Perturbation of the conformational equilibria in Ras by selective mutations as studied by $^{31}$P NMR spectroscopy

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Abstract Ras regulates a variety of different signal transduction pathways acting as molecular switch. It was shown by liquid and solid-state $^{31}$P NMR spectroscopy that Ras exists in the guanosine-5’-(β,γ-imido)triphosphate bound form in at least two conformational states interconverting in millisecond time scale. The relative population between the two conformational states affects drastically the affinity of Ras to its effectors. $^{31}$P NMR spectroscopy shows that the conformational equilibrium can be shifted specifically by point mutations, including mutations with oncogenic potential, thus modifying the effector interactions and their coupling to dynamic properties of the protein.

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1. Introduction

$^{31}$P NMR spectroscopy revealed that the guanine nucleotide-binding protein Ras occurs in two conformational states (state 1 and state 2) when it is complexed with the GTP analog guanosine-5’-(β,γ-imido)triphosphate (GppNHp) [1]. These two states are characterized by typical $^{31}$P NMR chemical shifts of the phosphate groups of the bound nucleotide and interconvert with rate constants in the millisecond time scale. NMR structural studies have shown that this dynamic equilibrium comprises mainly two regions of the protein called switch I and switch II [1–3]. $^{31}$P solid-state NMR shows that surprisingly even in single crystals of Ras(wt)·Mg$^{2+}$·GppNHp, the two conformational states can be observed which are in dynamic equilibrium at ambient temperatures [4].

One of these conformational states (state 2) corresponds closely to the conformation found in the complex with the Ras binding domains (RBD) of Raf-kinase [1], RalGDS [5], AF6 [6] and Byr2 [7]. In Ras-complexes with effectors, Thr35 is involved via its side chain hydroxyl in the coordination of the crucial metal ion and, via its main chain NH, in contacting the γ-phosphate of the nucleotide [8,9]. The same coordination pattern is most probably also characteristic for state 2 of uncomplexed Ras. Replacing this threonine by an alanine or serine residue leads to a complete shift of the equilibrium towards state 1 [1,10]. The structure of Ras(T35S)·Mg$^{2+}$·GppNHp was determined by X-ray crystallography [10]. Whereas the overall structure is very similar to wild-type, residues 31–37 and 64–67 from switch I and switch II are completely invisible, indicating that these parts of the structure are either disordered or mobile. Upon addition of Ras effectors, the $^{31}$P resonance lines of Ras(T35S) nucleotide complex but not of Ras(T35A) shift to positions corresponding to the binding conformation [10].

A conformational equilibrium in the interaction site with effectors seems to be a general property of small GTP-binding proteins. In the present study, we will investigate if and how the conformational equilibrium can be perturbed selectively by mutations of single amino acids in the P-loop, the switch I or switch II region of the protein, by C-terminal truncation of Ras, or by switching from H-Ras to K-Ras. Some of the studied mutants such as T35S and Y40C have been used in biological studies as partial loss-of-function mutants, because they are believed to interact specifically only with a certain subset of effectors; other mutants investigated are found in human tumors. For correlating kinetic data obtained with fluorescent N-methylanthraniloyl (mant)-derivates of the nucleotide analog GppNHp with NMR data, it is also necessary to know their potential effects on the conformational equilibrium of Ras. The NMR studies presented lead to a better understanding of the biological importance of the effector loop equilibrium for the effector interaction.

2. Materials and methods

2.1. Protein purification

Wild-type and mutants of human H-Ras (amino acids 1–189 or 1–166) were expressed in E. coli and purified as described before [11]. Nucleotide exchange to GppNHp or the mGppNHp was done using alkaline phosphatase treatment in the presence of excess GTP analog described by John et al. [12]. Free nucleotides and phosphates were removed by gel filtration. Final purity of the protein was >95% as judged from the sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Ras binding domains of human Raf-1 (Raf-RBD, amino acids 51–131) were expressed and purified as described by Herrmann et al. [13].

