Individual Rate Constants for the Interaction of Ras Proteins with GTPase-Activating Proteins Determined by Fluorescence Spectroscopy

Mohammad Reza Ahmadian,[‡] Ulrike Hoffmann,[§] Roger S. Goody,[§] and Alfred Wittinghofer*[,‡]

Abteilung Strukturelle Biologie and Abteilung Physikalische Biochemie, Max-Planck-Institut für molekulare Physiologie, Rheinlanddamm 201, 44139 Dortmund, Germany

Received October 11, 1996; Revised Manuscript Received January 23, 1997[⊗]

ABSTRACT: Individual rate constants for the interaction of H-, K-, and N-Ras with GAP-334 and NF1-333 were determined using fluorescent derivatives of guanine nucleotides at the active site of the Ras proteins. Stopped-flow experiments with NF1-333 show a fast concentration-dependent initial phase corresponding to the binding reaction followed by a slower phase, which corresponds to the hydrolysis reaction. With Ras bound to the nonhydrolyzable analogue mant-GppNHp, only the concentration-dependent first phase was observed. The Ras·mant-GppNHp·NF1-333 complexes were also used to measure dissociation rate constants of the Ras-GAP complexes. Using GAP-334 as the catalyst, the concentration-dependent first phase was too fast to be measured by the stopped-flow method, but the subsequent chemical cleavage reaction occurred at a similar rate ($5-10\ s^{-1}$) to that seen with NF1-333. With both GAP-334 and NF1-333, after rapidly reaching the initial equilibrium, there was no further time-dependent change on mixing GAPs with Ras·mant-GppNHp. The results obtained provide new insights into the individual steps of the GAP-catalyzed GTPase reaction on Ras. They do not require the postulation of a rate-limiting step occurring before GTP hydrolysis.

Ras proteins, the products of the H-, K-, and N-*ras* genes, function as molecular switches and cycle between the GDP-bound inactive state and GTP-bound active state. In the cell, the conformational state of these proteins is regulated by two kinds of interacting molecules, guanine nucleotide exchange factors (GEFs)[1] and GTPase-activating proteins (GAPs) (Lowy & Willumsen, 1993; Bourne *et al.*, 1991; Boguski & McCormick, 1993). GEFs promote the exchange of protein-bound GDP for GTP. Once activated, GTP-bound Ras can now interact with so-called effector molecules (Wittinghofer & Herrmann, 1995). Return to the inactive state is achieved by the interaction of GTP-bound Ras with GAPs, which accelerate the very slow intrinsic GTPase activity of Ras proteins by many orders of magnitude (Gideon *et al.*, 1992; Wiesmüller & Wittinghofer, 1992; Eccleston *et al.*, 1993).

Five Ras-specific GAPs have been described, which show a high degree of similarity in their GAP-related domains, GRD (Martin *et al.*, 1990). p120-GAP, the first to be isolated, is the prototype of this class of proteins (Trahey & McCormick, 1987; Trahey *et al.*, 1988; Vogel *et al.*, 1988). The second is neurofibromin (NF1), which is the product of the neurofibromatosis type I gene (Xu *et al.*, 1990a) and has also been shown to stimulate the GTPase of Ras (Martin *et al.*, 1990; Xu *et al.*, 1990b; Ballester *et al.*, 1990). This gene has been found to be frequently mutated in patients with the disease neurofibromatosis type I (Cawthon *et al.*, 1990; Wallace *et al.*, 1991; Viskochil *et al.*, 1990) but also, albeit less frequently, in solid tumors (Li *et al.*, 1992). $GAP1^m$, a mammalian homologue of the *Drosophila* GAP1 gene, has been described as the third form of Ras-GAP (Maekawa *et al.*, 1994). Further members of the Ras-GAP family are GAPIII, a brain-enriched isoform with high homology to $GAP1^m$ (Baba *et al.*, 1995), and a $GAP1^{IP4BP}$, which is a specific inositol 1,3,4,5-tetrakisphosphate (IP4)-binding protein with GAP activity toward both Ras and Rap (Cullen *et al.*, 1995).

In order to understand the mechanism of GAP-mediated GTP hydrolysis on Ras, it is important to understand the individual steps of the reaction. This is even more important considering the conflicting evidence as to the role of GAP. It has been postulated that the rate-limiting step of the intrinsic GTP hydrolysis is a conformational change which is accelerated by GAP (Neal *et al.*, 1990; Moore *et al.*, 1993). This implies that Ras is in principle capable of rapid GTP hydrolysis and only needs GAP for stabilizing a conformation competent to cleave GTP. On the other hand, evidence has been presented suggesting that the rate-limiting step is the chemical cleavage of GTP and that this is catalyzed by GAP (Rensland *et al.*, 1991). Moreover, it has recently been shown (Mittal *et al.*, 1996) that Ras needs equimolar amounts of GAP in order to bind AlF_4^-, which is supposed to mimic the transition state of the GTPase reaction (Sondek *et al.*, 1994; Coleman *et al.*, 1994). We present here kinetic data on the interaction of the catalytic domains of p120-GAP and NF1 with H-, K-, and N-Ras, which define and quantify individual rate constants. These data are also relevant for understanding the role of GAP in the enzymatic reaction on Ras.

* Corresponding author: Alfred Wittinghofer. Fax: 49-231-1206230. Phone: 49-231-1206280. E-mail: Alfred.Wittinghofer@mpi-dortmund-.mpg.de.

[‡] Abteilung Strukturelle Biologie.

[§] Abteilung Physikalische Biochemie.

[⊗] Abstract published in *Advance ACS Abstracts,* April 1, 1997.

