Conformational States of Human Rat Sarcoma (Ras) Protein Complexed with Its Natural Ligand GTP and Their Role for Effector Interaction and GTP Hydrolysis*

Michael Spoerner†, Konstantin Hosza‡, Johann A. Poetzl‡, Kerstin Reiss‡, Petra Ganser‡, Matthias Geyer§, and Hans Robert Kalbitzer††

From the ††Universität Regensburg, Institut für Biophysik und Physikalische Biochemie, Universitätsstrasse 31, 93053 Regensburg and the †‡Max-Planck-Institut für Molekulare Physiologie, Abteilung Physikalische Biochemie, Otto-Hahn-Str. 11, 44227 Dortmund, Germany

The guanine nucleotide-binding protein Ras exists in solution in two different conformational states when complexed with different GTP analogs such as GppNHp or GppCH₂p. State 1 has only a very low affinity to effectors and seems to be recognized by guanine nucleotide exchange factors, whereas state 2 represents the high affinity effector binding state. In this work we investigate Ras in complex with the physiological nucleoside triphosphate GTP. By polarization transfer ³¹P NMR experiments and effector binding studies we show that Ras(1–166)K12 contains two different conformational states, defined as states 1 and 2, which are in chemical equilibrium in solution (1–3). These conformational states are actually only defined for Ras with guanine triphosphate bound (T), a state that may be different from a state with guanine diphosphate bound (D). Therefore, we will denote the two states in the following as state 1(T) and 2(T) whenever the nucleotide ligand is of concern. The equilibrium between the two states is strongly influenced by the nature of the guanine nucleotide bound to Ras and can be shifted by interaction with effector proteins or regulators such as GTPase activation proteins (GAPs).² It was found that GTP analogs GppNHp and GppCH₂p partially shift the equilibrium toward state 1. For the complex of wild-type Ras with physiological GTP itself the existence of the two states could not be shown yet. However, for the slowly hydrolyzing mutants Ras(Q61H) and Ras(Q61L) a second weakly populated state could be observed. Because at this time functional data were not available, from an analysis of the chemical shift changes it was tentatively assigned to state 2, whereas the mainly populated state in Ras complexed with GTP was assumed to be state 1 (1).

When bound to guanosine triphosphates Ras exists in two different conformational states, defined as states 1 and 2, which are in chemical equilibrium in solution (1–3). These conformational states are actually only defined for Ras with guanine triphosphate bound (T), a state that may be different from a state with guanine diphosphate bound (D). Therefore, we will denote the two states in the following as state 1(T) and 2(T) whenever the nucleotide ligand is of concern. The equilibrium between the two states is strongly influenced by the nature of the guanine nucleotide bound to Ras and can be shifted by interaction with effector proteins or regulators such as GTPase activation proteins (GAPs).² It was found that GTP analogs GppNHp and GppCH₂p partially shift the equilibrium toward state 1. For the complex of wild-type Ras with physiological GTP itself the existence of the two states could not be shown yet. However, for the slowly hydrolyzing mutants Ras(Q61H) and Ras(Q61L) a second weakly populated state could be observed. Because at this time functional data were not available, from an analysis of the chemical shift changes it was tentatively assigned to state 2, whereas the mainly populated state in Ras complexed with GTP was assumed to be state 1 (1). The conformation of state 2 closely corresponds to the effector-bound state and becomes stabilized if Ras is bound to effectors (1, 4 – 6). Previously, similar results were shown for three other proteins belonging to the Ras superfamily, Ran, Cdc42, and Ral (7–9). Here in the guanosine triphosphate-bound state the protein also exists in two different conformational states. One of them also becomes stabilized by effector binding.

Different mutations in the switch I region of Ras or binding to the GTP analogs GppNHp or GppCH₂p shift the equilibrium between the two states toward state 1 (1, 2, 10). Binding studies using NMR spectroscopy as well as calorimetric or fluorescence-based methods show that these conformational equilibria directly influence the interaction between Ras and its effectors (3, 10, 11). Stabilizing state 1 by small ligands is a

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† To whom correspondence should be addressed. E-mail: hans-robert.kalbitzer@biologie.uni-regensburg.de.

‡ The abbreviations used are: GAP, GTPase activating protein; GppNHp, guanosine 5′-(β,γ-imido)triphosphate; GppCH₂p, guanosine 5′-(β,γ-methylene)triphosphate; Raf-RBD, Raf binding domain of Raf kinase (rapid fibrosarcoma); RaIGDS, Ras guanine nucleotide dissociation stimulator; Ras, protein product of the proto-oncogene ras (rat sarcoma); RBD, Raf binding domain; GTP$S$, guanosine 5′-3-O-(thio)triphosphate.
Conformational States of the Ras-GTP Complex

A novel approach to reduce the affinity of Ras for its effectors (12, 13). Very recent results lead to the assumption that state 1(T) of Ras is recognized by guanine nucleotide exchange factors and thus could represent an important conformational state in the Ras activation/inactivation cycle (14).

Mutation of a specific threonine residue (Thr-35 in Ras), totally conserved in all members of the Ras superfamily, to serine drastically decreases the affinity of Ras to its effectors, although this threonine residue is not directly involved in effector binding (15, 16). These mutations affect Ras to become a partial loss-of-function mutant, which can still trigger the MAP kinase pathway by interacting with Raf kinases but binds too weakly to RalGDS and thus cannot activate the Ral-dependent signaling pathway (17). Both the hydroxyl group and the methyl group of Thr-35 are necessary for Ras to stabilize its crucial effector binding conformation, which is essential for effector binding at physiological concentrations (11).

Because this conserved threonine residue plays such an important role in the switch between the active and inactive state we have studied its role in terms of the GTPase activity by investigating Ras mutants T35S and T35A, as well as other selected Ras variants containing mutations in the P-loop, the switch I and switch II regions, respectively. In this work we elucidate the importance of this dynamic behavior of Ras in the physiological GTP-bound state in terms of effector interactions as well as the intrinsic GTPase activity.

