# Solid-State $^{31}$P NMR Spectroscopy of Precipitated Guanine Nucleotide-Binding Protein Ras in Complexes with Its Effector Molecules Raf Kinase and RalGDS

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Liquid-state $^{31}$P NMR spectroscopy is a well-established method for the study of guanine nucleotide-binding proteins (GNB proteins) such as the proto-oncogene Ras. Solid-state $^{31}$P NMR spectroscopy could meanwhile also be used to study microcrystalline samples of Ras as well as its partial loss-of-function mutants Ras(T35S) and Ras(T35A). However, solid-state NMR studies of the latter mutants in complex with effector molecules such as RalGDS or Raf kinase were so far prevented, since it has been impossible to crystallize these complexes yet. The aim of the present contribution is to make such complexes accessible to solid-state $^{31}$P NMR spectroscopy by the application of precipitation methods. The complex formed by Ras(T35S) and Raf kinase is preserved during precipitation. In contrast, the weakly bound complex of Ras(T35S) with RalGDS is dissociated or at least perturbed by the precipitation procedure. Solid-state $^{31}$P NMR experiments on precipitates of these complexes deliver spectra of high resolution and signal-to-noise ratio which allows the application of two-dimensional techniques. Precipitates prepared using polyethylene glycol 6000 (PEG) as precipitant were found to exhibit spectra of maximum resolution and signal-to-noise ratio. Interestingly, the $^{31}$P signal due to the α-phosphate of GppNHp bound to Ras(T35S) in crystalline samples or aged precipitates has a significantly different isotropic chemical shift than in the liquid state or in freshly prepared precipitates. This directly indicates that the crystal structure differs from the equilibrium solution structure at least in the neighborhood of the α-phosphate group.

## Introduction

$^{31}$P NMR spectroscopy is a powerful method to investigate phosphate groups in proteins. This is due to the relatively high gyromagnetic ratio of $^{31}$P and its natural abundance of 100%. $^{31}$P NMR spectroscopic parameters such as the isotropic chemical shift of guanine nucleotides bound to guanine nucleotide-binding proteins (GNB proteins) sensitively depend on the structural features in the neighborhood of the $^{31}$P nuclei. Extended studies on members of the Ras superfamily such as Ras and Ran were carried out using liquid-state $^{31}$P NMR spectroscopy.[1−3] The isotropic $^{31}$P NMR chemical shift of the γ-phosphate group is a very sensitive probe for effector binding.

Meanwhile, solid-state NMR spectroscopy has also become a well-established method for the characterization of proteins.[4−10] The crystal structure[11] of the Ras protein does not identify the presence of conformational exchange between the two different states, 1 and 2, in the highly conserved loops of the switch I and switch II regions, which is crucial for the interaction with downstream effectors such as Raf kinase. Solid-state $^{31}$P NMR investigations, however, demonstrated the existence of two states in crystalline Ras if complexed with GTP analogs like GppNHp.[12] The presence of conformational exchange between these states could be shown in analogy to the liquid state. The state denoted as state 2 is related to the active form of Ras interacting with effector proteins. In contrast, state 1 shows a strongly reduced affinity toward effector molecules.[13]

Solid-state $^{31}$P NMR spectroscopy especially benefits from cross-polarization experiments transferring magnetization from the abundant $^{1}$H nuclei to $^{31}$P. In the liquid state, the repetition time for the NMR experiment is determined by the longitudinal relaxation time, $T_1$, of the phosphorus nuclei and amounts to several seconds up to minutes.[2] In contrast, the repetition time for the cross-polarization (CP) experiment in the solid state solely depends on the relaxation time of the protons which is of the order of tens of milliseconds. Thus, a considerable gain in sensitivity is obtained if $^{31}$P {$^{1}$H} CP MAS NMR spectroscopy is applied.[14] Numerous solid-state NMR investigations of proteins reported in the literature are carried out on micro- or nanocrystalline samples.[15−17] Although the requirements with respect to the size of the crystals are very moderate compared to X-ray crystallography, crystallization may take a considerable amount of time and resources. An alternative approach is based on the precipitation of protein samples[18] which is exploited for the solid-state $^{31}$P NMR investigations presented here. The well-known partial loss-of-function mutant Ras(T35S)[19] and, in particular, its complexes with the Ras-binding domains (RBDs) of the downstream effectors Raf kinase and RalGDS[2,13] are studied. These complexes are of special interest, since no crystallization procedures have been reported yet for Ras(T35S)·Raf-RBD and Ras(T35S)·RalGDS-RBD. Polyethylene glycol 6000 (PEG) and ammonium sulfate are employed as precipitants in the present study. It is generally assumed that these precipitants behave gently and perturb the protein conformation only weakly.