[1] Abbreviations: Ras or $p21^{ras}$, the protein product of the N-, K-, and H-*ras* gene, unless specified; GAP, GTPase-activating protein; GAP-334, the catalytic fragment of p120-GAP; GEF, guanine nucleotide exchange factor; NF1 or neurofibromin, the product of the neurofibromatosis type I gene; NF1-333: the catalytic fragment of neurofibromin; mant-GDP, mant-GTP, and mant-GppNHp, 2′,3′-*O*-(*N*-methylanthraniloyl)guanosine 5′-diphosphate, triphosphate, and (*β*,*γ*-imidotriphosphate); mant-dGTP, the 2′-deoxy derivative of mant-GTP.

S0006-2960(96)02556-1 CCC: $14.00 © 1997 American Chemical Society

MATERIALS AND METHODS

Recombinant H-Ras were prepared from *Escherichia coli* using the ptac-expression system as described (Tucker *et al*., 1986). K-Ras and N-Ras were prepared using the corresponding expression vectors (Trahey & McCormick, 1987; Skinner *et al*., 1991) and purification protocols as for the H-Ras protein. The catalytic domain of NF1, NF1-333, extending from amino acid E1198 to H1530, was isolated using the pLMM-expression system from *E. coli* as described previously (Ahmadian *et al*., 1996). GAP-334, the catalytic domain of p120-GAP spanning from the amino acid M714 to R1047, was prepared from *E. coli* using the vector ptrc99A as described (Gideon *et al*., 1992). The nucleotide-free forms of H-, K-, and N-Ras were prepared as described for H-Ras (John *et al*., 1990) and the fluorescent derivatives of GTP, dGTP and GppNHp, mant-GTP, mant-dGTP, and mant-GppNHp were synthesized as described (John *et al*., 1990). Ras·mant-GTP, Ras·mant-dGTP, and Ras·mant-GppNHp were prepared by loading nucleotide-free Ras proteins with a 1.5-fold molar excess of the fluorescent nucleotide. Excess nucleotide was separated from nucleotide-bound Ras by gel filtration on prepacked NAP-5 columns (Pharmacia, Uppsala, Sweden).

Stopped-flow experiments using mant-GTP, mant-dGTP, and mant-GppNHp were performed to monitor the interaction of NF1-333 and GAP-334 with Ras in an Applied Photophysics SX16MV apparatus. All the reactions were followed at 25 °C in 40 mM HEPES, pH 7.4, 5 mM DTE, and 5 mM $MgCl_2$ (standard buffer) using an excitation wavelength of 360 nm and a cut-off filter (408 nm) in front of the emission monochromator. Exponential and hyperbolic fits to the data were done using the program Grafit (Erithacus software). More complex kinetic situations were analyzed with the program Scientist. In particular, the process of interaction of NF1 with Ras·mant-GTP was modeled using a reversible association of the two proteins followed by an irreversible GTP hydrolysis with subsequent rapid dissociation of NF1. The situation was represented by a series of differential equations describing the change in concentration of each species with time and an algebraic equation relating the concentration and fluorescent yields of intermediates to the fluorescence signal measured. Curves were simulated by numerical integration of the set of differential equations using initial estimates for constants (rate constants and fluorescent yields), and the constants were varied and optimized by a least-squares procedure. For the interaction of NF1 with Ras·mant-GTP, a more extensive analysis was performed by simulaneously simulating and fitting of four data sets (Ras·mant-GTP concentration, 0.1 μM; NF1 concentrations, 0.2, 0.4, 0.6, and 0.8 μM) using the program Scientist.

The affinity between NF1-333 and Ras·mant-GppNHp was determined on an LS50B Perkin-Elmer spectrofluorometer by the guanine nucleotide dissociation inhibitor (GDI) assay, which uses the inhibition of the mant-GppNHp dissociation from Ras on complex formation with effectors as described recently for Ras and Rap and the Ras-binding domains of c-Raf-1 and Ral-GEF (Herrmann *et al*., 1995, 1996).

RESULTS AND DISCUSSION

Previous work has established that the fluorescent GTP analogue mant-GTP shows a decrease in fluorescence on GTP hydrolysis at the active site of Ras (Neal *et al*., 1990)

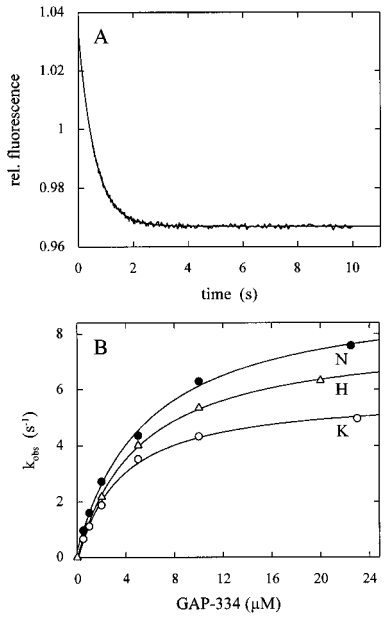

FIGURE 1: GTPase reaction of Ras catalyzed by GAP-334. (A) Fluorescence transient obtained by mixing 1 μM H-Ras·mant-GTP with 2 μM GAP-334 in standard buffer at 25 °C in a stopped-flow apparatus. The data were fitted to a single exponential. (B) The rate constants obtained from fluorescence transients as in panel A using 1 μM Ras·mant-GTP are plotted against the GAP concentrations. The three isoforms H-Ras (△), K-Ras (○), and N-Ras (●) were used as indicated. The data were fitted to a hyperbolic equation as outlined in Materials and Methods to obtain the K_D and k_{cat} values shown in Table 1.