2.2. Sample preparation

Typically, 1 mM Ras·Mg$^{2+}$·GppNHp was dissolved in 40 mM HEPES/NaOH, pH 7.4, 10 mM MgCl$_2$, 150 mM NaCl, 2 mM DTE and 0.1 mM DSS in 5% D$_2$O and 95% H$_2$O. For binding
studies, 5–7 mM Raf-RBD contained in the same buffer was added in appropriate amounts to the samples. When not stated otherwise full length H-Ras (amino acids 1–189) was used for the studies.

2.3. NMR spectroscopy

$^{31}$P NMR spectra were recorded with a Bruker DRX-500 NMR spectrometer operating at 202 MHz. Measurements were performed in a 10 mm probe using 8 mm Shigemi sample tubes at 278 K. $^{31}$P 70° pulses were used with a total repetition time of approximately 8 s (typical $T_1$ values for Ras-nucleotide complexes determined experimentally vary between 4 and 6 s [14]). Under our experimental conditions, the relative errors in the obtained integrals should be smaller than 10%. Typically, 1600 FIDs were summarized. Protons were decoupled during data acquisition by a GARP sequence [15] with a strength of the $B_1$-field of 830 Hz. A $\Delta$Z value of 0.4048073561 reported by Maurer and Kalbitzer [16] was used, which corresponds to 85% external phosphoric acid contained in a spherical bulb. Temperature was controlled by using the line separation (methylene-hydroxyl) of external ethylene glycol [17]. Thus, the absolute accuracy of the temperatures given in this study is better than ±0.5 K.

3. Results and discussion

3.1. Effect of different mutations on the conformational dynamics of Ras

$^{31}$P NMR spectroscopy allows to probe the conformational states of the Ras-protein, which in turn are related to the type of nucleotide present in the active center. In principle, whenever chemical shift changes are visible they indicate that there is a change of the environment of the phosphorus nuclei. For $^{31}$P NMR spectroscopy on nucleotides, it is known that two factors are the major determinants of chemical shift changes, namely a conformational strain and electric field effects polarizing the oxygens of the phosphate groups. In addition to these direct factors, long range effects may occur due to structure-dependent changes of the anisotropy of the magnetic susceptibility. Here, ring current effects may be the most dominant contribution.

Ras(wt) · $\text{Mg}^{2+}$ · GppNHp exists in solution in (at least) two conformational states, state 1 and state 2. The two states are primarily defined on a spectroscopic basis where in state 1 the resonances assigned to the $\alpha$- and $\gamma$-phosphate group are shifted downfield relative to those of state 2 by 0.5 ppm and 0.73 ppm, respectively. The resonances of the $\beta$-phosphate group in the two states cannot be separated at a $^{31}$P frequency of 202 MHz [1,10]. From the spectroscopic view, the conformational states in which the mutants occur are defined by the chemical shifts. Comparing the $^{31}$P NMR spectra of the various Ras mutants, we can mainly observe two effects of the mutations: changes in the chemical shift values for each phosphate resonance of the bound GppNHp and a shift of the equilibrium between state 1 and state 2. In addition, linewidths can be somewhat influenced by the local environment of the phosphate groups and strongly when exchange between more than one state leads to an exchange broadening. In the most extreme case, only one state is detectable (Fig. 1). The assignment is straightforward in cases where only small changes in chemical shifts are observed or where two spectroscopic states exist (Figs. 1 and 2, Table 1). In these cases, it is very likely that the major structural features are similar.

Mutations of Gly12 in the P-loop, such as to valine or asparagine, are known to inhibit GTP-hydrolysis and thus lead to an oncogenic activation of Ras. The $^{31}$P NMR spectra of Ras(G12D) [1] were re-evaluated and show, just as for Ras(G12V), two states in the $\text{Mg}^{2+}$ · GppNHp complex. At 278 K, only one wide resonance line is observed for the $\alpha$-phosphate of GppNHp of Ras(G12D), but the $\gamma$-phosphate resonance splits into two lines. In Ras(G12V), clearly two pairs of resonance lines are observed for the $\alpha$-phosphate as well as the $\gamma$-phosphate resonance. The equilibrium is slightly shifted towards state 1 (Figs. 1–3, Table 1). Compared to the wild-type protein, the chemical shifts of the $^{31}$P resonances are also considerably influenced by these mutations. The separation of resonances corresponding to the $\alpha$-phosphate in the two states is smaller and that of the $\gamma$-phosphate is larger compared to the
Table 1