## Experimental Section

**Protein Purification.** The mutant Ras(T35S) of truncated human H-Ras (amino acids 1−166) was expressed in *Escherichia coli* and purified as described previously.[20] Nucleotide

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exchange with GppNHp was performed following the method described by John et al.[21] Free nucleotides and phosphates were removed by gel filtration. Ras-binding domains of human RalGDS (RalGDS-RBD, amino acids 11−97) and human Raf-1 (Raf-RBD, amino acids 51−131) were expressed in *E. coli* and purified following previous reports.[22,23]

**NMR Spectroscopy.** Solid-state NMR experiments were carried out on a Bruker Avance 300 spectrometer operating at 300.13 MHz for $^{1}$H and 121.49 MHz for $^{31}$P. A commercial double resonance MAS NMR probe (2.5 mm outer rotor diameter, 35 kHz maximum sample spinning rate) was used. Ramped $^{1}$H−$^{31}$P cross-polarization was employed. The $^{31}$P NMR spectra were referenced relative to 85% $H_3PO_4$ by setting the signal of solid $NaH_2PO_4 \cdot H_2O$ to 2.3 ppm. The phosphorylated amino acid *O*-phospho-L-tyrosine was used as a model compound for optimizing the CP experiments.[24] TPPM decoupling (phase difference $\phi = 15°$, $\tau_p = 8\ \mu$s) was applied during signal acquisition.[25] The $\pi/2$ pulse lengths for $^{31}$P and $^{1}$H were 2.1 and 1.5 $\mu$s, respectively. The relaxation delay was adjusted to 0.5 s, the contact time to 3 ms, and the sample spinning rate to 10 kHz. It should be noted that the spectral resolution observed for $^{31}$P {$^{1}$H} CP MAS NMR spectra of several reference compounds (phosphorylated amino acids, $NaH_2PO_4 \cdot H_2O$) at high magnetic fields (17.62 T) was only slightly better than that at the magnetic field used in the present study (7.05 T). This behavior indicates that the residual line width of the $^{31}$P MAS NMR signals[26] is dictated by the distribution of the isotropic chemical shift and/or the isotropic magnetic susceptibility of the sample particles. In other words, the limiting "natural" line width is achieved even at the lower magnetic field as long as efficient $^{1}$H decoupling is applied. In contrast, the sensitivity, that is, the signal-to-noise ratio, considerably increases at higher magnetic field strength. Due to the larger chemical shift anisotropy, however, the samples need to be spun faster at high magnetic field in order to fully benefit from this sensitivity gain. It should further be noted that the samples were centrifuged down into the rotor directly after precipitation (see below) without any freeze-drying or other procedures which may influence the hydration state.

Liquid-state $^{31}$P NMR spectra were recorded on a Bruker Avance 500 NMR spectrometer operating at 202.46 MHz for $^{31}$P. The measurements were performed at 277 K using a dedicated $^{31}$P 10 mm probe and 8 mm Shigemi sample tubes. Seventy degree pulses together with a total repetition time of 7 s were used. Protons were decoupled during data acquisition by a GARP sequence.[27] Spectra were referenced indirectly.[28]

**Precipitation Using PEG 6000.** Solutions of 0.7−1.7 mM Ras(T35S) or Ras(T35S)−effector complex were incubated at 273 K and slowly adjusted to 20% PEG 6000 by adding a solution of 40% PEG 6000 in Tris buffer (50 mM Tris/HCl, pH 7.6, 10 mM $MgCl_2$, 2 mM DTE, 40% (w/v) PEG 6000). The final 1:1 mixture of protein solution and Tris buffer, therefore, contained 20% PEG 6000.

