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Rapid assignment of solution ^{31}P NMR spectra of large proteins by solid-state spectroscopy ☆

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Abstract

The application of the ^{31}P NMR spectroscopy to large proteins or protein complexes in solution is hampered by a relatively low intrinsic sensitivity coupled with large line widths. Therefore, the assignment of the phosphorus signals by two-dimensional NMR methods in solution is often extremely time consuming. In contrast, the quality of solid-state NMR spectra is not dependent on the molecular mass and the solubility of the protein. For the complex of Ras with the GTP-analogue GppCH$_2$p we show solid-state ^{31}P NMR methods to be more sensitive by almost one order of magnitude than liquid-state NMR. Thus, solid-state NMR seems to be the method of choice for obtaining the resonance assignment of the phosphorus signals of protein complexes in solution. Experiments on Ras·GDP complexes show that the microcrystalline sample can be substituted by a precipitate of the sample and that unexpectedly the two structural states observed earlier in solution are present in crystals as well.

Keywords: ^{31}P NMR; Phosphate-binding proteins; Solid-state NMR; Liquid-state NMR; Two-dimensional techniques

Phosphorylation, especially of serine, threonine, and tyrosine residues, belongs to the most common post-translational modifications observed on peptides and proteins in nature (see, e.g. [1]). Phosphorylation of so-called silaffins, a novel class of peptides involved in the formation of the highly siliceous and regularly structured cell walls of diatoms, was shown to be responsible for their ability to phase-separate and to precipitate silica [2]. Guanine nucleotide-binding (GNB) proteins such as the heterotrimeric G-proteins and proteins of the Ras-superfamily are responsible for signal transduction between transmembrane receptors and cellular effectors. They usually switch between their active and inactive state by binding to GTP and GDP, respectively [3]. For these reasons, ^{31}P NMR spectroscopy currently gains increasing interest in protein NMR spectroscopy. Numerous liquid-state ^{31}P NMR spectroscopic studies were meanwhile carried out on members of the Ras-superfamily such as Ras and Ran complexed with GTP-analogues like GppCH$_2$p or GppNHp (see, e.g. [4–6]). Liquid-state ^{31}P NMR spectroscopy was also used to investigate the self-aggregation of silaffins [2].

If more than one phosphate group is present in a protein complex, assignment problems may arise since the corresponding ^{31}P NMR shifts are strongly dependent on the environment and the degree of protonation, which often prevents an assignment solely based on chemical shifts. For guanine nucleotide-binding proteins it is barely possible to adopt the assignment of the phosphate resonances of the free nucleotide or of the free Mg^{2+}-nucleotide complexes to the resonances derived from measurements on the complex bound to the protein [6]. In principle, modern two-dimensional (2D) NMR techniques could be used to solve the assignment problem. However, indirect detection of the phosphorus nuclei via protons is often impossible. In

☆ *Abbreviations:* DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; DTE, 1,4-dithioerythritol; GTP, guanosine-5′-triphosphate; GppCH$_2$p, guanosine-5′-(β,γ-methyleno)triphosphate; GppNHp, guanosine-5′-(β,γ-imido)triphosphate; Ras, protein product of the human protooncongene H-ras (rat sarcoma).

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phosphate groups, no ^{1}H nuclei are directly bound to the ^{31}P nucleus. The *J*-coupling constants governing a possible polarisation transfer are rather small even for phosphate groups of phosphorylated serine and threonine as well as the α-phosphate group of GTP. For phosphorylated tyrosine and the β- or γ-phosphate groups, however, the ^{1}H–^{31}P *J*-coupling constants are negligible.