<table>
<thead>
<tr>
<th>Ras·Mg(^2+)·GppNHp-complex</th>
<th>(\gamma)-Phosphate</th>
<th>(\beta)-Phosphate</th>
<th>(\gamma)-Phosphate</th>
<th>(K_{12})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(d_1) (ppm)</td>
<td>(d_2) (ppm)</td>
<td>(d_1) (ppm)</td>
<td>(d_2) (ppm)</td>
</tr>
<tr>
<td>H-Ras(wt)(^a)</td>
<td>-11.20</td>
<td>-11.70</td>
<td>-0.25</td>
<td>-2.59</td>
</tr>
<tr>
<td>Ras(wt) (mantGppNHp)</td>
<td>-10.92</td>
<td>-11.72</td>
<td>-0.49</td>
<td>-2.53</td>
</tr>
<tr>
<td>c' Ras(V29G)(^b)</td>
<td>-11.15</td>
<td>-11.71</td>
<td>-0.27</td>
<td>-2.54</td>
</tr>
<tr>
<td>Ras(Y32W)(^c)</td>
<td>-11.08</td>
<td>-11.71</td>
<td>-0.30</td>
<td>-2.52</td>
</tr>
<tr>
<td>Ras(wt) (\text{mantGppNHp})</td>
<td>-11.49</td>
<td>-12.05</td>
<td>-0.34</td>
<td>0.07</td>
</tr>
<tr>
<td>Ras(Y32R)</td>
<td>-11.00</td>
<td>-11.36</td>
<td>-0.24</td>
<td>0.11</td>
</tr>
<tr>
<td>Ras(Y32W)</td>
<td>-12.02</td>
<td>-0.21</td>
<td>-2.53</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Ras(T35A)(^d)</td>
<td>-11.00</td>
<td>-0.26</td>
<td>-2.49</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Ras(T35S)</td>
<td>-11.10</td>
<td>-0.26</td>
<td>-2.57</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Ras(I36G)</td>
<td>-11.00</td>
<td>-0.26</td>
<td>-2.57</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Ras(Y40C)</td>
<td>-11.08</td>
<td>-0.23</td>
<td>-2.46</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>c'Ras(E62H)(^e)</td>
<td>-12.18</td>
<td>-11.82</td>
<td>-0.38</td>
<td>-0.24</td>
</tr>
<tr>
<td>c'Ras(S65P)</td>
<td>-11.28</td>
<td>-11.78</td>
<td>-0.37</td>
<td>-0.23</td>
</tr>
</tbody>
</table>

\(a\)Data were recorded at various temperatures, the shifts actually given were taken from spectra recorded at 278 K and pHe 7.4 with an estimated error of ±0.05 ppm. The equilibrium constant \(K_{12}\) between state 1 and state 2 is calculated from the integrals of the \(\gamma\)-resonances defined by \(K_{12} = \frac{[2]}{[1]}\). State 1 and state 2 are two conformational states of the Ras·nucleotide complexes with different chemical shifts. In the Ras(wt)·Mg\(^2+\)·GppNHp complex, state 1 is defined as the state where the \(\gamma\)-phosphate group is also perturbed significantly. As in the wild-type protein, a significant exchange broadening can be observed at low temperatures.

Mutations in the hinges of the effector loop which replace amino acids with more bulky side chains by glycine, such as the single and double mutants V29G, I36G, V29G/I36G, induce a complete shift to state 1 probably because the loop becomes much more mobile. As already reported [10], the same result has been obtained for the partial loss-of-function mutants T35S and T35A, since Thr35 stabilizes state 2 by hydrogen bonds to the \(\gamma\)-phosphate of GppNHp and to the metal ion. As shown here, also the other well-known partial

**Fig. 3.** Correlation of population shifts with the site of mutation. (A) Amino acids which were mutated are indicated. The color code represents the shift of the conformational equilibrium as follows: green, \(K_{12} < 0.1\); yellow, \(K_{12} \approx 1\); and orange, \(0.1 < K_{12} < 0.8\) depending on the mutation. (B) Equilibrium constants of the \(K_{12} = [2]/[1]\) of state 1 and state 2 of different Ras mutants.
loss-of-function mutant Ras(Y40C) is characterized by a complete shift towards state 1.