**EXPERIMENTAL PROCEDURES**

**Protein Purification**—Wild-type and mutants of full-length human H-Ras (residues 1–189) and the truncated variant Ras(1–166) (residues 1–166) were expressed in *Escherichia coli* and purified as described before (18). The final purity of the protein was >95% as judged from the SDS-PAGE. Nucleotide exchange from GDP to GTP was performed by incubation of Ras in the presence of 200 mM (NH₄)₂SO₄ and 50-fold excess of GTP as described by John et al. (19). Free nucleotides were removed by gel filtration. The Ras concentration was determined by measuring the concentration of bound nucleotide using C18-reversed phase chromatography with a calibrated detector system. Ras-binding domains of human RalGDS (RalGDS-RBD, residues 11 to 97) and human Raf-1 (Raf-RBD, residues 51 to 131) were expressed in *E. coli* and purified as described before (20, 21).

**NMR Spectroscopy**—Typically 1 mM Ras-Mg²⁺-GTP was dissolved in buffer A (40 mM HEPE/NaOH, pH 7.4, 10 mM MgCl₂, 150 mM NaCl, 2 mM 1,4-dithioerythritol, 0.1 mM 2,2-dimethyl-2-silapentane-5-sulfonate in 5% D₂O, 95% H₂O).

For binding studies aliquots of a highly concentrated (5–7 mM) Ras-RBD solution contained in the same buffer was added in appropriate amounts to the samples.

³¹P NMR spectra were recorded with a Bruker Avance-500 NMR spectrometer operating at ³¹P frequency of 202 MHz. Measurements were performed in a 10-mm probe using 8- or 10-mm Shigemi sample tubes at various temperatures. Two-dimensional ³¹P-³¹P NOESY (22) spectra were recorded with mixing times of 2.5 s and a total repetition time of 10 s. Protons were decoupled during t₂ evolution by a proton 180° pulse and during t₁ by a GARP (23) sequence with a strength of the B₁-field of 900 Hz.

³¹P longitudinal relaxation times (T₁) were determined at 278 K by an inversion recovery sequence using a repetition time of 20 s. The obtained signal integrals were fitted by a three parameter fit to the Equation 1,

\[ M_z(t) = M_0 + (M_1(t) - M_0)e^{-\frac{t}{T_1}} \]  

(Eq. 1)

with \( M_z(t) \) the z-magnetization (signal integral) at time \( t \), and \( M_0 \) the magnetization in thermal equilibrium. Protons were decoupled during magnetization recovery and data acquisition by GARP (23) sequence.

The ³¹P NMR saturation transfer experiments were performed at 278 K using a strength of the B₁ field of 18 Hz. A total repetition time of 20 s was used for each scan. For identifying the resonance frequency of state 1, a presaturation time of 1 s was used. To determine the rate of exchange, presaturation was applied for 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, and 1.6 s at the frequency of the low populated state of the γ-phosphorus. 100 cycles of the experiments, each with 8 scans, were added to avoid instability effects of the sample or the spectrometer, respectively. For determination of direct saturation effects the same experiments was performed applying presaturation at corresponding frequencies up- or downfield of the observed signal, respectively.

If no direct saturation effects can be observed, data can be fitted by Equation 2.

\[ I_A(\tau) = \frac{I_A(0)}{1 + \frac{1}{\tau_A}e^{-\frac{\tau}{\tau_A}}} \left( 1 + \frac{1}{T_{1A}} \right) \]  

(Eq. 2)

With \( I_A(\tau) \) the area of the non-saturated signal after saturation of resonance B for the time \( \tau \), and \( T_{1A} \) the longitudinal relaxation time of signal A (estimated from inversion recovery experiment). The mean lifetime \( \tau_A \) of the protein in state A is related to the rate constant \( k_{AB} \) of the transition from state A to state B by \( k_{AB} = \frac{1}{\tau_A} \) (see e.g. Ref. 24). The effects of direct saturation can be approximated by Equation 3.

\[ I_A(\tau) = \frac{I_A(0)}{1 + \frac{1}{\tau_A}e^{-\frac{\tau}{\tau_A}}} \left( 1 + \frac{1}{T_{1A}} \right) \]  

(Eq. 3)

The value for the correction factor \( a \) can be calculated from a fit of Equation 3 and be inserted into Equation 4.

\[ I_A(\tau) = \frac{I_A(0)}{1 + \frac{1}{\tau_A}e^{-\frac{\tau}{\tau_A}}} \left( a + \frac{1}{T_{1A}} \right) \]  

(Eq. 4)

For indirect referencing of the ³¹P NMR resonances to DSS, a Ξ-value of 0.4048073561 reported by Maurer and Kalbitzer (25) was used that corresponds to 85% external phosphoric acid contained in a spherical bulb. Temperature was controlled using the line separation of the methylene and hydroxyl group of external ethylene glycol for calibration (26).
Conformational States of the Ras-GTP Complex

Kinetics of Hydrolysis—To determine the kinetics of intrinsic GTPase reaction by Ras, 100 μM Ras-GTP in 40 mM HEPES/NaOH, pH 7.4, 150 mM NaCl, 10 mM MgCl2, 2 mM 1,4-dithioerythritol were incubated at 310 K. Care was taken that free nucleotide was completely removed to ensure the single turnover reaction. At different times of incubation samples were frozen in liquid nitrogen, and the nucleotides afterward were separated by HPLC using a C18 reversed phase column with 100 mM phosphate buffer, pH 6.5, 10 mM tetra butyl-ammonium bromide, 7% acetonitrile as buffer. The absorbance was measured at a wavelength of 254 nm and the lines corresponding to GDP and GTP were integrated. The concentration of GTP compared with the total nucleotide at different incubation times was determined. A single exponential function was fitted to the values. For each Ras variant the rates of hydrolysis were determined as a function of the concentrations of RBD added. Hydrolysis rates given are measured at saturation that was usually observed when the RBD concentration was 1.5 times the Ras concentration. Therefore the given maximum rate constant should represent the values of the Ras variants totally complexed with effector. The only exception was Ras(V29G/I36G), which shows an estimated affinity to Raf-RBD of ~1 mM as derived from the 31P NMR titration and GTPase activation experiments.