Two-dimensional techniques using direct ^{31}P detection are, however, extremely time consuming due to sensitivity problems arising from the large longitudinal ^{31}P relaxation times. Commonly, T_1-values of the order of 2–30 s are observed for phosphate groups bound to proteins as they are found in covalent modifications of amino acids or nucleotides bound to proteins and peptides. The repetition time applicable in liquid-state ^{31}P NMR experiments can be, therefore, of the order of minutes. Correspondingly long acquisition times are the consequence that limits the sensitivity. The low sensitivity can usually not be compensated by high phosphorus concentrations. In contrast to most small molecules such as free nucleotides which can be measured at concentrations of 100 mM, protein samples like GNB-proteins are normally soluble and stable only at concentrations below 1–3 mM. At external magnetic fields $B_0 \geqslant 5$ T, transverse ^{31}P relaxation is governed by the chemical shift anisotropy (CSA) [7]. The correlation time τ_C for molecular reorientation is higher than 5 ns for proteins with a molecular mass exceeding 10 kDa dissolved in water at room temperature. CSA relaxation rates R_2^{CSA} beyond $30\ s^{-1}$ follow from this correlation times corresponding to a contribution to the line width (full width at half maximum) exceeding 10 Hz for $\Delta\sigma = 150$ ppm and $\eta = 0.5$, the average values observed [8] for the ^{31}P NMR signals of Ras(wt)·Mg^{2+}·$GppCH_2p$ ($M = 19$ kDa), at a ^{31}P resonance frequency of 202.46 MHz ($B_0 = 11.74$ T). Since R_2^{CSA} is proportional to the square of the chemical shift anisotropy $\Delta\sigma$, this contribution rapidly increases at higher field strengths. In other words, liquid-state ^{31}P NMR spectra exhibit a decreasing spectral resolution at higher B_0. It is, therefore, impossible to apply higher magnetic field strengths in order to solve the sensitivity problem.

On the other hand, recent methodological progress makes solid-state NMR increasingly applicable in protein NMR spectroscopy (see, e.g. [8–13]). In the present contribution we compare liquid- and solid-state ^{31}P NMR spectra of Ras·Mg^{2+}·$GppCH_2p$ especially with respect to 2D spectroscopic techniques.

Materials and methods

NMR spectroscopy. Liquid-state ^{31}P NMR NOESY experiments were performed on a Bruker Avance 500 spectrometer operating at a ^{31}P resonance frequency of 202.46 MHz using 8 mm Shigemi tubes in a 10 mm probe. Seven hundred microliters of a 1.6 mM solution of Ras(wt)·Mg^{2+}·$GppCH_2p$ or a 1.3 mM solution of Ras(T35S)·Mg^{2+}·$GppCH_2p$ in 40 mM Hepes/NaOH, pH 7.4, 10 mM $MgCl_2$, 2 mM DTE, 0.1 mM DSS, and 95% H_2O/5% D_2O was measured at different temperatures. 128 t_1 increments were recorded. The mixing time was varied between 0.2 and 3.5 s. The delay between 2 scans was 10 s, which corresponds to 3–4 times T_1. Protons were decoupled during t_1 evolution by a proton 180° pulse and during t_2 by a GARP sequence.

T_1 relaxation times of the phosphorus nuclei of the bound nucleotides were obtained by a standard inversion recovery experiment. The delay between the 180° and 90° pulse was varied between 3 ms and 16 s. The delay between 2 scans was 21 s.

The solid-state ^{31}P NMR experiments were performed on a Bruker Avance 300 spectrometer operating at 300.13 MHz for ^{1}H and 121.44 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. Heteronuclear TPPM decoupling [14] was applied during data acquisition.

Crystallisation of Ras-nucleotide complexes. Ras(wt)·Mg^{2+}·$GppCH_2p$ was prepared and crystallised as described in [8]. Crystals of Ras(wt)·Mg^{2+}·GDP were grown following the sitting drop method. Approximately 15–20 mg of crystalline protein was required for the NMR experiment. Seventeen microliters of 50 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithioerythrtiol (DTE), and 56% (w/v) polyethylene glycol 400 were added to 15 µl of 1.2 mM protein solution in each well of the plates. The plates (3 Well Spot Plate, Hampton Research) were sealed with transparent tape and incubated in a darkroom at 291 K. Crystals grew within 2 days.