Table 1: Rate Constants for the Reaction of mant-GTP-Bound Ras Proteins with GAP-334

	K_M (μM)	k_{cat} (s^{-1})
H-Ras·mant-GTP	5.0	8.0
K-Ras·mant-GTP	3.4	5.9
N-Ras·mant-GTP	5.8	9.3

and that this signal can be used to monitor the GAP-catalyzed reaction (Moore *et al*., 1993; Rensland *et al*., 1991). A similar change with the uncleavable analogue mant-GppNHp has been interpreted to be associated with a slow conformational change preceding the hydrolysis step although this has been disputed (Rensland *et al*., 1991). Figure 1A shows that there is a relatively rapid decrease of fluorescence on mixing H-Ras·mant-GTP with GAP-334, and the rate constant for this change, which is believed to represent the chemical cleavage reaction (see below), can be fitted as a single exponential. The rate constant increases in a hyperbolic fashion as a function of the GAP concentration (Figure 1B). Fitting a hyperbolic curve to the points leads to an apparent K_D of 5 μM and a maximal rate of 8 s^{-1}. Since this interaction is relatively weak and correspondingly has a fast dissociation rate constant, it seems likely that the value of 5 μM represents a genuine K_D value, not influenced by the relatively slow cleavage reaction. The results are tabulated in Table 1 and are similar to those obtained for K-Ras and N-Ras using the same method (Eccleston *et al*., 1993; Moore *et al*., 1993) and by using a fluorescence anisotropy method (Brownsbridge *et al*., 1993). Table 1 shows the basic kinetic constants for the interaction. The results are in quite good agreement with values of K_M and k_{cat} obtained using catalytic amounts of GAP and varying the concentration of Ras·mant-GTP (Gideon *et al*., 1992).

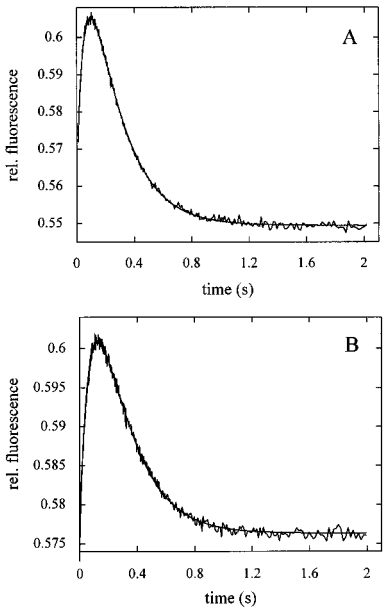

FIGURE 2: Fluorescence transients obtained on mixing (A) 0.1 μM H-Ras·mant-GTP with 0.2 μM NF1-333 ($k_{on} = 5.3 \times 10^7$ M^{-1} s^{-1} and $k_{cat} = 5.4$ s^{-1}) and (B) 0.1 μM H-Ras·mant-dGTP with 0.2 μM NF1-333 ($k_{on} = 6.2 \times 10^7$ M^{-1} s^{-1} and $k_{cat} = 4.4$ s^{-1}), in standard buffer at 25 °C. The lines through the data points were obtained by fitting with a numerical integration fitting procedure as described in the Materials and Methods section.

The fluorescent transient observed on the interaction of Ras with neurofibromin was quite different. Figure 2A shows the results of mixing H-Ras·mant-GTP with NF1-333. Instead of the simple exponential decrease seen with GAP, there is an initial rapid increase in fluorescence followed by a decrease at a rate similar to that seen at high GAP concentrations. The first phase of the reaction is concentration-dependent, whereas the second is less obviously so. This suggests that an initial association between the proteins leads to an increase in fluorescence, which then decreases on mant-GTP hydrolysis (and consequent dissociation of the proteins) to a level below that of the starting level, in keeping with the observation that the fluorescence of Ras·mant-GDP is lower than that of Ras·mant-GTP. This interpretation means that the mechanism cannot be treated as a very rapid initial equilibration followed by a relatively slow cleavage step, which is the underlying assumption in the analysis of the data obtained with GAP. An indication of this type of behavior in the interaction between neurofibromin and Ras·mant-GTP was reported earlier, but was not pursued (Eccleston *et al*., 1993). As the concentration of NF1 was increased, the rate of the first phase increased noticeably, whereas that of the second phase soon reached a maximum. To ensure accurate collection of data for the very different time domains for these two phases, a logarithmic time base was used, effectively increasing the time resolution at the start of the transient and progressively decreasing it as slow changes occurred. We have evaluated individual experimental data sets using a numerical integration fitting procedure from the program package Scientist as decribed in Materials and Methods.

Scheme 1 describes a minimal reaction scheme for the GAP-catalyzed GTPase reaction. The program was written to allow the three kinetic constants (k_{+1}, k_{-1}, $k_{+2} = k_{cat}$) of Scheme 1 as well as the fluorescent yields of Ras·mant-GTP, Ras·mant-GTP·NF1, and Ras·mant-GDP to be fitted to the data. The value of k_{-1} was not well determined by individual data sets, and the fits used to determine k_{+1} and k_{+2} were obtained by using a nonvarying value of k_{-1}, which was experimentally determined for the interaction of NF1-333 with Ras·mant-GppNHp (see below). Similar data were obtained with H-Ras, K-Ras, and N-Ras, and the results are shown in Table 2. The association rate constants are in the range $4-5 \times 10^7$ M^{-1} s^{-1} in all three cases, and the dissociation rate constants are between 7 and 10 s^{-1}. The rate constants for GTP hydrolysis are in the same range. This implies that there is an approximately equal chance of hydrolysis and dissociation from the Ras·GTP·NF1-333 complex, or in other words that GTP hydrolysis occurs on average on every second contact between neurofibromin and Ras·GTP. The constants for the cleavage reaction (k_{cat}) are higher than those obtained by using catalytic amounts of NF1-333 (enzyme) and saturating concentration of Ras·GTP (the substrate) (Wiesmüller & Wittinghofer, 1992) and support earlier results (Eccleston *et al*., 1993). The discrepancy probably arises because in the multiple turnover measurements using catalytic amounts of NF1-333, but not in the single turnover experiments shown here and earlier (Eccleston *et al*., 1993), the quality of the protein preparation is crucial for the value of k_{cat}.