**Precipitation Using Ammonium Sulfate.** Solutions of 0.7−1.7 mM Ras(T35S) or Ras(T35S)−effector complex were incubated at 273 K and slowly adjusted to 3.2 M $(NH_4)_2SO_4$ by adding solid $(NH_4)_2SO_4$.

## Results and Discussion

**Effector Binding Observed in the Solid State.** Figure 1A demonstrates the aforementioned sensitivity gain obtained by $^{31}$P {$^{1}$H} CP MAS NMR spectroscopy for the PEG-precipitated Ras(T35S)·Raf-RBD complex. The signal-to-noise ratio of the solid-state $^{31}$P NMR spectrum is 50 times higher than that of the liquid-state $^{31}$P NMR spectrum measured under comparable conditions, that is, using identical amounts of sample and measurement time. This advantage is particularly striking for larger proteins or protein complexes as investigated here, because line broadening is observed for increasing molecular mass in solution leading to a further decreasing signal-to-noise ratio. In contrast, the line width of the solid-state NMR spectra does not depend on the size of the molecules/complexes.

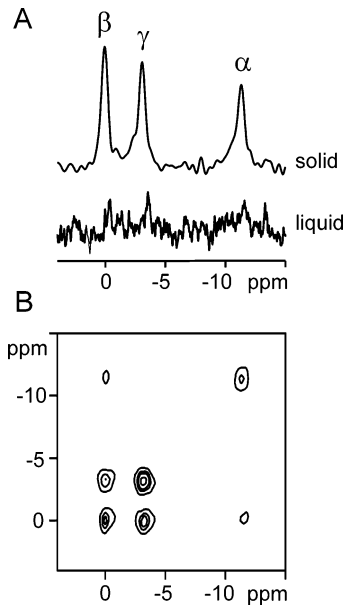


**Figure 1.** (A) (top) $^{31}$P {$^{1}$H} CP MAS NMR spectrum of PEG-precipitated Ras(T35S)·$Mg^{2+}$·GppNHp complexed with Raf-RBD (10 000 scans acquired). (bottom) Liquid-state $^{31}$P NMR spectrum of Ras(T35S)·$Mg^{2+}$·GppNHp complexed with Raf-RBD (840 scans acquired). A 5 mg portion of Ras(T35S) $Mg^{2+}$·GppNHp was used with twofold excess of Raf-RBD in both experiments. Measurement time: 1.5 h; $T = 277$ K for both spectra. (B) 2D $^{31}$P−$^{31}$P $^{1}$H-driven spin diffusion CP MAS NMR spectrum of Ras(T35S)·$Mg^{2+}$·GppNHp·Raf-RBD at a mixing time of 100 ms, a recycle delay of 0.5 s, and 128 $t_1$ increments (2200 scans). The sample spinning rate was 10 kHz. The CP contact time was set to 3 ms. A 10 mg portion of Ras(T35S)·$Mg^{2+}$·GppNHp was used with twofold excess of Raf-RBD for PEG precipitation. Measurement time: 48 h.

Although the $^{31}$P NMR spectra of GNB proteins contain only a few spectral lines, signal assignment is by far not trivial.[14] It has been shown, for example, that the isotropic chemical shift is highly pH dependent, occasionally leading to a crossover of spectral lines during a pH titration.[2] It is, therefore, important to have reliable experiments to assign the signals, usually two-dimensional experiments. The described sensitivity gain makes solid-state $^{31}$P NMR spectroscopy particularly well-suited for two-dimensional $^{31}$P NMR experiments. As an example, Figure 1B shows the two-dimensional $^{1}$H-driven spin diffusion spectrum for Ras(T35S) in complex with Raf-RBD. This spectrum confirms the signal assignment of Figure 1A and shows that solid-state $^{31}$P NMR spectroscopy is a sensitive and well-suited method to study the protein complexes of interest within the present paper.