Precipitation of Ras(wt)·Mg^{2+}·GDP. A solution of 1.5 mM Ras was incubated at 273 K and slowly adjusted to 3.2 M $(NH_4)_2SO_4$ by adding solid $(NH_4)_2SO_4$. Precipitation employing $(NH_4)_2SO_4$ turned out to be superior to the use of polyethylene glycol 400 and polyethylene glycol 6000 as precipitant in terms of spectral quality. Approximately 5 mg of precipitated protein was used for the NMR experiment.

Results and discussion

Nucleoside triphosphates are usually bound to proteins in the presence of Mg^{2+}. Binding to Ras or other small GTPases often leads to large ^{31}P chemical shift changes compared to the free nucleotide. This prevents an assignment of these signals based on the chemical shifts of the free nucleotide on its own [6]. The assignment problem can, in principle, be overcome by nuclear Overhauser effect spectroscopy (NOESY) or by correlation spectroscopy (COSY or TOCSY). Since the ^{31}P–^{31}P *J*-couplings are small (see above) compared to the line widths, the COSY cross-peaks disappear due to their anti-phase structure. This prevents the application of the COSY experiment. The homonuclear 2D ^{31}P–^{31}P NOESY spectrum of Ras(wt)·Mg^{2+}·$GppCH_2p$ is shown in Fig. 1A. Since there is no ^{1}H nucleus directly attached to phosphorus, efficient spin polarisation transfer from ^{1}H nuclei to ^{31}P cannot be performed. To optimise the acquisition parameters, T_1 relaxation times were determined for each of the ^{31}P nuclei of the bound nucleotide by a standard inversion recovery experiment (data not shown). The obtained values of the ^{31}P nuclei are 1.5 ± 0.1 s of the α-phosphate, 2.9 ± 0.2 s of the β-phosphate, and 3.2 ± 0.2 s of the γ-phosphate group at 293 K. The repetition time used in the ^{31}P–^{31}P NOESY experiment was 10 s. The time required to measure the 2D spectrum shown in Fig. 1A was ca. 3 days. As can be seen from the diagonal peaks at 2.6 and 16.8 ppm, the supposedly non-hydrolysable GTP-analogue even started to hydrolyse during the long duration of this measurement at room temperature. The mixing time was varied between