RESULTS

Assignment of the Conformational States of Ras in the GTP-bound Form—In the GTP-bound form Ras(wt) seems to exist exclusively in one conformational state corresponding to three single signals in the 31P NMR spectrum, one for each phosphorus of the bound nucleotide (Fig. 1A). The 31P NMR spectrum of Ras(T35A) shows one set of resonances too, but with different chemical shift values compared with that obtained for wild-type Ras. In contrast, the serine mutant Ras(T35S) shows a pair of resonance lines for the γ-phosphate group, indicating an equilibrium of two states in solution (Fig. 1A). The chemical shift value of the more intense line corresponds to that found in Ras(T35A), the weaker line corresponds to that found in the wild-type protein (Table 1).

The 31P resonances of Ras-Mg2+ were assigned earlier by comparison of the chemical shifts of free Mg2+ -GTP with bound GTP (1, 27). Because binding of nucleotides to proteins can induce large chemical shift changes, a direct transfer of resonance assignments from the unbound to the bound state can be precarious. We therefore performed a two-dimensional 31P-31P NOESY experiment on Ras(T35A)-Mg2+ -GTP. The spectrum is shown in Fig. 1B. The assignment of the resonance of the β-phosphate can be directly deduced from the cross-peak patterns because only that shows cross-peaks to the two other phosphate 31P resonances. The chemical shift of the β-phosphate of bound nucleotide (~15.23 ppm) largely

![FIGURE 1.](image)

**TABLE 1**

| Protein-complex | α-Phosphate | β-Phosphate | γ-Phosphate | K12 | ΔG12 | kcat |
|-----------------|-------------|-------------|-------------|-----|------|------|
| Ras(1-166)/Mg2+ -GTP | (-11.2) | (-11.71) | (-15.2) | -14.85 | -6.63 | -7.95 | 11.3 | -5.6 | 0.028 |
| Ras(wt)/Mg2+ -GTP | - | - | - | - | - | - | - | - | - |
| + Raf-RBD | -11.58 | -14.81 | -8.08 | 0.029 |
| + RafGDS-RBD | -11.68 | -14.86 | -7.91 | - |
| Ras(T35S)/Mg2+ -GTP | -11.14 | -15.23 | -6.39 | <0.04 | >5.3 | 0.005 |
| + Raf-RBD | -10.67 |-15.3 | -6.37 | 0.005 |
| + RafGDS-RBD | -11.16 | -15.24 | -6.37 | 0.005 |
| Ras(T35S)/Mg2+ -GTP | -11.21 | -15.18 | -3 | -6.6 | -7.92 | 0.29 | 2.1 | 0.011 |
| + Raf-RBD | -11.59 | -14.78 | -8.00 | 0.031 |
| + RafGDS-RBD | -11.81 | -14.85 | -7.81 | - |
| GTp | -11.07 | -22.31 | -7.50 | <0.001 |
| Mg2+ -GTP | -10.57 | -19.22 | -5.58 | <0.001 |

* Measurements were performed at 278 K at pH 7.4. Samples contained 2 mM GTP, 0.1 mM 2,2-dimethyl-2-silapentane-5-sulfonate in 5% D3O, 95% H2O. The chemical shifts for Mg2+ GTP were measured at saturation concentrations of 3 mM MgCl2. Chemical shifts correspond to the fourfold negatively charged nucleotide found at neutral and basic pH values.

* Values determined for the complex were determined at various concentrations of Raf-RBD. The obtained maximum rate is given, which should represent saturation of Ras with effector-RBD. The temperature was 310 K (for details, see “Experimental Procedures”).

* The resonance position could not be determined because of the signal overlap and/or the low signal-to-noise ratio but most probably corresponds to that observed in the effector complex.

* Even at the mM concentration used in the titration experiment there seems to be no binding of RafGDS-RBD, which one would expect to detect as line broadening.

* Difference in free energy between states 1 and 2: ΔG12 = −RT lnK12.
Conformational States of the Ras-GTP Complex

In free Mg$^{2+}$-GTP the chemical shifts of the $\alpha$ - and $\gamma$-phosphates are 10.57 and $-5.58$ ppm, respectively, at pH 7.4, as used herein and in all other experiments performed. The frequencies of the other resonances at $-11.14$ and $-6.39$ ppm are rather close to the corresponding chemical shift values and can thus be assigned to the $\alpha$ - and $\gamma$-phosphates of the bound GTP. With these experiments, the earlier used chemical shift assignment could thus be confirmed.

In analogy to the results obtained for several GTP analogs complexed with Ras, the conformation of Ras(T35A)-Mg$^{2+}$-GTP should therefore correspond to state 1. In Ras(T35S)-Mg$^{2+}$-GTP, two lines are observed for the $\gamma$-phosphate group, one with the same frequency as that observed in Ras(T35A)-Mg$^{2+}$-GTP and thus assigned to state 1, although the high-field shifted resonance line is assigned to state 2 (Fig. 1A). Correspondingly, we conclude that Ras(wt)-Mg$^{2+}$-GTP occurs predominantly in state 2.

Equilibrium Constants and Exchange Rates in Ras Complexed with GTP—For Ras(T35S)-Mg$^{2+}$-GTP, the equilibrium constant $K_{12}$ can be directly determined from the integrals of the two $\gamma$-phosphate lines under the experimental conditions used here. At 278 $K$, $K_{12}$ is 0.29 ± 0.2 (Table 1). For Ras(T35A)-Mg$^{2+}$-GTP and Ras(wt)-Mg$^{2+}$-GTP such a determination is more difficult, because direct inspection of the spectra does not allow the unambiguous identification of lines assignable to an alternate state. However, the frequencies where these resonances are to be expected can be deduced from the spectrum of the Ras(T35S) mutant. For the $\gamma$-phosphate line of Ras(T35A)-Mg$^{2+}$-GTP a resonance line for state 2 is expected at $-8.6$ ppm. Here, no resonance line is visible. From the signal-to-noise ratio an upper limit for $K_{12}$ can be given as 0.04. For Ras(wt)-Mg$^{2+}$-GTP a very weak signal is visible at the position where the resonance of state 1 is to be expected (Fig. 1A).