In contrast to the wildtype protein, the mutant Ras(T35S) exclusively exists in the conformation corresponding to state 1 in solution if the GTP analog GppNHp is bound to the protein. This is a state with low affinity to effectors. If complexed with effector molecules, however, state 2 is stabilized, as was also found for Ras(wt)−effector complexes.[13] Since the $\gamma$-phosphate $^{31}$P NMR chemical shifts for the two states of the molecule are different,[1−3] effector binding can easily be detected by a shift of the $\gamma$-phosphate signal of GppNHp. Figure 2 summarizes

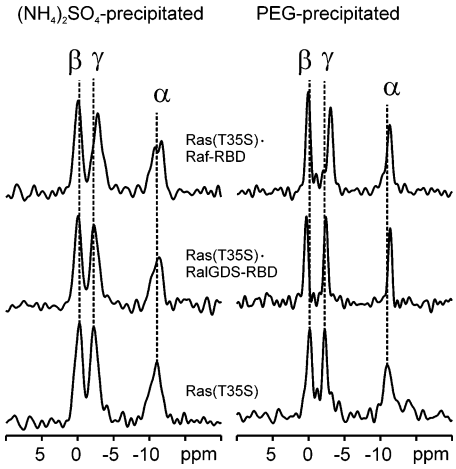


**Figure 2.** $^{31}$P {$^1$H} CP MAS NMR spectra measured at 277 K and a sample spinning rate of 10 kHz. A 5 mg portion of Ras(T35S)·$Mg^{2+}$·GppNHp was used. Effectors were added in twofold excess. Samples were measured immediately after precipitation. All spectra were recorded and processed under identical conditions. Left: Ammonium sulfate precipitates of Ras(T35S) (bottom), Ras(T35S)·RalGDS-RBD (middle), and Ras(T35S)·Raf-RBD (top). 80 000 scans were collected for each experiment (measurement time 12 h). Right: PEG 6000-precipitated Ras(T35S) (bottom), Ras(T35S)·RalGDS-RBD (middle), and Ras(T35S)·Raf-RBD (top). 10 000 scans were collected for each experiment (measurement time 1.5 h).

**TABLE 1: Comparison of Isotropic $^{31}$P NMR Chemical Shifts Measured for Ras(T35S) as Well as Its Complexes with Raf-RBD and RalGDS-RBD in the Solid State (The Data Include Measurements for the Two Different Precipitation Methods as Well as Microcrystalline Samples)**

| | $\delta$/ppm | | |
|---|---|---|---|
| | $(NH_4)_2SO_4$-precip. (±0.2 ppm) | PEG-precipitated (±0.1 ppm) | microcrystals[a] (±0.1 ppm) |
| | Ras(T35S) | | |
| α-phosphate | −11.1 | −11.1 | −9.7 |
| *β*-phosphate | −0.2 | −0.2 | 0.0 |
| *γ*-phosphate | −2.2 | −2.3 | −1.7 |
| | Ras(T35S)·Raf-RBD | | |
| α-phosphate | −11.2 | −11.3 | *b* |
| *β*-phosphate | −0.1 | 0.0 | *b* |
| *γ*-phosphate | −2.8 | −3.1 | *b* |
| | Ras(T35S)·RalGDS-RBD | | |
| α-phosphate | −11.3 | −11.4 | *b* |
| *β*-phosphate | −0.1 | 0.3 | *b* |
| *γ*-phosphate | −2.1 | −2.4 | *b* |

[a] Data from Iuga et al.[12] [b] Samples could not be crystallized yet.

the $^{31}$P {$^1$H} CP MAS NMR spectra acquired for Ras(T35S) and its complexes with the Ras-binding domains (RBDs) of the effector proteins Raf kinase and RalGDS employing ammonium sulfate (left) as well as polyethylene glycol 6000 (PEG, right) as precipitants. The isotropic chemical shifts are summarized in Table 1.