Scheme 1

$$\text{Ras•mant-GTP} + \text{NF1} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{NF1•Ras•mant-GTP} \xrightarrow{k_{+2}} \text{NF1•Ras•mant-GDP} + P_i$$

Table 2: Rate Constants for the Reaction of mant-GTP-Bound Ras Proteins with NF1-333

	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (μM)	K_M (μM)	k_{cat} (s^{-1})
H-Ras·mant-GTP	5.3×10^7		0.13	0.23	5.4
H-Ras·mant-GppNHp	4.4×10^7	6.8	0.15		0
K-Ras·mant-GTP	5.2×10^7		0.14	0.28	6.9
K-Ras·mant-GppNHp	5.0×10^7	7.5	0.15		0
N-Ras·mant-GTP	3.7×10^7		0.28	0.52	8.7
N-Ras·mant-GppNHp	5.8×10^7	10.5	0.18		0

For H-Ras, a more detailed investigation of the kinetics of NF1 binding and mant-GTP cleavage was performed by varying the NF1 concentration. As mentioned, k_{-1} was not well determined in individual curves, but fitting four different concentrations of NF1 simultaneously led to a value of 0.46 s^{-1}, with k_{+1} having a value of 10^8 M^{-1} s^{-1} and k_{+2} a value of 4.9 s^{-1}. This would suggest that the dissociation rate constant appears to be significantly lower than measured directly for Ras·GppNHp. Although the fit obtained was stable, in the sense that it always converged to the values mentioned independent of the starting values, it is clear that when k_{-1} is much smaller than k_{+2}, k_{-1} cannot be determined accurately by the method used. However, the global fit obtained with a value of k_{-1} fixed at 6.8 s^{-1} was worse than that obtained with the fitted value of 0.46 s^{-1}, so we conclude that k_{-1} is significantly smaller than 6.8 s^{-1}. Nixon *et al.* (1995), using a different signal, estimated k_{-1} to be ca. 2 s^{-1} at 30 °C (with k_{+2} equal to 11.5 s^{-1}) so that there is reasonable agreement between the two studies on this point. The reliability of the value of 2 s^{-1} given by Nixon *et al*. is difficult to judge, since a proper fitting procedure was not used.

Experiments of the type shown in Figure 2A were repeated with 3′-mant-dGTP instead of the mixture of isomers of mant-GTP used so far and in most other studies of this type. This derivative does not show a fluorescence change at the active site of Ras on GTP hydrolysis (Rensland, 1992), but there was a readily measured transient increase of fluorescence on mixing with NF1 (Figure 2B), which could be fitted as in Figure 2A. The results were essentially similar to those with mixed isomers, with the main differences that the starting and finishing levels for the fluorescence signal are identical, whereas with mixed isomers, the end point was lower. This is in keeping with the observations with Ras alone and shows that the transient fluorescence signals seen on mixing mant-GTP or mant-dGTP with NF1 arise primarily from interaction between the two proteins. The rate constant for cleavage in this case is 4.4 s^{-1}.

What is the reason for the difference in the fluorescence transients seen on mixing Ras·mant-GTP with GAP (Figure 1A) on the one hand and NF1 on the other (Figure 2A)? One possibility is that, unlike with NF1-333, there is no fluorescence change due to the interaction of GAP-334 with Ras·mant-GTP, but the observed signal is caused by the conformational change on Ras due to GTP hydrolysis, as in the absence of GAP-334 (Neal *et al*., 1990; Rensland *et al*., 1991; Moore *et al*., 1993), albeit at a much faster rate. The second possibility is that a fluorescence increase does occur on binding of GAP-334 to Ras·mant-GTP, but that it is too fast to be measured in the stopped-flow apparatus. Two observations favor the second interpretation. The first is that the amplitudes of the fluorescence changes seen in the experiment reported in Figure 1 increase hyperbolically as the GAP-334 concentration is increased. This can be understood in light of the NF1 experiments if we assume that the first step is a more rapid equilibrium for GAP than for NF1. The second observation is that although there is no fluorescence change associated with the hydrolysis of mant-dGTP at the active site of Ras (Rensland, 1992), the fluorescent decrease shows an increasing amplitude with increasing GAP concentration, with a rate constant at high GAP concentration similar to that seen with mant-GTP (data not shown). These pieces of evidence argue strongly in favor of the hypothesis that a rapid increase in fluorescence occurs on interaction of Ras·mant-GTP with GAP-334 and that this change occurs within the dead-time of the stopped-flow machines used (ca. 2 ms). On the basis of the experiments which can be performed, we cannot exclude a mechanism in which an isomerization reaction of Ras·mant-GTP precedes rapid binding of either GAPs. Even in this case, the initial slope of the line in the NF1 experiments would define an effective second-order rate constant for binding, but it would have a different significance, as discussed by Bagshaw *et al*. (1974).