Provided that the resonance line in question for Ras(wt)-Mg$^{2+}$-GTP is indeed at $-6.6$ ppm, we performed a $^{31}$P NMR saturation transfer experiment. Fig. 2A shows the integrals of the $\gamma$-phosphate resonance of state 2 as a function of the saturation frequencies used. Indeed, a clear minimum of the integral for a saturation frequency at $-6.59$ ppm was obtained. This indicates that a polarization transfer from the saturated signal to the observed signal takes place that confirms the existence of a second state in dynamic equilibrium with state 2 in full-length wild-type Ras although at a low population. For a weak, long saturation a Lorentzian response function is to be expected. However, for frequencies close to the reporter resonance additional direct saturation of the resonance line leads to a distortion of response curve as it observed in Fig. 2A. The same experiment was performed for frequencies positioned symmetrically on the other side of the reporter signal as control for direct saturation effects. Performing the analogous experiments using the T35A mutant with presaturation frequencies at the resonance position expected (Fig. 1A).
Conformational States of the Ras-GTP Complex

| Complex                  | T/K  | \(\tau_m/\text{ms}\) | \(\tau_s/\text{ms}\) | \(k_{12}/s^{-1}\) | \(k_{21}/s^{-1}\) | \(k_{11}/s^{-1}\) |
|--------------------------|------|----------------------|----------------------|-----------------|-----------------|-----------------|
| Ras(1–166)(wt)-Mg\(^{2+}\)-GTP | 278  | 175 ± 45             | 2000 ± 500           | 5.7 ± 1.5       | 0.5 ± 0.1       | 7 ± 2           |
| Ras(wt)-Mg\(^{2+}\)-GppNHp\(^{a}\) | 278  | 13 ± 2               | 24 ± 4               | 80 ± 5          | 42 ± 5          | 122 ± 10        |
| Ras(wt)-Mg\(^{2+}\)-GppCH\(_2\)P\(^{a}\) | 278  | 13 ± 2               | 26 ± 4               | 80 ± 5          | 39 ± 5          | 119 ± 10        |

\(^{a}\) Data from Spoerner et al. (2).

The lifetime of states 1 and 2 in wild-type Ras-complexed with Mg\(^{2+}\)-GTP

Experimental conditions are as described in the legend to Fig. 2. The exchange rate \(k_{ex}\) is defined as \(k_{ex} = 1/\tau_{ex} = 1/\tau_s + 1/\tau_m\).

| Complex                  | T/K  | Relaxation times \(T_1/\text{s}\) of the resonances |
|--------------------------|------|---------------------------------------------------|
| Mg\(^{2+}\)-GDP\(^{a}\) | 278  | 0.53 ± 0.01                                       |
| Mg\(^{2+}\)-GTP\(^{a}\) | 278  | 0.45 ± 0.01                                       |
| Ras(wt)-Mg\(^{2+}\)-GDP\(^{a}\) | 278  | 4.8 ± 0.3                                         |
| Ras(wt)-Mg\(^{2+}\)-GppNHp\(^{a}\) | 278  | 4.5 ± 0.4                                         |
| Ras(wt)-Mg\(^{2+}\)-GppCH\(_2\)P\(^{a}\) | 278  | 4.3 ± 0.3                                         |

\(^{a}\) The sample contained 10 mM nucleotide in 40 mM Hepes/NaOH with an final pH of 7.4, 30 mM MgCl\(_2\), 10% D\(_2\)O, 90% H\(_2\)O.

\(^{b}\) The sample contained 2 mM Ras in buffer A (see "Experimental Procedures").

\(^{c}\) Data from Sponsor et al. (2).

**3\(^{13}\)P Longitudinal Relaxation Times of Ras Nucleotide Complexes—**

The relaxation time \(T_1\) is mainly determined by chemical shift anisotropy and the dipolar interaction that are dependent on the structure of the environment of the phosphorous nucleus under consideration and thus on the protein conformation. Therefore we determined \(T_1\) relaxation times of Mg\(^{2+}\)-GTP and Mg\(^{2+}\)-GDP bound to Ras(wt) and Ras(T35A), which both represent predominantly just one of the two conformational states, respectively (Table 3). As a reference, we also measured the relaxation times for the free nucleotides. Compared with the unbound state, Mg\(^{2+}\)-GDP and Mg\(^{2+}\)-GTP showed about 10-fold higher \(T_1\) relaxation times when bound to Ras, as would be expected due to the higher rotational correlation time of the protein-nucleotide complex. Mg\(^{2+}\)-GTP complexed with either Ras(wt) or Ras(T35A) show similar \(T_1\) relaxation times for the \(\alpha\)- and \(\beta\)-phosphate group. An exception holds for the \(\gamma\)-phosphate group. Here a significantly shorter \(T_1\) value is found in complex with the mutant compared with the wild-type protein (Table 3). Values obtained for wild-type Ras (5.5 s) essentially represent state 2, whereas those for Ras(T35A) (4.4 s) represent state 1, respectively.

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**Complex Formation between Ras-Mg\(^{2+}\)-GTP and Ras Mutants with Effectors—**

Binding of the Ras-binding domain (RBD) of Raf to Ras(wt)-Mg\(^{2+}\)-GTP leads to a line-broadening by a factor of 1.5, corresponding to the expected increase of the molecular mass from 21 to 30 kDa in the effector complex. However, the line positions of all phosphate groups remain almost unchanged (Fig. 3, Table 1). This indicates that GTP-bound Ras(wt) exists predominantly in the same conformation, which is also found for Ras-GppNHp in the effector bound state (state 2). The resonances of the alanine mutant Ras(T35A) are somewhat broadened when the RBD of Raf kinase is present in a molar ratio of 1:2 (Ras to RBD), but there are only slight changes in chemical shifts of the \(\gamma\)- and \(\beta\)-phosphate. A somewhat larger shift change is observed for the \(\alpha\)-phosphate group that surprisingly is further upfield but not downfield as expected for a more state 2-like conformation (Table 1). In Ras(T35S) the intensity of the \(\gamma\)-phosphate...
line assigned to state 1 disappears, whereas simultaneously the population of the other γ-phosphate line increases. This is to be expected when the populations of the two states are shifted completely to the effector binding state 2. The data are thus consistent with our earlier assignments of states 1 and 2. As previously observed for Ras complexed with GTP analogs (11), Ras(T35A):Mg$^{2+}$-GTP binds RasGDS only weakly and remains also in state 1 in complex with Raf-RBD (Fig. 3). For the binding of Raf to Ras(T35A):Mg$^{2+}$-GppNHp a dissociation constant of 7 μM was estimated earlier (11). The chemical shift values of phosphate signals of Ras-Mg$^{2+}$-GTP in complex with effector RBDs of Raf kinase and RasGDS are summarized in Table 1.