As already mentioned, binding of the effector domains Raf-RBD and RalGDS-RBD to Ras(T35S) can be monitored by measuring the isotropic chemical shift of the *γ*-phosphate signal. The expected complexation-induced chemical shift change $\Delta\delta = -0.8$ ppm found in the liquid state[2] is exactly observed for PEG precipitates of Ras(T35S) in complex with Raf-RBD (see Table 1). A slightly smaller value of $\Delta\delta = -0.6$ ppm is measured for the ammonium sulfate-precipitated sample. The latter deviation does not, however, exceed the experimental error for $\Delta\delta$.

In contrast, no such shift appears within the experimental error for Ras(T35S) in complex with RalGDS-RBD, whereas a shift of $\Delta\delta = -0.7$ ppm[2] could be measured in the liquid state. This observation can be explained by the fact that the dissociation constant, $K_D$, for the Ras(T35S)·RalGDS-RBD complex in solution of ∼360 $\mu$M is more than 2 orders of magnitude larger than that for the Ras(T35S)·Raf-RBD complex ($K_D = 1.2$ $\mu$M).[13] Obviously, the weaker Ras(T35S)·RalGDS-RBD complex is not preserved during precipitation. It dissociates if ammonium sulfate is used for precipitation, since the sample containing RalGDS-RBD in twofold excess shows almost the same chemical shifts as pure Ras(T35S). This may be due to the introduction of a high ionic strength when ammonium sulfate is used. The *γ*-phosphate chemical shift of the PEG precipitate of Ras(T35S)·RalGDS-RBD also remains constant within the experimental error. However, a significant change of the isotropic *β*-phosphate chemical shift of $\Delta\delta = 0.5$ ppm is observed. This suggests a perturbation of the complex for the PEG-precipitated sample which leads to a state different from the native complex observed in liquid-state $^{31}$P NMR investigations.[13] Hence, we conclude that sufficiently strong protein−protein interactions remain conserved in precipitates. Such precipitates deliver well-resolved solid-state $^{31}$P NMR spectra consistent with the results of liquid-state $^{31}$P NMR spectroscopy, as was shown here for Ras(T35S)·Raf-RBD. In contrast, weakly bound complexes are perturbed or even dissociated. Two observations are readily made if the two precipitants, namely, ammonium sulfate and PEG, are compared: Both the spectral resolution and the signal-to-noise ratio are significantly improved for PEG-precipitated samples. Since the same amount of protein was used in both series of experiments, the observation of an almost 3 times higher signal-to-noise ratio must be the result of the narrower lines in combination with a higher $^{31}$P {$^1$H} cross-polarization efficiency obtained for PEG-precipitated samples. It should be noted that the ammonium sulfate-precipitated samples seem to show asymmetries for several signals. The α-phosphate signal of Ras(T35S)·Raf-RBD is even split into two components. This indicates an inhomogeneous state of the protein molecules in the ammonium sulfate precipitates. In contrast, no such effects are observed in the well-resolved spectra of PEG-precipitated samples. In addition to the structural inhomogeneities causing the signal asymmetries and splittings, internal dynamics[16] may occur and contribute to the line broadening.[29] This possibility would also explain the decreased cross-polarization efficiency of the ammonium sulfate precipitates.

Another frequently used solid-state NMR spectroscopic parameter is the chemical shift anisotropy (CSA) which could possibly be influenced by effector molecule binding. In order to check this possibility, the chemical shift anisotropy, $\Delta\sigma$, of pure Ras(T35S) was measured and compared with the values observed for Ras(T35S)·Raf-RBD (see Figure 3 and Table 2). Visual inspection of the spectra shows that the spinning sideband pattern is indeed influenced by both the formation of the so-called "crystal-like" state (see below) and effector binding. The absolute values of $\Delta\sigma$ measured for Ras(T35S)·Raf-RBD were all bigger than those observed for pure Ras(T35S) in the "liquid-like" state found in fresh precipitates (see below). However, the experimental error for $\Delta\sigma$ (see Table 2) is still larger than the observed binding-induced changes of $\Delta\sigma$ for all three signals. That means the $^{31}$P NMR chemical shift anisotropy cannot, under the present experimental conditions, be used in order to detect effector molecule binding of Ras proteins in contrast to the isotropic chemical shift.

