution of GAP to the binding of AlF$_4^-$ to p21$^{Ha-ras}$. According to these authors, GAP supplies one or more critical positive charges to the y-phosphate-binding site of p21$^{Ha-ras}$, allowing the binding of the phosphate analog in the binding site. The presence of GAP, providing additional positive charges, creates an affinity for the phosphate analog that is 3–4 orders of magnitude higher than in its absence, i.e., a magnitude similar to the acceleration of the hydrolysis reaction by GAP (30). The fluorescence properties of GTP-p21$^{Ha-ras}$ and GDP-BeF$_3^-p21^{Ha-ras}$ are almost identical, indicating that the conformation is very close or identical to the active conformation.
the experimental curves, it can be deduced that the fluorescence change arises almost completely from the first step of the reaction, i.e., the binding of the ligand to the site. Another remarkable fact is that the bimolecular rate constant (146 M$^{-1}$ s$^{-1}$ at 25 °C) is far from the value of $\sim$10$^{10}$ M$^{-1}$ s$^{-1}$ expected for a diffusion-controlled reaction, even if we correct for the number of collisions arriving at the correct angle (approximated by the ratio between the surfaces of the colliding molecules and estimated to be 1:100) (31). This suggests indeed that not all the collisions are efficient, and after collision at the correct environment, a process of recognition has to take place.

This suggests that the first step of the reaction has to be decomposed in a fast formation of a collisional complex of low affinity, followed by a recognition step. The observed pseudo first-order rate constant for binding can thus be considered to be as follows: $k_{1}([BeF_3]_1) = k_{rec}(K_c/([BeF_3](1 + K_c/[BeF_3])))$, where $K_c$ is the association constant for the collisional complex, and $k_{rec}$ is the rate constant for recognition. Since the concentration dependence is linear, $K_c/([BeF_3]_1 < 1$. The fluorescence change is supposed to occur upon the recognition step. Otherwise, $BeF_3$ would be a collisional quencher.

Thermodynamics of Binding of $BeF_3$ to $p21^{ras}$—With the knowledge of the kinetic parameters of both observed steps of the binding reaction, it is possible to obtain the thermodynamic parameters of the reaction. Fig. 6 shows the Arrhenius plot of the four observed individual rate constants, i.e., the binding of the complex to the protein, its dissociation, and both directions of the conformational transition. Both the binding of the anion to the protein and the conformational transition to the final state are endothermic and entropy-driven. The thermodynamic parameters obtained from the Arrhenius analysis as well as the entropy values obtained from the temperature dependence of the free energy change are summarized in Table III.

It is actually interesting to compare the kinetic and thermodynamic parameters obtained from the binding of nucleotides to the empty site of the same mutant of $p21^{ras}$ (15). The kinetics also show a two-step binding mechanism; nevertheless, the binding constant of the site for the nucleotide is much larger than for the phosphate analog. This is not surprising given the large number of interactions involved in the binding of a nucleotide as compared with the phosphate analog. The values obtained for the rate constants are of comparable magnitude: the conformational transition to the GTP-bound state has a rate constant of 21.8 s$^{-1}$ at 25 °C (15), while the conformational transition from the GDP-$BeF_3$-$p21^{ras}$ state to the GDP-$BeF_3$-$p21^{ras}$ state has a value of 4.65 s$^{-1}$. This shows that this kind of internal rearrangement in the $p21^{ras}$ molecule is relatively slow. The kinetic constant of the backwards transition is quite variable, depending on the nucleotide that is bound to the site, with values ranging from $< 10^{-5}$ to 3.3 s$^{-1}$, reflecting the difference in stability of the bound forms.

The small value of the binding constant of the complex for
Activation of p21^Hauto-ras by Beryllium Trifluoride

23143

carboxinational pathway of the transition between the two ac-
kinetics of binding can be a very useful tool in the study of the
bound (32, 33). The results obtained show that the study of the
i.e. oncogenic (32).

suggestions.

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the GDP-p21ras molecule should be just a reflection of the small
difference in affinities of the molecule for GDP and GTP (of the
order of 2 (15)) enhanced by the higher affinity of the phosphate
analog for the site as compared with phosphate itself (16). The
small value of the equilibrium constant obtained for the step of
the conformational change suggests that the transition could
be very finely controlled by very small changes. Given an equi-
librium constant of ~3.0 for the conformational transition, the
free energy change is 2.5 kJ mol⁻¹ for the change between the
active and inactive conformations at 25 °C. This fact fits very
well with the observation that mutants of p21^Ha-ras can become
oncogenic (i.e. active) even in the absence of any nucleotide
bound (32, 33). The results obtained show that the study of the
kinetics of binding can be a very useful tool in the study of the
conformational pathway of the transition between the two ac-
tive and inactive states of a molecular switch like p21^Ha-ras,
allowing (using site-directed mutagenesis) the validation of
theoretical data obtained from pathway calculations (34).