**Importance of the Conformational Equilibria to the Intrinsic GTPase Activity of Ras**—The dynamic conformational equilibria effects the actual chemical environment of the nucleotide and may thus also influence the intrinsic GTPase activity. Fig. 4 demonstrates that under single turnover conditions at 37 °C Ras(T35A) hydrolyzes bound GTP with \( k_{\text{cat}} \) of 0.005 min$^{-1}$, which is 5 times slower than wild-type Ras with a \( k_{\text{cat}} \) of 0.028 min$^{-1}$. A similar result was first reported by John et al. (28). In contrast, the T35S mutant hydrolyzes GTP with a rate constant of 0.011 min$^{-1}$, which is between those determined for wild-type Ras and Ras(T35A), respectively. This agrees with a simple model where the two conformational states have different intrinsic GTPase activity and the observed enzymatic activity is the population weighted average of the two rate constants because the exchange rate between the two conformational states is more than 4 orders of magnitude faster than the observed hydrolysis rates. Using the rate constants of C-terminal-truncated Ras(wt):Mg$^{2+}$-GTP (with the equilibrium constant \( K_{12} = 11.3 \)) and Ras(T35A):Mg$^{2+}$-GTP as typical examples for the two states, the \( k_{\text{cat}} \) of the pure state 2 should be 0.030 min$^{-1}$ and for the pure state 1 it is 0.005 min$^{-1}$ (Table 1).

**Intrinsic GTP Hydrolysis of Ras in the Presence of Effector-RBD**—Because complex formation between Ras(T35S) and Raf-RBD stabilizes conformational state 2 (Fig. 3), we investigated the GTPase activity in the presence of Raf-RBD. Fig. 4 shows that the intrinsic GTPase activity of Ras(wt), which exists predominantly in state 2 is not (or only slightly) accelerated by addition of Raf-RBD in a concentration where complete complex formation is to be expected. The same results could also be obtained by Herrmann and colleagues (20). Also GTP hydrolysis by the alanine mutant in Ras(T35A) seems not to be affected by effector binding and remains at the low intrinsic value. This mutant cannot switch completely toward state 2 by binding of Raf-RBD, a rate constant very similar to that of wild-type Ras is observed, which confirms that the two different effector states of triphosphate-
Effects of Mutations in the P-loop, in Switch I and Switch II on Conformational Equilibria, Effector Binding, and GTPase Activity—To further characterize this two-state model we have investigated selected P-loop, switch I and II mutants in terms of conformational equilibria, GTPase activity, as well as effector binding. The two P-loop mutants investigated, namely Ras(G12V) and Ras(G13R), exist predominantly in state 2 respective to their chemical shifts and the response to effector interaction (Fig. 5A). Only slight chemical shift changes and some line broadening was observed. In contrast to the oncogenic Ras(G12V) mutant, Ras(G13R) shows GTPase activity similar to wild-type Ras whose GTP hydrolysis is not accelerated by effector binding. The switch I mutants of Ras V29G/I36G, Y32C/C118S, Y32F, Y32R, and Y32W most likely exist predominantly in state 2 (Fig. 5B). Whereas effector binding to the glycine double mutant shows the same behavior as Ras(T35S) by stabilizing state 2, the response of the Tyr-32 mutants on effector binding is not unequivocal because the chemical shift response, the intrinsic GTPase activity and the increase of the GTPase activity by effector binding do not follow a simple scheme. The missing tyrosine ring current effects probably lead to smaller chemical shift changes between the $^{31}$P resonances of states 1 or 2. Arginine and tryptophan in amino acid position 32 lead to a significant increase of the intrinsic GTPase activity (Fig. 6). Effector binding to Ras(Y32R) accelerates hydrolysis $\sim$13-fold compared with wild-type Ras. According to their chemical shifts, switch II mutants Ras(Q61A), Ras(Q61L), Ras(Q61H), and Ras(Y64A) predominantly exist in state 2 when GTP is bound (Fig. 5C). The only exception is Ras(G60A), which exists in the two states with almost identical populations (Fig. 5C). The GTPase activity of Ras(Y64A) and Ras(G60A) increases somewhat with binding of Raf-RBD up to 0.013 min$^{-1}$ in contrast to that of the oncogenic mutant Ras(Q61A) (Table 4 and Fig. 6). The hydrolysis rates were determined as a function of the Raf concentration, the rates given are the rates when saturation is reached (no significant further increase of the rates with increasing Raf concentration). In principle, a binding constant for Raf could be determined by fitting the data with an appropriate model. However, since the expected affinities are in the micromolar to nanomolar range, at the protein concentration of 10 $\mu$M Ras used here an accurate binding constant cannot be determined. Nevertheless, an upper limit of the $K_D$ of the order of 10 $\mu$M can be estimated based on the $^{31}$P NMR titration and/or GTPase acceleration experiments for all Ras variants investigated, with the exception of the double mutant Ras(V29G/I36G) with an estimated $K_D$ value of 1 mM.
TABLE 4