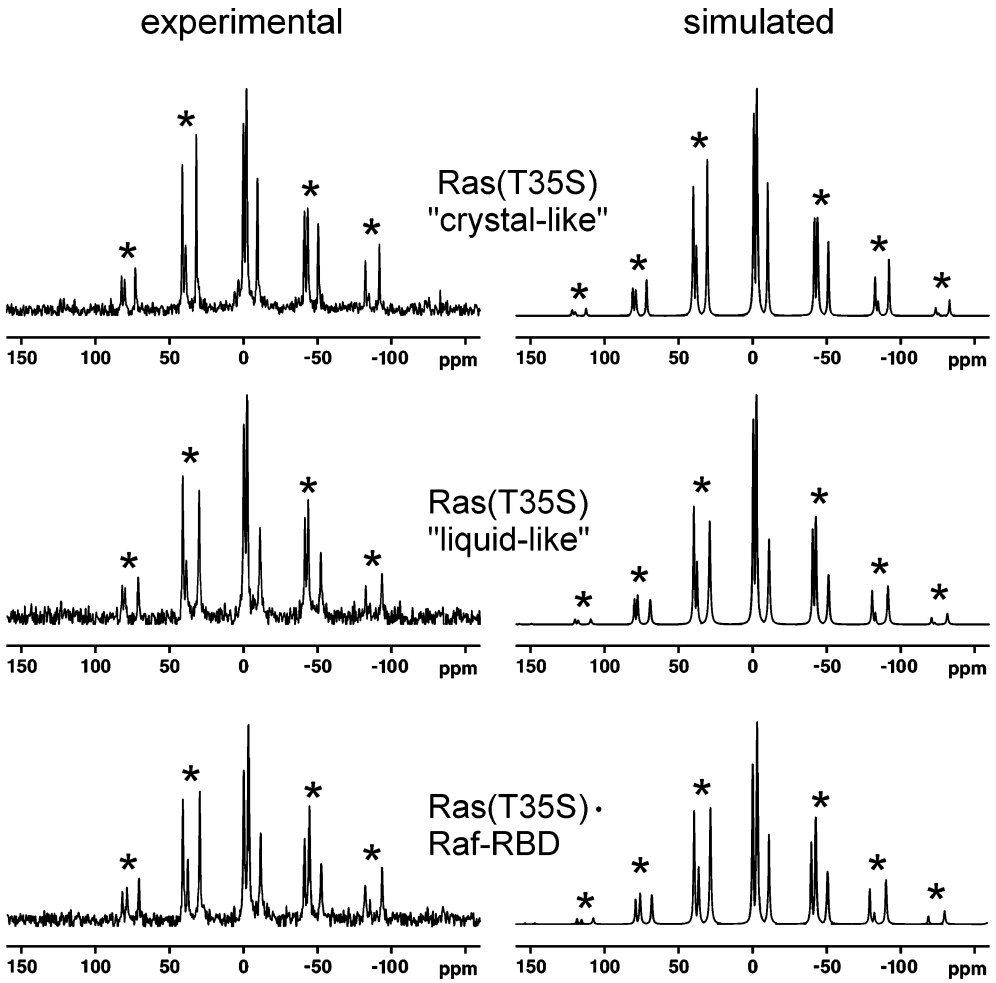


**Figure 3.** Experimental and simulated $^{31}$P {$^{1}$H} CP MAS NMR spectra of precipitated Ras(T35S)·$Mg^{2+}$·GppNHp and its complex with Raf-RBD at a 5 kHz sample spinning rate. The spectra were simulated using the SIMPSON software[30] in order to determine the chemical shift anisotropy and the asymmetry parameter. Top: PEG precipitate of pure Ras(T35S) older than 5 h (crystal-like form). Middle: PEG precipitate of Ras(T35S) with BSA added (liquid-like form). Bottom: PEG-precipitated Ras(T35S)·Raf-RBD. The asterisks denote spinning sidebands.