In order to address both this question and the more important one about the nature of the rate-limiting step in the GTPase mechanism, the interaction of NF1 with Ras bound to the nonhydrolyzable fluorescent analogue mant-GppNHp was examined in detail. With all three Ras isoforms, there was a rapid increase in fluorescence due on mixing (Figure 3), which was concentration-dependent, after which the signal was stable for at least 500 s. Thus, there was no sign of a fluorescent decrease at a rate corresponding to that seen with mant-GTP. If the hypothesis that there is a fluorescence change associated with a rate-limiting step

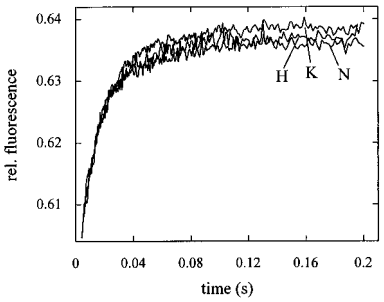

FIGURE 3: Fluorescence transients seen mixing 0.1 μM Ras·mant-GppNHp with 1 μM NF1, with three isoforms of Ras as indicated. Conditions as in Figure 1.

before GTP hydrolysis holds and that this occurs in an NF1 or GAP sensitive manner even in the absence of GTP hydrolysis, we would expect to see this as a drop in fluorescence on a time scale of seconds in the present experiment. However, two points should be borne in mind here. One is that the amplitude of this signal would be expected to be significantly smaller than the increase seen in the first phase, since it would be due to the isomerization reaction alone and not due to dissociation of the proteins. Secondly, it is conceivable that an isomerization reaction could occur in the ternary complex without a fluorescence change, even though a change is seen in the binary complex. We have therefore repeated experiments of the type reported before using substoichiometric amounts of NF1 with Ras·mant-GppNHp and see no evidence of a NF1-dependent slow fluorescence transient, as we also reported earlier for GAP (Rensland *et al*., 1991).

The association reactions between NF1 and mant-GppNHp-bound H-, K-, and N-Ras show similar rate constants of about $4-6 \times 10^7$ M^{-1} s^{-1}. It is of interest to compare these rate constants with those obtained for the Ras·mant-GTP complexes from experiments of the type shown in Figure 2A. Table 2 shows that there is quite good agreement between the association rate constants for the reaction between NF1 and either the mant-GTP or the mant-GppNHp complexes of H-Ras, K-Ras, and N-Ras.

A more detailed examination of the kinetics of the first phase in the interaction of NF1 with Ras·mant-GTP was performed by varying the concentration of NF1 between 0.2 and 10 μM in the stopped-flow experiments. A different fitting strategy was used in which the first reaction was treated as pseudo-first-order in the numerical integration program, and this led to the data shown in Figure 4, where the pseudo-first-order rate constant is plotted against the NF1 concentration. It can be seen that there is not the simple linear dependence of the rate constant on NF1 concentration expected from a simple second-order reaction, but a hyperbolic dependence. Fitting a hyperbolic curve to the data leads to a maximal rate of 493 s^{-1}, with a K_D value of 4.1 μM. The simplest interpretation of this behavior is that the binding process occurs in two steps, with an initial rapid and weak equilibrium being followed by a relatively rapid isomerization reaction. This two step binding mechanism for the interaction of NF1 with Ras·mant-GTP is included in the kinetic mechanism shown in Scheme 2.

The first step in the association reaction is a rapid equilibrium with an equilibrium constant ($K_D = k_{-1}/k_{+1}$) of 4.1 μM. k_{+2} has a value of 493 s^{-1} and k_{-2} of 0.46 s^{-1} from the analysis given above. There is an increase in the

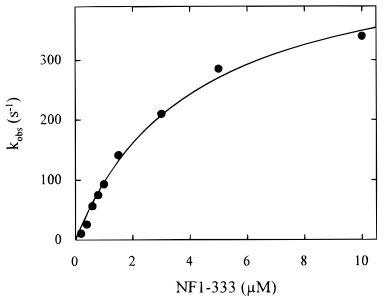

FIGURE 4: Concentration-dependence of the pseudo-first-order rate constant for the initial phase of the fluorescence transients obtained from the interaction of H-Ras·mant-GTP with NF1-333, as shown in Figure 2. The fitted line is described by a hyperbolic function with an apparent K_D of 4.1 μM and a maximal rate constant of 493 s^{-1}. Conditions as in Figure 1.

Scheme 2

$$\text{Ras•mant-GTP} + \text{NF1} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{NF1•Ras•mant-GTP} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}}$$

$$\text{NF1•Ras*•mant-GTP} \xrightarrow{k_{+3}} \text{NF1•Ras•mant-GDP} + P_i$$

emission intensity of the methylanthraniloyl group at this step. k_{+3}, the rate of GTP hydrolysis, is 5.4 s^{-1} for H-Ras (see Table 2). The value for the reverse rate constant is not known. As a consequence of GTP hydrolysis, we assume there is rapid dissociation of Ras·mant-GDP from its complex with NF1, since this complex has been shown to have a low affinity (Brownsbridge *et al*., 1993). It has also been shown earlier that the release of inorganic phosphate is not rate-limiting (Nixon *et al*., 1995). The fact that the fitted hyperbola in Figure 4 appears to pass through the origin of the graph is in good agreement with the low value of k_{-2} determined from the previous analysis. The line should intercept the *y*-axis at a value corresponding to k_{-2}, and the quality of the data at low concentrations of NF1 would be good enough to detect an intercept of 6−7 s^{-1} (the value for Ras·mant·GppNHp·NF1) but not for an intercept of ca. 0.5 s^{-1}.