| Protein complex                  | δα/ppm | δβ/ppm | δγ/ppm | δα/ppm | δβ/ppm | δγ/ppm | K12  | K10  |
|----------------------------------|--------|--------|--------|--------|--------|--------|------|------|
| Ras(G12V)-Mg2⁺-GTP              | -11.54 | -14.69 | -7.59  | >10    | 0.02   |        |      |      |
| +RaF-RBD                        | -11.44 | -14.78 | -7.85  | >0.02  | 0.02   |        |      |      |
| Ras(G13R)-Mg2⁺-GTP              | -11.59 | -14.58 | -8.33  | >10    | 0.025  |        |      |      |
| +RaF-RBD                        | -11.45 | -14.52 | -8.52  | >0.02  | 0.025  |        |      |      |
| Ras(V29G/I36G)-Mg2⁺-GTP         | -10.38 | -15.38 | -5.92  | <0.1   | 0.008  |        |      |      |
| +RaF-RBD                        | -11.00 | -14.84 | -7.82  | >0.1   | 0.020  |        |      |      |
| Ras(I1-166)/Y32/C118S+-Mg2⁺-GTP | -10.99 | -15.27 | -5.88  |        |        |        |      |      |
| +RaF-RBD                        | -10.75 | -15.01 | -6.11  | >0.8   | 0.014  |        |      |      |
| Ras(Y32F)-Mg2⁺-GTP              | -11.23 | -15.37 | -5.92  |        |        |        |      |      |
| +RaF-RBD                        | -11.38 | -14.70 | -6.44  |        |        |        |      |      |
| Ras(V32R)-Mg2⁺-GTP              | -10.86 | -15.28 | -5.58  | >0.8   | 0.048  |        |      |      |
| +RaF-RBD                        | -10.86 | -15.00 | -5.87  |        |        |        |      |      |
| Ras(Y32W)-Mg2⁺-GTP              | -12.04 | -15.03 | -5.76  | >0.9   | 0.074  |        |      |      |
| +RaF-RBD                        | -12.21 | -14.95 | -5.87  |        |        |        |      |      |
| Ras(G66A)-Mg2⁺-GTP              | -11.27 | -15.29 | -7.31  |        |        |        |      |      |
| +RaF-RBD                        | -12.02 | -15.42 | -7.76  |        |        |        |      |      |
| Ras(Q61A)-Mg2⁺-GTP              | -11.65 | -15.04 | -8.31  | >20    | <0.001 |        |      |      |
| +RaF-RBD                        | -11.52 | -14.94 | -8.43  |        | <0.001 |        |      |      |
| Ras(Q61H)-Mg2⁺-GTP              | -11.78 | -15.67 | -7.75  | 4.8    | ND     |        |      |      |
| +RaF-RBD                        | -11.84 | -15.75 | -7.73  | 4.3    | ND     |        |      |      |
| Ras(Y64A)-Mg2⁺-GTP              | -11.61 | -14.70 | -7.32  |        |        |        |      |      |
| +RaF-RBD                        | -11.46 | -14.83 | -8.24  |        |        |        |      |      |
| Ras(Y65C)-Mg2⁺-GTP              | -11.62 | -14.87 | -6.62  |        |        |        |      |      |
| +RaF-RBD                        | -11.62 | -14.83 | -8.24  |        |        |        |      |      |

*α* Values for the complex were determined at various concentrations of RaF-RBD. The obtained maximum value at saturation is given. The temperature was 310 K (for details, see "Experimental Procedures").

*β* The affinity between RaF-RBD and Ras(V29G/I36G) is in the *ms* range thus Ras was not totally complexed with RaF-RBD at a RBD concentration of 700 μM.

*γ* On the basis of chemical shift values alone one cannot distinguish between the two states. From data obtained using GppNHp complexes of these Ras variants one would expect that the equilibrium is shifted towards state 2 (10).

*δ* The resonances position could not determined because of the signal overlap.

*ε* Data from Geyer et al. (1). Chemical shifts were corrected for the reference used in this paper.

*Φ* ND, not determined.

**DISCUSSION**

Conformational States of Ras Bound to GTP—When Ras is complexed with nucleoside triphosphates (T), it exists in two conformations, designated as state 1(T) and state 2(T) that were first characterized by the chemical shifts of their γ-phosphate resonance in the complex with GppNHp. In state 2 the α- and γ-phosphate resonance is shifted upfield relatively to the position found in state 1. State 1 was identified as a state that only weakly interacts with effectors but seems to be stabilized by guanine nucleotide exchange factors, whereas state 2 strongly interacts with effectors (1, 7, 11, 14). Binding of effectors usually shifts the equilibrium toward state 2, which is the high-field shifted α- and γ-phosphate resonances gain in intensity after effector binding. An additional feature of the intrinsic Ras dynamics is the observation that mutation of Thr-35 to alanine or serine shifts the equilibrium toward state 1 (11).

Later we could show that a similar behavior is observed for nucleotide analog GppCH₂p where again the α- and γ-phosphate resonances are high-field shifted in state 2 (2). For the complex of wild-type Ras with the analog GTPpS (3) or GTP itself (1) only one set of resonance lines was observed. The resonances of GTPpS bound to wild-type Ras shift only slightly after binding of effectors indicating that the protein already occurs in the strong effector binding state 2. An analysis of the corresponding Thr-35 mutants and their NMR response to effector binding confirmed the assignment to state 2 (3).

The presence of two conformational states in Ras-GTP was first proposed for the mutant Q61H (1). However, whereas the initial assignment of the phosphate resonances is in agreement with the two-dimensional NOESY spectrum recorded here (Fig. 1B), the assignment of the two states compared with RasGppNHp was based on the shift of the two-resonance signal relative to each other. Now, a closer inspection of a spectrum of Ras(1–160)(wt)-Mg2⁺-GTP recorded with a Cryo-QNB probe at a 31P frequency of 242 MHz (Bruker, Rheinstetten, Germany) clearly reveals that a second set of weak resonances exists also in the C-terminal-truncated wild-type protein with an equilibrium constant *K*₂ of 11.3 (Fig. 2B). For the weak resonance downfield from the strong γ-phosphate resonance we can show a conformational exchange by a 31P saturation transfer. The chemical shifts of the α- and γ-resonances indicate that the main conformation of Ras(wt)-Mg2⁺-GTP corresponds indeed to state 2.