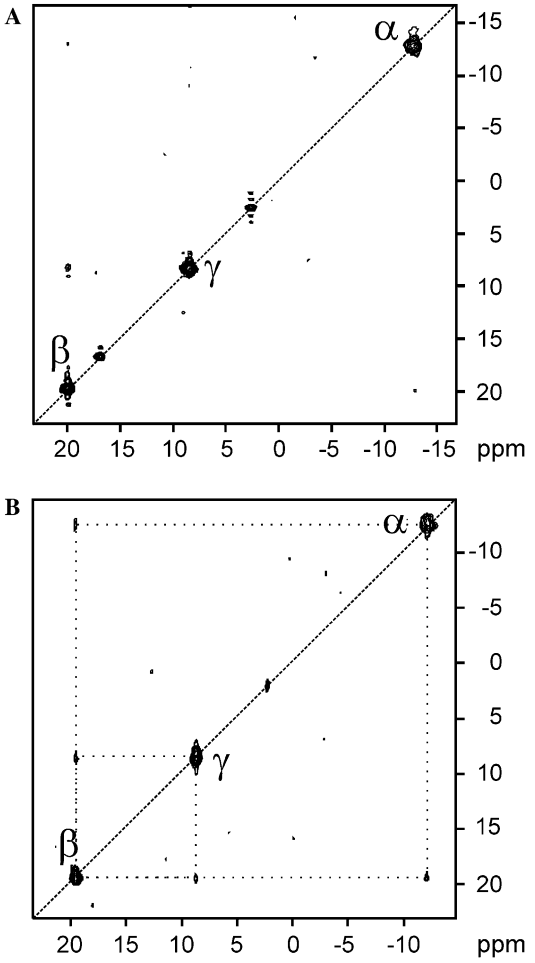

Fig. 1. (A) Liquid-state ^{1}H decoupled ^{31}P–^{31}P 2D NOESY spectrum of 700 μl 1.6 mM Ras(wt)·Mg^{2+}·$GppCH_2p$ measured at 202.4 MHz ^{31}P resonance frequency at 293 K. 160 scans were acquired for each of the 128 t_1 increments using a mixing time of 1.3 s and a repetition time of 10 s. Resulting measurement time: ca. 65 h. (B) Liquid-state ^{1}H decoupled ^{31}P–^{31}P 2D NOESY spectrum of 700 μl 1.3 mM Ras(T35S)·Mg^{2+}·$GppCH_2p$ measured at 202.4 MHz ^{31}P resonance frequency at 278 K. 192 scans were acquired for each of the 128 t_1 increments using a mixing time of 2 s and a repetition time of 10 s. Resulting measurement time: ca. 78 h. Note that the unassigned signals in these spectra are due to hydrolysis products of the nucleotide.

0.2 and 3.5 s, thereby it turned out that the mixing time of 1.3 s provided best results. Whereas the diagonal peaks could be seen, cross-peaks can hardly be detected. The experiment was, therefore, repeated with a well-known Ras mutant, Ras(T35S), in complex with the same GTP-analogue. In contrast to the wild-type [6], no broadening of the ^{31}P NMR signals due to conformational exchange is observed at 278 K. It is, therefore, possible to cool this sample to 278 K in order to slow down the hydrolysis of the nucleotide. The resulting ^{31}P–^{31}P NOESY spectrum acquired within ca. 3 days is shown in Fig. 1B. Weak cross-peaks show up in this spectrum.

A solid-state NMR spectroscopic analogue of the 2D NOESY experiment, the proton-driven ^{31}P–^{31}P spin-diffusion spectrum [15], is shown in Fig. 2 for microcrystalline Ras(wt)·Mg^{2+}·$GppCH_2p$. The sample contained approxi-

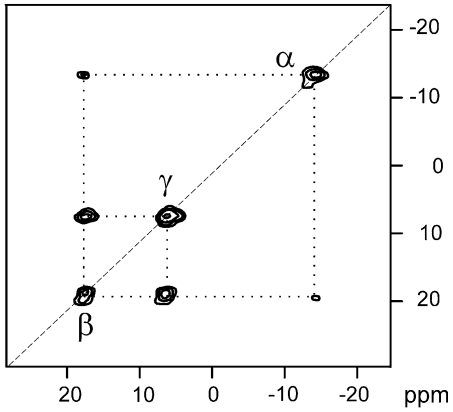

Fig. 2. ^{1}H driven spin-diffusion experiment on microcrystals of Ras(wt)·Mg^{2+}·$GppCH_2p$ at 298 K with a mixing time of 3 ms, a sample spinning rate of 10 kHz, and a recycle delay of 0.3 s. 864 scans were acquired for each of the 256 t_1 increments. Resulting measurement time: ca. 18 h.

mately the same amount of protein as used in Fig. 1A. The solid-state spectrum measured within 18 h (instead of 65 h, required for the spectrum shown in Fig. 1A) exhibits a considerably better signal-to-noise ratio compared to the liquid-state NMR spectrum especially for the cross-peaks. In the presented experiments the signal-to-noise of the $^{31}P^{\beta}$–$^{31}P^{\gamma}$ cross-peak is 7 times better than in solution taking into account the measurement period (Table 1). The resolution of the two spectra is comparable. It is, furthermore, important to note that the GTP-analogue does not hydrolyse during the measurement time in contrast to the liquid state. The improvement in sensitivity in the solid-state NMR spectrum is caused by two effects: (1) in contrast to liquid-state NMR, spin polarisation could be transferred from neighbouring ^{1}H to ^{31}P nuclei by conventional ramped cross-polarisation. The repetition rate is, therefore, governed by the short T_1 of the ^{1}H nuclei which was found to be of the order of 50–100 ms. (2) A further increase in sensitivity is, of course, obtained by the polarisation transfer from ^{1}H to ^{31}P due to the higher magnetogyric ratio of ^{1}H. The resulting sensitivity of solid-state ^{31}P NMR opens the way to the application of many other 2D techniques to phosphate containing proteins or protein complexes as it is illustrated in Fig. 3. This figure shows the 2D ^{1}H–^{31}P heteronuclear correlation (HETCOR) [16] spectrum measured under phase-modulated Lee-Goldburg (PMLG) decoupling [17] during t_1 evolution. As can be seen, dipolar contact between the ^{31}P nuclei and neighbouring ^{1}H nuclei could be detected. Apart from the expected cross-peaks between the ^{1}H nuclei of the CH_2 group of $GppCH_2p$ and the neighbouring $^{31}P^{\beta}$ and $^{31}P^{\gamma}$ another cross-peak is observed around 10 ppm for all three phosphate groups. At the actual spectral resolution it is impossible to decide if these cross-peaks are due to a coupling to the same proton(s) or to protons with similar chemical shifts. In the first case, the amino group of Lys16 would be a plausible candidate since it is assumed to be close to the phosphate groups. In the latter case, the existence of