To test the conclusions made above concerning the fluorescence change occurring on the interaction of NF1 with Ras·mant-GTP, the interaction of GAP with Ras·mant-GppNHp was examined. It was found that a rapid increase of fluorescence occurred inside the dead-time of the stopped-flow apparatus. This increase was ca. 10% of the original intensity, similar to that seen with NF1. The change could be reversed, again within the dead-time of the stopped-flow machine, which is ca. 2 ms, by mixing with an excess of Ras·GppNHp. We therefore conclude that the rate constant for dissociation of the Ras·mant-GppNHp·GAP complex is at least 1000 s^{-1} (data not shown). Taken together with the K_D for this complex as determined here (Table 1), somewhat lower as compared to other reports (Gideon *et al*., 1992; Eccleston *et al*., 1993; Brownsbridge *et al*., 1993), we come to the conclusion that the association rate constant must be greater than 10^8 M^{-1} s^{-1}, approaching the diffusion-controlled limit.

Displacement of Ras·mant-GppNHp from its complex with NF1 by the nonfluorescent Ras·GppNHp complex leads to an easily measurable signal for dissociation (Figure 5). The values for the dissociation rate constants are shown in Table 2. K_D values for the Ras·mant-GppNHp and Ras·mant-GTP complexes have been calculated from the association and dissociation rate constants. Since the rate of the cleavage reaction is of the same order of magnitude, or even higher than, the dissociation rate constant in the case of the NF1-catalyzed GTPase reaction, K_M, given by $(k_{diss} + k_{cat})/k_{on}$, is different from K_D and is also shown in Table 2.

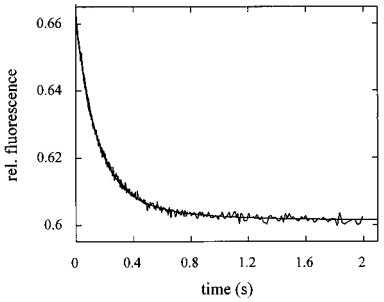

FIGURE 5: Displacement of 0.1 μM H-Ras·mant-GppNHp from its complex with NF1-333 (1 μM) by 10 μM H-Ras·GppNHp. The fitted curve was obtained using a single exponential equation (k_{off} = 6.8 s^{-1}). Conditions as in Figure 1.

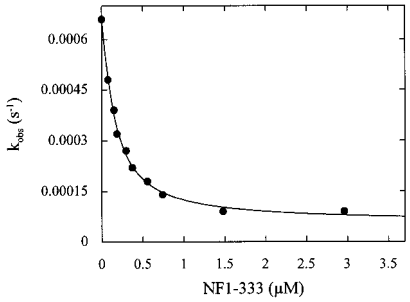

FIGURE 6: Dependence of the rate constant for dissociation of mant-GppNHp from its complex with H-Ras on the concentration of NF1-333. H-Ras·mant-GppNHp (0.1 μM) was incubated with increasing concentration of NF1-333 and the dissociation of the fluorescent analogue in the presence of a large excess of nonfluorescent GppNHp was measured. The observed rate constants, obtained from a single exponential fit, were plotted against the NF1-333 concentration as indicated (K_D = 0.115 μM). The fitted line was obtained using an equation describing the transition from a rapidly dissociating species to a more slowly dissociating species on binding NF1, as described (Herrmann *et al*., 1996). Conditions as in Figure 1.

The affinity of NF1-333 to Ras·mant-GppNHp could also be determined in another manner. On complex formation between Ras·mant-GppNHp and NF1-333 a guanine nucleotide dissociation inhibitor effect can be observed, which leads to the reduction of the rate constant for mant-GppNHp dissociation from Ras. Such an effect is also observed for the interaction between Ras·GppNHp and its downstream effectors and has been used to measure the affinity constants for such complexes (Herrmann *et al*., 1995, 1996). Figure 6 shows the dependence of this effect on the concentration of NF1-333. Evaluation of this experiment according to Herrmann *et al*. (1996) leads to a value of 0.115 μM for the K_D between NF1 and Ras·mant-GppNHp, in good agreement with the value obtained from the measurement of the association and dissociation rate constants. The limiting value for the rate of mant-GppNHp dissociation from the Ras·mant-GppNHp·NF1-333 complex is 5.7 × 10^{-5} s^{-1} or a factor of ca. 10 slower than from the binary complex with Ras.

CONCLUSION

We have investigated the interaction of the catalytic fragments of p120-GAP and neurofibromin, GAP-334 and

NF1-333, with the three isoforms of Ras, and find that there is no significant difference in the kinetics or mechanism between N-, H-, and K-Ras. There is however a very significant difference between the NF1 and the GAP-catalyzed reaction on the three Ras proteins. As noted before (Gideon *et al*., 1992; Wiesmüller & Wittinghofer, 1992; Eccleston *et al*., 1993; Moore *et al*., 1993; Bollag & McCormick, 1991), there is a large difference in the affinity between Ras in the triphosphate form and the two GAP molecules. We show here that this difference is due in part to an estimated severalfold difference in the association rate constants and that the dissociation rate constants are estimated to be different by 2−3 orders of magnitude. The results of Nixon *et al*. (1995) using a P_i-monitor to follow release of P_i on interaction of Ras·GTP with GAP and NF1 also confirmed the difference in affinity between the two GTPase activating proteins. As in the present study, it was not possible to measure the association rate of GAP and Ras·GTP, but the measured rate constant for NF1 association with Ras·mant-GTP (5×10^7 M^{-1} s^{-1}) was in good agreement with the values measured in the present work. The results obtained by Nixon *et al*. were interpreted in terms of a model in which a rate-limiting conformational change occurs before a rapid cleavage step, but the data can be equally well explained assuming, as in the present work, that cleavage itself is rate-limiting.