**TABLE 2: $^{31}$P NMR Chemical Shift Anisotropies, $\Delta\sigma$, Measured for PEG-Precipitated Ras(T35S) as Well as Its Complex with Raf-RBD[a]**

| | $\Delta\sigma$/ppm | | |
|---|---|---|---|
| | α-phosphate | β-phosphate | γ-phosphate |
| Ras(T35S) crystal-like (older than 5 h) | 183 ± 7 | 144 ± 7 | −124 ± 7 |
| Ras(T35S) liquid-like (BSA added) | 186 ± 7 | 138 ± 7 | −128 ± 3 |
| Ras(T35S)·Raf-RBD | 191 ± 7 | 150 ± 7 | −135 ± 6 |

[a] Note that the measurement for pure Ras(T35S) was carried out on a sample older than 5 h as well as a sample containing BSA as coprecipitant. The former sample is comparable to the microcrystalline state of Ras(T35S), while the latter resembles the liquid state.

**Structural Changes Induced by Crystallization.** The $^{31}$P NMR chemical shifts measured for microcrystals of the wildtype Ras protein complexed with GppNHp are very close to the chemical shifts observed in the solution $^{31}$P NMR experiments.[12] A corresponding behavior was also observed for $^{13}$C and $^{15}$N NMR signals of other proteins.[15,18] In contrast, solid-state $^{31}$P NMR spectra of the microcrystalline Ras(T35S) and Ras(T35A) mutants showed significantly different chemical shifts compared with the liquid state. In particular, the chemical shift of the α-phosphate group changed by 1.4 ppm: −9.7 ppm was found for microcrystals[12] and −11.1 ppm for solutions.[13] This observation indicates that the crystal structure is different from the solution structure at least in the neighborhood of the α-phosphate group. Probably, crystal contacts influence the environment of the nucleotide. As the α-phosphate group is not located at the outer surface of the molecule, direct intermolecular interactions are unlikely to be the reason of this effect. Instead, changes in the hydrogen bond network surrounding the α-phosphate group are the more likely reason. In contrast to microcrystals, freshly prepared precipitates of Ras(T35S) exhibit a chemical shift of −11.1 ppm (see Figure 2 and Table 1) which is exactly the value observed in solution. This indicates that the precipitation process preserves the solution structure as far as the direct environment of the phosphate groups of the nucleotide is concerned. However, the isotropic chemical shift of the α-phosphate group in the PEG precipitate of pure Ras(T35S) changes within the first few hours after precipitation, as is illustrated in Figure 4. A chemical shift of −9.7 ppm is observed for the α-phosphate signal after 5 h which is exactly the value recorded earlier for microcrystals.[12] This surprising observation suggests the growth of small crystals or domains of crystal-like state within the PEG precipitate, a process which obviously takes place on a time scale of several hours. After 5 h, it is virtually completed, since the chemical shift does then exactly correspond to the value found for microcrystalline samples. Afterward, the chemical shift remains constant. However, the assumed small crystals could not be visualized using a light microscope with 100-fold magnification. It is, therefore, concluded that these crystals or crystal-like domains

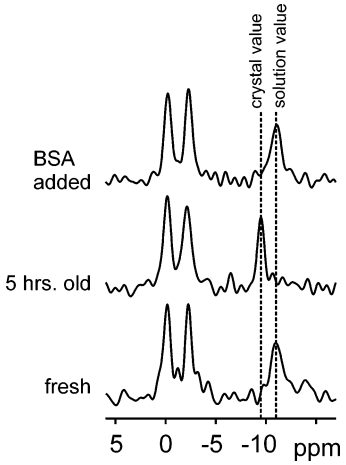


**Figure 4.** $^{31}$P {$^{1}$H} CP MAS NMR spectra of precipitated Ras(T35S)·$Mg^{2+}$·GppNHp. Bottom: Immediately after precipitation employing PEG 6000. Middle: 5 h after precipitation. Note that the sample was kept in a refrigerator during these 5 h. Hence, the observed effect cannot be due to the influence of MAS during that time. Top: 1 day after precipitation if 5 mg of BSA was introduced as an impurity. A 5 mg portion of Ras(T35S) was used for each experiment. 10 000 scans were acquired (measurement time 1.5 h).