Table 1
Signal-to-noise ratio in solid and liquid state[a]

^{31}P cross-peak	NOESY (Fig. 1A)			1H-SD (Fig. 2)		
	Amount of protein (mg)	S/N	S/N*[b] (mg^{-1} h^{-1})	Amount of protein (mg)	S/N	S/N*[b] (mg^{-1} h^{-1})
α–α	20	8.3	0.006	~20	4.9	0.014
α–β		**2.0**	**0.001**		**2.5**	**0.007**
β–β		56.3	0.043		4.2	0.012
β–γ		**2.5**	**0.002**		**5.0**	**0.014**
γ–γ		32.7	0.025		8.3	0.023

The bold letters characterise cross-peaks.
[a] The signal-to-noise ratio was determined in the spectrum shown in Figs. 1A and 2 using an exponential filtering with 20 and 115 Hz, respectively, which corresponds to half of the mean line width in liquid and solid state, respectively.
[b] The normalized S/N* was calculated by division of S/N by the amount of protein and the measurement time used.

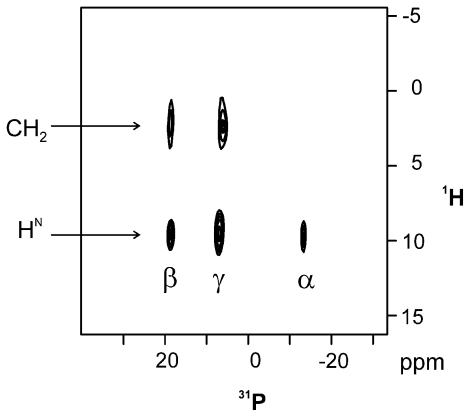

Fig. 3. 2D PMLG decoupled ^{1}H–^{31}P LG-CP HETCOR spectrum of Ras(wt)·Mg^{2+}·$GppCH_2p$ recorded at a sample spinning rate of 16 kHz. The data were obtained with a LG-CP contact of 2 ms. 128 t_1 increments (2300 scans for each increment) were acquired.

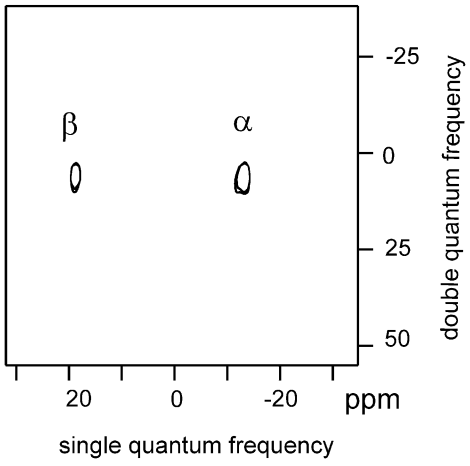

Fig. 4. ^{31}P 2D refocused INADEQUATE spectrum of Ras(wt)·Mg^{2+}·$GppCH_2p$ recorded at a sample spinning rate of 10 kHz with a ^{31}P{^{1}H} cross-polarisation mixing time of 3 ms, a τ delay of 5 s, and a recycle delay of 0.5 s. 64 t_1 increments (6900 scans for each increment) were acquired. This 2D experiment correlates double-quantum coherence with single-quantum coherence. The double-quantum coherence is created during the evolution period (2τ) under J-coupling. The maximum double-quantum coherence is created for $\tau = 1/(4J)$.

contacts is in agreement with the X-ray structure of the protein predicting a variety of hydrogen bonds between the phosphate groups and backbone H^N groups [18].