Mutations of Tyr32 are always characterized by a shift of the equilibrium to state 1 but the magnitude of the shift is dependent on the amino acid introduced in position 32. A small effect on the equilibrium could possibly be explained by the interaction between the aromatic ring hydroxyl group and the γ-phosphate of bound GppNHp. In Ras(Y32F) and Ras(Y32R), the equilibrium constant for the dynamic interchange between the conformations 1 and 2, $K_{12} = [2]/[1]$, drops from 1.9 for wild-type Ras to 0.7 and 0.8, respectively. Ras(Y32W), a mutant often used in fluorescence based binding studies, occurs in only one state. From the chemical shift of the γ-phosphate resonance, it represents most probably state 1. In contrast, replacement of GppNHp in the Ras(wt) complex by the fluorescent mant-analog mGppNHp has only a slight effect on the phosphorus chemical shifts and the population of the different states (Table 1), which is important to know as the mant group is often used in kinetic and thermodynamic studies with effectors and regulators. In contrast, the 31P NMR data presented here suggest that Ras(Y32W) is in a different conformational equilibrium compared to wild-type Ras, suggesting that caution must be applied in the use of this mutation in effector binding studies.

The switch II mutants Ras(E62H) and Ras(S65P) investigated by Geyer et al. [1] show similar spectra as the wild-type but with a slight shift of the equilibrium towards state 1 (Table 1).

### 3.2. Effects of truncation and change of isoforms

Most 31P NMR experiments were performed with H-Ras(1–189) but in the literature also the C-terminal truncated H-Ras(l–166) is used. From 31P NMR spectroscopy point of view, truncation does not have a significant effect on the chemical shifts or the conformational equilibrium (Table 1). Since NMR-spectroscopy is very sensitive against small structural changes, this supports earlier conclusions based on biochemical data that this truncation does not perturb any biochemical property of the protein [18]. This is different for K-Ras: here the equilibrium constant $K_{12}$ is significantly reduced and drops from 1.9 in H-Ras(wt) to 1.2 in K-Ras(wt).

### 3.3. Complex formation between Ras effectors and different Ras mutants

Binding of effectors to Ras(wt) · Mg2+ · GppNHp normally leads to the disappearance of the 31P NMR resonances corresponding to state 1 and the appearance of resonances with shifts that correspond closely to state 2 (Fig. 4, Table 2).

#### Table 2

<table>
<thead>
<tr>
<th>Ras · Mg2+ · GppNHp-complex</th>
<th>Effector</th>
<th>α-Phosphate (δ, ppm)</th>
<th>β-Phosphate (δ, ppm)</th>
<th>γ-Phosphate (δ, ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ras(wt)b</td>
<td>Raf-RBD</td>
<td>−11.55</td>
<td>−0.22</td>
<td>−3.50</td>
</tr>
<tr>
<td>Ras(wt)b</td>
<td>RalGDS-RBD</td>
<td>−11.54</td>
<td>−0.37</td>
<td>−3.40</td>
</tr>
<tr>
<td>Ras(wt)b</td>
<td>AF6-RBD</td>
<td>−11.70</td>
<td>−0.30</td>
<td>−3.60</td>
</tr>
<tr>
<td>Ras(wt)b</td>
<td>Byr2-RBD</td>
<td>−11.54</td>
<td>−0.37</td>
<td>−3.36</td>
</tr>
<tr>
<td>Ras(G12V)b</td>
<td>Raf-RBD</td>
<td>−11.60</td>
<td>−0.23</td>
<td>−4.46</td>
</tr>
<tr>
<td>Ras(V29G/I36G)b</td>
<td>Raf-RBD</td>
<td>−10.79</td>
<td>−0.08</td>
<td>−3.42</td>
</tr>
<tr>
<td>Ras(T35A)b</td>
<td>Raf-RBD</td>
<td>−11.10</td>
<td>−0.31</td>
<td>−2.49</td>
</tr>
<tr>
<td>Ras(T35A)b</td>
<td>RalGDS-RBD</td>
<td>−11.11</td>
<td>−0.31</td>
<td>−2.49</td>
</tr>
<tr>
<td>Ras(T35S)b</td>
<td>Raf-RBD</td>
<td>−11.60</td>
<td>−0.27</td>
<td>−3.42</td>
</tr>
<tr>
<td>Ras(T35S)b</td>
<td>RalGDS-RBD</td>
<td>−11.54</td>
<td>−0.29</td>
<td>−3.31</td>
</tr>
<tr>
<td>Ras(Y32R)</td>
<td>Raf-RBD</td>
<td>−11.33</td>
<td>+0.15</td>
<td>−3.23</td>
</tr>
<tr>
<td>Ras(Y32W)</td>
<td>Raf-RBD</td>
<td>−12.03</td>
<td>−0.11</td>
<td>−2.80</td>
</tr>
<tr>
<td>Ras(Y40C)</td>
<td>Raf-RBD</td>
<td>−11.60</td>
<td>−0.21</td>
<td>−3.52</td>
</tr>
</tbody>
</table>