must be smaller than 2 $\mu$m which prevents their resolution by the microscope. In contrast, the spectrum of the ammonium sulfate-precipitated sample remains constant over time. The addition of another protein (bovine serum albumin, BSA) to the Ras(T35S) solutions used for the preparation of the precipitates prevents the described crystallization within the PEG precipitates (Figure 4). In agreement with the latter observation, the isotropic chemical shifts of the spectral lines due to the α-phosphate also remain constant at −11.3 and −11.4 ppm over time for Ras(T35S) in complex with Raf-RBD and RalGDS-RBD, respectively, precipitated using PEG (cf. Figure 2). It is, furthermore, important to note within this context that the protein solution used for the growth of pure Ras(T35S) crystals to be analyzed by X-ray crystallography contained PEG 1500.[13] Martin et al.[17] have described a procedure for the preparation of protein nanocrystals from PEG-containing solutions. These nanocrystals were grown within a characteristic time of less than 1 h and delivered solid-state $^{13}$C NMR spectra of similar quality as larger crystals.

Since the published crystal structure of uncomplexed Ras(T35S)·GppNHp has only a resolution of 2.8 Å, no quantitative correlation between the observed change in the isotropic α-phosphate chemical shift and possible crystallization-induced changes in the local structure can be established on the basis of the available data. Possibly, the hydrogen bond network surrounding the nucleotide is influenced by the crystallization leading to the observed shift of the α-phosphate signal from −11.1 to −9.7 ppm. This assumption is supported by observations made for the mutant Ras(V29G)[31] as well as mouse Ras (M-Ras).[32] The solution $^{31}$P NMR spectra of these molecules exhibit α-phosphate chemical shifts of −10.5 ppm for Ras(V29G)[1] and −10.4 ppm for M-Ras.[32] In the crystal structure of M-Ras, a hydrogen bond from the ribose of the nucleotide to a backbone carbonyl group (Val39) is missing. This hydrogen bond is, however, present in the crystal structure of wildtype H-Ras. It is also missing in the crystal structure of human Ras(T35S).[13] It is, therefore, tempting to speculate that the crystallization-induced absence of this hydrogen bond in crystalline human Ras(T35S) causes the described change of the α-phosphate chemical shift compared with the liquid state or with wildtype H-Ras. This is further corroborated by recent experiments on the H-Ras mutant Ras(G60A)[33] which prefers state 1 in solution in analogy to Ras(T35S). If crystallized, this mutant does also not exhibit the aforementioned hydrogen bond.[33]

## Conclusions

From the point of view of the signal-to-noise ratio, solid-state NMR spectroscopy is the method of choice to observe $^{31}$P NMR signals of larger proteins or protein complexes. Protein crystallization, which has not yet been established for the complexes studied here, is not necessarily required, since the samples can be studied in the precipitated state. As was shown for Ras(T35S) and its complexes with effectors, precipitation has the advantage of minimizing crystallization-induced effects. PEG precipitates deliver solid-state $^{31}$P NMR spectra of higher resolution and signal-to-noise ratio than samples precipitated with ammonium sulfate. Weakly bound protein complexes were found to dissociate in the presence of the precipitant, as observed for Ras(T35S) complexed with RalGDS-RBD, while the stronger bound Ras(T35S)·Raf-RBD complexes remain preserved. Interestingly, the $^{31}$P signal due to the α-phosphate of GppNHp bound to Ras(T35S) has a different isotropic chemical shift in crystals compared with the liquid state or freshly prepared precipitates. This directly indicates that the crystal structure deviates from the equilibrium solution structure at least in the environment of the α-phosphate group. Our observations show that crystal-like domains are formed in PEG precipitates of pure Ras(T35S) after several hours. This process can be prevented by the addition of other proteins such as BSA or effector molecules to the solution used for precipitation.

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