In agreement with this assumption the addition of the effectors RalGDS and Raf (Table 1 and Fig. 3) leads only to small chemical shifts changes after binding. The line positions of the weak resonances of Ras(wt)-Mg2⁺-GTP closely corresponds to that of Ras(T35A)-Mg2⁺-GTP found to exist mainly in state 1 when GTP analogs are bound. The latter is confirmed by comparing the spectra of Ras(T35A)-Mg2⁺-GTP with those of the serine mutant. Ras(T35S)-Mg2⁺-GTP shows clearly two sets of resonance lines at the positions found in Ras(T35A)-Mg2⁺-GTP (assigned to state 1) and Ras(wt)-Mg2⁺-GTP (assigned to state 2) with *K*₂ = 0.29.
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agreement with this assignment, addition of effectors to Ras(T35S): Mg\(^{2+}\)-GTP shifts the lines completely to the position dominantly found in Ras(wt): Mg\(^{2+}\)-GTP. In line with this assignment is the observation of weak resonances observed earlier in the GTP complexes of mutants Ras(Q61H) and Ras(Q61L) (1) that can now unambiguously be assigned to state 1. The differences of free energies can be calculated from the integrals of the resonance lines for the GTP complexes. For the wild-type protein \(\Delta G_{12} \approx -5.6 \text{ kJ mol}^{-1}\).

An interesting observation is that in full-length Ras(1–189) a higher equilibrium constant \(K_{12}\) is found compared with Ras(1–166), that is truncation of the C terminus leads to a shift of the conformational equilibrium toward state 1. This equilibrium may be further shifted to the effector binding state 2(T) when the C terminus is farnesylated and palmitoylated. Physiologically, this would make sense because the interaction with effectors (state 2) should be strongly preferred compared with the guanine nucleotide exchange factor interaction (state 1) when Ras is bound to the membrane.

Previously, investigations comparing the dynamic behavior of wild-type and (T35S)Ras complexed to GTP was also performed by time-resolved FTIR using a caged GTP compound (29). Here, the rate for formation of the conformation corresponding to state 2 was determined to be 5 s\(^{-1}\) after cleavage of the caging pH modification for wild-type Ras at 260 K. For Ras(T35S) the transition into that conformation was only detected in the presence of Raf-RBD, which we show to stabilize state 2 in this mutant. The obtained rate constant (measured at 260 K) is rather similar to that determined by us at 278 K but should be correlated, as discussed, with complex formation with the effector protein RBD. It would be interesting to determine how Ras(T35A) would behave in such an experiment, which remains in state 1 in complex with effectors. In contrast to the FTIR experiments, the equilibria between the two conformational states in Ras complexed with GTP can be directly observed by \(^{31}\)P NMR spectroscopy and it can be shown that the dominant conformation in wild-type or T35S mutant in complex with effectors is state 2.

The conformation of state 2 should be similar to the structure found by x-ray crystallography of Ras(wt): Mg\(^{2+}\)-GppNHp (30), although it could be demonstrated by single crystal and MAS \(^{31}\)P NMR spectroscopy that a conformational equilibrium also exists in crystals of Ras(wt): Mg\(^{2+}\)-GppNHp (31, 32). Whereas in the x-ray structure of Ras(T35S): Mg\(^{2+}\)-GppNHp solved by us earlier, both switch regions are not resolved (11), a structure possibly corresponding to state 1 could be determined in the GppNHp complexes of the Ras mutant Ras(G60A) (33) and in another Ras isoform M-Ras (34). In a most recent structure of Ras(T35S) the switch regions are visible and the hydrogen bonding pattern around the phosphate groups is revealed (35). In all these state 1 structures the interactions of the amide and the hydroxyl groups of the conserved residue Thr-35 with the \(\gamma\)-phosphate and the Mg\(^{2+}\)-ion are disrupted. In addition, in Ras(T35S) in state 1 two slightly different forms could be found, one with the hydrogen bond of Gly-60 with the \(\gamma\)-phosphate intact and one with the hydrogen bond broken. Another feature concerns the \(\alpha\)-phosphate group, where the interaction of the side chain of Glu-31 via a water molecule is disrupted. In contrast, the hydrogen bonding pattern around the \(\beta\)-phosphate is identical in the two states. This qualitatively fits well to the observed chemical shift changes. We had argued earlier (10) that the phosphorus chemical shift changes observed in Ras and its mutants may be dominated by the bond polarization via hydrogen bonding or electric charges and only to a weaker degree by the other two mechanisms, conformational strain and magnetic anisotropy effects. The downfield shift of the \(\gamma\)-phosphate group in Ras(T35S) in complex with GppNHp (10) by \(-0.7 \text{ ppm}\) in state 1 fits to the breakage of the hydrogen bond to Gly-60 and Ser-35 (Thr-35) observed in the crystal structure (35) of Ras(T35S). In this structure, hydrogen bonding to the \(\beta\)-phosphate is unper- turbed as it is chemical shift but again the interaction of Glu-31 with the \(\alpha\)-phosphate is perturbed and the \(\alpha\)-phosphate resonance is shifted downfield in state 1 relative to state 2 by \(-0.5 \text{ ppm}\). Interestingly, the chemical shift changes also include the \(\beta\)-phosphate when GTP is bound indicating that details of the two structural states may vary somewhat when the natural ligand GTP is bound.

In the complex of Ras(T35S) with GTP\(\gamma\)S we observed two substates of state 1 we named 1(a) and 1(b) (3) that we assigned to the different rotamers of the \(\gamma\)-phosphate group. Especially, in the rotamer where the sulfur is directed toward the amino group of Lys-16 the hydrogen bond with Gly-60 should be weakened substantially. In line with the solution NMR experiments, in the new crystal structures of Ras(T35S) (35) two forms are also observed, one with Gly-60 hydrogen bonded to \(\gamma\)-phosphate, and a second one with this hydrogen bond cleaved.

Dynamics of the Conformational Transitions—The exchange rate between the two states was determined by saturation transfer experiments for the Ras(1–166) as \(7 \text{ s}^{-1}\) at 278 K. This value is substantially smaller than those observed for GTP analogs GppNHp or GppCH\(_2\)p (Table 2). The main difference between GTP and these analogs is the replacement of the oxygen bridging the \(\beta\)- and \(\gamma\)-phosphate group by groups that cannot accept hydrogen bonds. If the possible hydrogen bonding of Gly-13 with this oxygen has to be broken for the transition between two structural states, it can explain the reduced exchange rate when GTP is bound instead of the analogs. Because the rate constant \(k_{21}\) is decreased and simultaneously the equilibrium is shifted to state 2, such an interaction may be found only in state 2 but not state 1.