aData were recorded at 278 K and pH 7.4. Published data were reassigned according to Spoerner et al. [14]. The estimated error of the given chemical shift values is less than ± 0.1 ppm.

aData from Spoerner et al. [10].

aData from Linnemann et al. [6].

aData from Gronwald et al. [7].
represents the effector-bound state and state 1 that of free
tion experiments (see Section 2) with known concentrations of
the equilibrium towards state 1 (Figs. 1–3). This is especially
respectively.

As described for the α-phosphate resonance, a shift to higher
fields is usually observed for the α-phosphate resonance after
effector binding. The largest effect is seen for the mutant Ras(Y40C) with ~0.52 ppm. Also, large effects are found for the β-phosphate resonances of Ras(G12V) with an upfield shift of 0.22 ppm. The Ras(Y32R)-Raf-RBD complex shows a large downfield shift of all three resonances compared to the wild-
type Ras-effector complex.

From 31P NMR titration experiments, we estimate for the interaction between Ras(T35S) andRalGDS-RBD an equilibrium dissociation constant of about 350 μM and a affinity less
than 10 mM in the case of Ras(T35A) [10]. From investigations

Mutations within switch I region most often lead to a shift of
the equilibrium towards state 1 (Figs. 1–3). This is especially
important for the partial loss-of-function mutants Ras(T35S),
which can interact with Raf-kinase but not any more with Ral-
GDS, and for Ras(Y40C), which interacts with PI3-kinase but
neither with Raf-kinase nor with RalGDS. From this study,
we see that in principle the complex formation between
Ras(Y40C) and the Ras-binding domain of Raf (Raf-RBD)
as well as Ras(T35S) andRalGDS-RBD is possible but the
affinity drops drastically, so that under physiological concen-
trations there is almost no binding any more (Fig. 4).

4. Conclusion

Ras exists in (at least) two conformational states, which can be identified by NMR spectroscopy. One of the states (state 2)
represents the high affinity state for effectors. The other state (state 1) represents a different (GDP-like?) state of the protein with a strongly reduced affinity to effectors. All investigated mutations but especially mutations in switch I shift the equilib-
rium between the states towards state 1 followed by a decrease of binding affinity to effectors. These mutations seem to dis-
turb directly or indirectly the interactions of Thr35 with the Mg2+ ion and the γ-phosphate group. In general, the 31P chemical
shifts after effector binding are close to that of state 2 of
free Ras(wt) but nevertheless different. This means that upon
effector binding, a limited structural re-arrangement occurs
(induced fit) which selects one of the possible substrates of state
2. This is in line with the general theory of effector interaction
described by Spoerner et al. [14]. State 1 could represent a new
target for the development of anti-cancer drugs, since its stabil-
ization would reduce the strength of the effector interaction
considerably.

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sional NMR spectroscopy, Biochemistry 33, 3515–3531.