The difference between GAP and NF1 can only be partially explained by the finding that the properties of the catalytic domain of p120-GAP are influenced by the N-terminal two-thirds of the protein (Gideon *et al*., 1992; Bryant *et al*., 1996), in contrast to what is found for neurofibromin (Gideon *et al*., 1992; Bollag *et al*., 1993). It is clear from the experiments reported here that the GAP-catalyzed GTPase reaction on Ras is preceded by a fast equilibrium between Ras and p120-GAP, whereas in the reaction with neurofibromin the initial binding reaction is slower and that dissociation of the Ras·NF1 complex and the chemical cleavage take place on a similar time scale. It is likely that these mechanistic differences are brought about by structural differences between the two proteins, as suggested by proteolysis experiments (Ahmadian *et al*., 1996). The recent X-ray structure determination of GAP-334 (Scheffzek *et al*., 1996) has shown that this fragment of p120-GAP consists of two domains, one of which corresponds to the minimal catalytic domain as defined by proteolysis experiments and by sequence homology (Ahmadian *et al*., 1996), whereas the other one might in fact be different between different Ras-GAPs and contribute to the differences described above. Which, if any, of these mechanistic and structural differences contributes to the different biological roles of these Ras-GAPs as suggested by gene disruption experiments (Henkemeyer *et al.*, 1995; Vogel *et al.*, 1995; Jacks *et al.*, 1994) remains unclear.

The similarities in the interaction of GAP and NF1 with Ras·GTP suggest that the basic mechanism of activation of the GTPase activity is similar for the two proteins. The results reported are in keeping with the idea that their effect is exerted directly at the stage of GTP cleavage by Ras rather than on a rate-limiting conformational change preceding an intrinsically rapid hydrolysis step. The hypothesis that GAP may contribute residues essential for the hydrolysis reaction is supported not only by the data reported here, but most dramatically by the demonstration that Ras·GDP can form a stable complex with aluminum tetrafluoride and GAP or NF1, in contrast to the situation with Ras·GDP alone (Mittal *et al.*, 1996). It therefore seems highly likely that Ras and GAPs cooperate intermolecularly in a similar manner to the Ras-like G domain and the helical domain of the α-subunits of heterotrimeric G-proteins, which function intramolecularly (Sondek *et al.*, 1994; Coleman *et al.*, 1994; Sternweiss & Gilman, 1982; Biday *et al.*, 1985). As suggested before, GAPs probably contribute one or more arginines to the active site of Ras to stabilize the transition state of the GTPase such that the reaction rate is accelerated by several orders of magnitude (Mittal *et al.*, 1996; Wittinghofer *et al.*, 1993). With the methods to measure individual rate constants at hand, together with the knowledge of the three-dimensional structure of GAP-334, it will be possible, by using site-specific mutants, to further define how particular residues are involved in GAP-mediated GTP hydrolysis.

ACKNOWLEDGMENT

We thank Iris Simon and Christian Lenzen for helpful discussions; Patricia Stege, Andrea Beste, and Dorothee Vogt for expert technical assistance; and Rita Schebaum for valuable secretarial assistance.

REFERENCES

Ahmadian, M. R., Wiesmüller, L., Lautwein, A., Bischoff, F. R., & Wittinghofer, A. (1996) *J. Biol. Chem. 271*, 16409−16415.
Baba, H., Fuss, B., Urano, J., Poullet, P., Watson, J. B., Tamanoi, F., & Macklin, W. B. (1995) *J. Neurosci. Res. 41*, 846−858.
Bagshaw, C., Eccleston, J. F., Eckstein, F., Goody, R. S., Gutfreund, H., & Trentham, D. R. (1974) *Biochem. J. 141*, 351−364.
Ballester, R., Marchuk, D., Boguski, M., Saulino, A., Letcher, R., Wigler, M., & Collins F. (1990) *Cell 63*, 851−859.
Bigay, J., Deterre, P., Pfister, C., & Chabre, M. (1985) *FEBS Lett. 191*, 181−185.
Boguski, M. S., & McCormick, F. (1993) *Nature 366*, 643−654.
Bollag, G., & McCormick, F. (1991) *Nature 351*, 576−579.
Bollag, G., McCormick, F., & Clark, R. (1993) *EMBO J. 12*, 1923−1927.
Bourne, H. R., Sanders, D. A., & McCormick, F. (1991) *Nature 349*, 117−127.
Brownbridge, G. G., Lowe, P. N., Moore, K. J. M., Skinner, R. H., & Webb, M. R. (1993) *J. Biol. Chem. 268*, 10914−10919.
Bryant, S. S., Mitchell, A. L., Collins, F., Miao, W., Marshall, M., & Jove, R. (1996) *J. Biol. Chem. 271*, 5195−5199.
Cawthon, R. M., Weiss, R., Xu, G. F., Viskochil, D., Culver, M., Stevens, J., Robertson, M., Dunn, D., Gesteland, R., O'Connel, P., & White, R. (1990) *Cell 62*, 193−201.
Coleman, D. E., Berguis, A. M., Lee, E., Linder, M. E., Gilman, A. G., & Sprang, S. R. (1994) *Science 265*, 1405−1412.
Cullen, P. J., Hsuan, J. J., Truong, O., Letcher, A. J., Jackson, T. R., Dawson, A. P., & Irvine, R. F. (1995) *Nature 376*, 527−530.
Eccleston, J. F., Moore, K. J. M., Morgan, L., Skinner, R. H., & Lowe, P. N. (1993*) J. Biol. Chem. 268*, 27012−27019.
Gideon, P., John, J., Frech, M., Lautwein, A., Clark, R., Scheffler, J. E., & Wittinghofer, A. (1992) *Mol. Cell. Biol. 12*, 2050−2056.
Henkemeyer, M., Rossi, D. J., Holmyard, D. P., Puri, M. C., Mbamalu, G., Harpal, K., Shih, T. S., Jacks, T., & Pawson, T. (1995) *Nature 377*, 695−701.
Herrmann, C., Martin, G. A., & Wittinghofer, F. (1995) *J. Biol. Chem. 270*, 2902−2905.
Herrmann, C., Horn, G., Spaargaren, M., & Wittinghofer, A. (1996) *J. Biol. Chem. 271*, 6794−6800.
Jacks, T., Shih, T. S., Schmidt, E. M., Bronson, R. T., Bernards, A., & Weinberg, R.A. (1994) *Nat. Genet. 7*, 353−361.
John, J., Sohmen, R., Feuerstein, J., Linke, R., Wittinghofer, A., & Goody, R. S. (1990) *Biochemistry 29*, 6058−6065.