Conformational States and Effector Binding—In agreement with data obtained for Ras in complex with different GTP analogs, Ras variants existing in state 1 show drastically decreased affinity for their effector proteins. Thus wild-type and Ras(G12V) in concentrations used for NMR experiments are completely effector-bound in the presence of Raf with a Raf: Ras ratio of about 1:1, whereas for mutants such as Ras(V29G/I36G) we can estimate a \(K_D\) value of approximately 700 \(\mu\)M for Raf binding from the \(^{31}\)P NMR titrations, taking into account that for these mutants Ras in state 2 reflects the effector-bound fraction. In analogy to Ras complexed with other guanine nucleotide triphosphates the binding of effectors shifts the equilibrium toward state 2 in Ras(T35S)-GTP or
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Ras(V29G/I36G)-GTP (Table 4) but not in Ras(T35A) missing the side chain hydroxyl, which interacts with the Mg$^{2+}$-ion. Mutation of Tyr-32 leads to chemical shift changes that cannot unambiguously be assigned to either states 1 or 2 by chemical shift analysis alone, because the ring current of the aromatic side chain is partially responsible for the chemical shift difference between states 1 and 2 (1). All Tyr-32 mutants investigated in this work, namely Y32C, Y32F, Y32R, and Y32W, show accelerated GTP hydrolysis rates after effector binding, thus it seems that mutation of this amino acid destabilizes state 2 as well.

In addition to the decrease in GTPase activity strongly transforming Gln-61 mutants of Ras show a high affinity state to effectors (36) required for an efficient activation of the Ras pathways. This observation can be generalized, normal high affinity for effectors is a general property for oncogenic Ras mutants. Because state 1 has a low affinity for effectors, mutants that cannot be switched to state 2 in the presence of effectors as Ras(T35A) cannot be oncogenic. In addition, some of the state 1 mutants initially have a low GTPase activity but show a high GTPase activity in the presence of effectors. These mutants are probably also not oncogenic because they show potentially, but not necessarily a sufficiently high GTPase activity in the presence of GAP. An example in our case would be Ras(T35S).

Conformational Equilibria Affect the GTPase Activity of Ras—The conformational states of Ras exhibit different rates in GTP hydrolysis. This can be shown best in the comparison between Ras(wt) and the two Thr-35 mutants investigated. It is not surprising that hydrolysis is faster in the conformation found in wild-type Ras, because here we should find the optimized structure for intrinsic as well as GAP accelerated GTPase activity. Various reasons could be responsible for that: (i) different chemical properties of the γ-phosphate, such as an increased pK$_{a}$ of the γ-phosphate (37, 38), (ii) localization of the nucleophilic water molecule that attacks the γ-phosphate and thus initiates the reaction (39), or (iii) the orientation of the γ-phosphate or Gln-61 by perturbation of the main chain contacts of Thr-35 and probably also Gly-60 of Ras. In fact, in the recent crystal structure of Ras(T35S) the attacking water is not visible and the correct orientation of the side chain of Gln-61 is disturbed.

Mutation of Tyr-32 to other amino acids shows different effects on GTPase activity, thus Ras(Y32C/C118S) and Ras(Y32F) show a decrease in the rate of hydrolysis, but after effector binding they are both 2–3 times faster than wild-type. For Ras(Y32F), a 2.3-fold increase of GTPase activity in the presence of Raf-RBD-CRD could be demonstrated but only up to the rate similar to that found at wild-type was observed using γ-32P-labeled GTP based assay at 30 °C (40). An explanation could be that the 1:1 ratio between effector and Ras at submicromolar concentrations used in that study, saturation of Ras binding was not achieved. The mutation of Tyr-32 in the Ras-Raf complex could partially mimic the Ras-GAP complex in which Tyr-32 is interacting with GAP and thus directs away from the phosphate groups (41). Mutation of Tyr-32 to tryptophan or arginine showed an increase in activity already in the non-effector bound state. A further increase was observed after effector binding in the arginine mutant, Ras(Y32R), thus in the effector complex a k$_{cat}$ value of 0.36 min$^{-1}$ at 37 °C is reached that is about 13 times higher than the one obtained for the wild-type protein. In the Ras-effector complex the position of Tyr-32 is stabilized close to the phosphate groups. One can assume that in the corresponding complex the arginine of Ras at position 32 partly adopts the function of the arginine finger in GAP. This acceleration could be confirmed determining the dissociation of inorganic phosphate as a product of GTP hydrolysis by means of a luminescent terbium complex (42). Finally, the differential intrinsic hydrolysis activity of two triphosphate-bound states might be generalized to explain the very slowly hydrolyzing activity of the Ran. Ran-GTP was previously shown to exist in two different states interacting in the slow exchange regime, with prevalence for state 1 at high temperatures (7). If state 1 would be similarly slower hydrolyzing than state 2 as shown for Ras, this observation could explain the high stability of Ran in its triphosphate-bound form. Because at higher temperatures the equilibrium is shifted toward state 1 one would expect an abnormal temperature dependence of the GTPase activity with an unusually slow increase or a decrease of the GTPase activity with temperature. Unfortunately, there are no such data at low temperatures available so far.

Conclusion—Ras bound to guanine nucleoside triphosphates exists in an equilibrium of at least two different conformational states. This equilibrium could be shifted by the use of different nucleotides or by specific mutations in the switch I and II regions of the protein. Bound to GTP or GTP·γS, Ras(wt) predominantly exists in a conformation similar to the effector bound state (state 2). For the naturally occurring complex Ras(1–166)-Mg$^{2+}$-GTP we finally could determine the free enthalpy difference ΔG$_{12}$ as ~5.6 kJ mol$^{-1}$. Replacing the β,γ-phosphate bridging oxygen by the NH or CH$_{2}$ group shifts the equilibrium toward the conformation with low affinity to effectors (state 1). Ras molecules existing in conformational state 1 show a decrease in intrinsic GTP hydrolysis activity. Binding of effectors can stabilize state 2 and hence increase the rate of hydrolysis in these mutants. Replacing Tyr-32 by other amino acids seems to shift the equilibrium toward state 1 because the GTP hydrolysis is always increased in the presence of the effector Raf known to stabilize state 2.

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