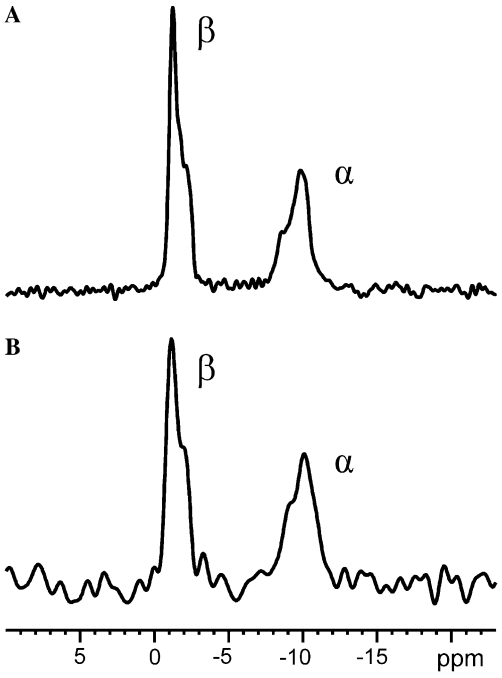

Fig. 5. Ramped ^{1}H–^{31}P cross-polarisation spectra on (A) crystalline Ras(wt)·Mg^{2+}·GDP, 90000 scans (experimental time: 14 h) and (B) precipitated Ras(wt)·Mg^{2+}·GDP, 240000 scans (experimental time: 36 h). TPPM decoupling was applied during signal acquisition. Relaxation delays were adjusted to 0.5 s, contact time to 3 ms, and MAS spinning rate to 10 kHz.

Finally, the ^{31}P 2D refocused INADEQUATE (incredible natural abundance double-quantum transfer experiment [19]) spectrum of microcrystalline Ras(wt)·Mg^{2+}·$GppCH_2p$ is shown in Fig. 4. Obviously, the correlation between the α- and β-phosphate group of the nucleotide is reflected by the corresponding cross-peaks. Note that the absolute value of the J-coupling constant between the α- and β-phosphate group in the free nucleotide amounts to 26.1 Hz. However, no cross-peaks due to the coupling between β- and γ-phosphate groups are observed due to the small J-coupling constant (8.9 Hz for the free nucleotide in the liquid state).

Although only small crystallites are necessary for the solid-state investigations, the crystallisation could be the limiting factor. Therefore, the question was if it is also pos-

sible to obtain results of comparable quality with precipitated protein as well. Fig. 5 shows the solid-state ^{31}P NMR spectra of Ras(wt)·Mg^{2+}·GDP in crystallised form and in precipitated form. Obviously, the precipitated protein delivers a spectrum of similar resolution as the crystalline sample. In both samples, the existence of two states can be detected as it was shown earlier by liquid-state ^{31}P NMR and EPR investigations on Ras·GDP complexes [20]. This fact is, however, not detected by the X-ray crystallography. The difference in signal-to-noise compared to the crystallised sample is caused by the lower concentration of the protein in the precipitated form.

Conclusion

In summary, we can state that solid-state ^{31}P NMR spectroscopy is clearly superior to liquid-state ^{31}P NMR for larger phosphorylated proteins or nucleotide-binding proteins with respect to sensitivity. It can be very helpful or even represent the only possibility to assign and interpret solution ^{31}P NMR-spectra of protein complexes. Although in the present example a microcrystalline sample was used, this is most probably not mandatory since high-quality solid-state NMR spectra can often be obtained by precipitation of the protein. In case of Ras(wt)·Mg^{2+}·GDP, the resolution of the spectra is very similar in the two forms. In general, a combination of liquid-state with solid-state NMR data seems to be a promising avenue to solve assignment problems of large proteins when indirect detection is not possible.

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