Li, Y., Bollag, G., Clark, R., Stevens, J., Conroy, L., Fults, D., Ward, K., Friedman, E., Samowitz, W., Robertson, M., Bradley, P., McCormick, F., White, R., & Cawthon, R. (1992) *Cell 69*, 275−281.

Lowy, D. R., & Willumsen, B. M. (1993) *Annu. Rev. Biochem. 62*, 851−891.

Maekawa, M., Li, S., Iwamatsu, A., Morishita, T., Yokota, K., Imai, Y., Kohsaka, S., Nakamura, S., & Hattori, S. (1994) *Mol. Cell. Biol. 14*, 6879−6885.

Martin, G. A., Viskochil, D., Bollag, G., McCabe, P. C., Crosier, W. J., Haubruck, H., Conroy, L., Clark, R., O'Connel, P., Cawthon, R. M., Innis, M. A., & McCormick, F. (1990) *Cell 63*, 843−849.

Mittal, R., Ahmadian, M. R., Goody, R. S., & Wittinghofer, A. (1996) *Science 273*, 115−117.

Moore, K. J. M., Webb, M. R., & Eccleston, J. F. (1993) *Biochemistry 32*, 7451−7459.

Neal, S. E., Eccleston, J. F., & Webb, M. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3562−3565.

Nixon, A. E., Brune, M., Lowe, P. N., & Webb, M. R. (1995) *Biochemistry 34*, 15592−15598.

Rensland, H. (1992) Ph.D. Thesis, Ruprecht-Karls-Universität Heidelberg, Germany.

Rensland, H., Lautwein, A., Wittinghofer, A., & Goody, R. S. (1991) *Biochemistry 30*, 11181−11185.

Scheffzek, K., Lautwein, A., Kabsch, W., Ahmadian, M. R., & Wittinghofer, A. (1996) *Nature 384*, 591−596.

Skinner, R. H., Bradley, S., Brown, A. L., Johnson, N. J. E., Rhodes, S., Stammers, D. K., & Lowe, P. N. (1991). *J. Biol. Chem. 266*, 14163−14166.

Sondek, J., Lambright, D. G., Noel, J. P., Hamm, H. E., & Sigler, P. B. (1994) *Nature 369*, 621−628.

Sternweiss, P. C., & Gilman, A. C. (1982) *Proc. Natl. Acad. Sci. USA 79*, 4888−4891.

Trahey, M., & McCormick, F. (1987) *Science 238*, 542−545.

Trahey, M., Wong, G., Halenbeck, R., Rubinfeld, B., Martin, G. A., Ladner, M., Long, C. M., Crosier, W. J., Watt, K., Koths, K., & McCormick, F. (1988) *Science 242*, 1697−1700.

Tucker, J., Sczakiel, G., Feuerstein, J., John, J., Goody, R. S., & Wittinghofer, A. (1986) *EMBO J. 5*, 1351−1358.

Viskochil, D., Buchberg, A. M., Xu, G., Cawthon, R. M., Stevens, J., Wolff, R. K., Culver, M., Carey, J. C., Copeland, N. G., Jenkins, N. A., White, R., & O'Connel, P. (1990) *Cell 62*, 187−192.

Vogel, K. S., Brannan, C. I., Jenkins, N. A., Copeland, N. G., & Parada, L. F. (1995) *Cell 82*, 733−742.

Vogel, U., Dixon, R. A. F., Schaber, M. D., Diehl, R. E., Marshall, M. S., Scolnick, E. M., Sigal, I., & Gibbs, J. B. (1988) *Nature 335*, 90−93.

Wallace, M. R., Andersen, L. B., Saulino, A. M., Gregory, P. E., Glover, T. W., & Collins, F. S. (1991) *Nature 353*, 864−866.

Wiesmüller, L., & Wittinghofer, A. (1992) *J. Biol. Chem. 267*, 10207−10210.

Wittinghofer, A., & Herrmann, C. (1995) *FEBS Lett. 369*, 52−56.

Wittinghofer, F., Pai, E. F., & Goody, R. S. (1993) GTPases in Biology I, in *Handbook of Experimental Pharmacology* (Dickey, B. F., & Birnbaumer, L., Eds.) 108/I, Chapter 14, pp 195−212, Springer-Verlag, Berlin-Heidelberg.

Xu, G. F., O'Connel, P., Viskochil, D., Cawthon, R., Robertson, M., Culver, M., Dunn, D., Stevens, J., Gesteland, R., White, R., & Weiss, R. (1990a) *Cell 62*, 599−608.

Xu, G. F., Lin, B., Tanaka, K., Dunn, D., Wood, D., Gesteland, R., White R., Weiss, R., & Tamanoi, F. (1990b) *Cell 63*, 835−841.

BI962556Y