Review

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What vibrations tell us about GTPases

Abstract: In this review, we discuss how time-resolved Fourier transform infrared (FTIR) spectroscopy is used to understand how GTP hydrolysis is catalyzed by small GTPases and their cognate GTPase-activating proteins (GAPs). By interaction with small GTPases, GAPs regulate important signal transduction pathways and transport mechanisms in cells. The GTPase reaction terminates signaling and controls transport. Dysfunctions of GTP hydrolysis in these proteins are linked to serious diseases including cancer. Using FTIR, we resolved both the intrinsic and GAP-catalyzed GTPase reaction of the small GTPase Ras with high spatiotemporal resolution and atomic detail. This provided detailed insight into the order of events and how the active site is completed for catalysis. Comparisons of Ras with other small GTPases revealed conservation and variation in the catalytic mechanisms. The approach was extended to more nearly physiological conditions at a membrane. Interactions of membrane-anchored GTPases and their extraction from the membrane are studied using the attenuated total reflection (ATR) technique.

Keywords: biomolecular simulations; caged compounds; catalysis; proteins; reaction mechanism; vibrational spectroscopy.

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Introduction: small GTPases

Small GTPases are molecular switches that regulate and propagate cellular signals depending on the bound nucleotide GTP or GDP. Comprehensive details regarding this class of proteins have been discussed in several excellent reviews (Vetter and Wittinghofer, 2001; Wittinghofer and Vetter, 2011; Ligeti et al., 2012; Cherfils and Zeghouf, 2013). Figure 1 shows the complete GTPase cycle including the critical reactions and interactions. The switch region conformation depends on the bound nucleotide species. The switch is in the 'on' state or 'off' state when GTP or GDP is bound, respectively. In the GTPase reaction, the hydrolysis of GTP to GDP switches the small GTPases off, terminating the interaction with effector proteins. Dysfunction in the 'switch off' reaction leads to uncontrolled signaling and is involved in several severe diseases. Therefore, understanding the 'switch off' mechanism is of great interest. This reaction can be monitored using time-resolved FTIR spectroscopy by means of caged GTP [labeled (1) in Figure 1]. In water, GTP hydrolyzes at a rate of 5×10^{-8} s^{-1} at 303 K, whereas Ras-bound GTP has a rate of 6.8×10^{-4} s^{-1} at 303 K (Kötting and Gerwert, 2004). This rate is still too slow to regulate signal transduction pathways. Therefore, the rate is enhanced by GTPase-activating proteins (GAPs) to 7.8 s^{-1} at 303 K. GAPs play an important role in the regulation of signal transduction pathways (Phillips et al., 2003). Furthermore, GTPases can interact with guanine exchange factors (GEFs), which induce the 'switch on' conformation by exchanging GDP for GTP. The GTP-bound 'on' state interacts with downstream effectors and transduces the signal.

Guanine nucleotide dissociation inhibitors (GDIs) can also regulate GTPase signal transduction. Despite the name, GDIs' main function is soluble complex formation. Many GTPases have lipid anchor post-translational modifications on their C-terminus (e.g., farnesyl, geranylgeranyl, and/or palmitoyl), and membrane targeting plays an important role in GTPase regulation and function (Hodges-Loaiza et al., 2011). GDIs can both detach GTPases from or deliver them to the membrane. ATR-FTIR spectroscopy allows us to investigate GTPase effector and GTPase GDI interactions [labeled (2) and (3) in Figure 1].

Structurally, all small GTPases share a common G-domain consisting of five α-helices and a six-stranded β-sheet. GTPases are involved in a large number of cellular signaling and transport mechanisms. They are grouped into five subfamilies. Ras GTPases are mainly involved in

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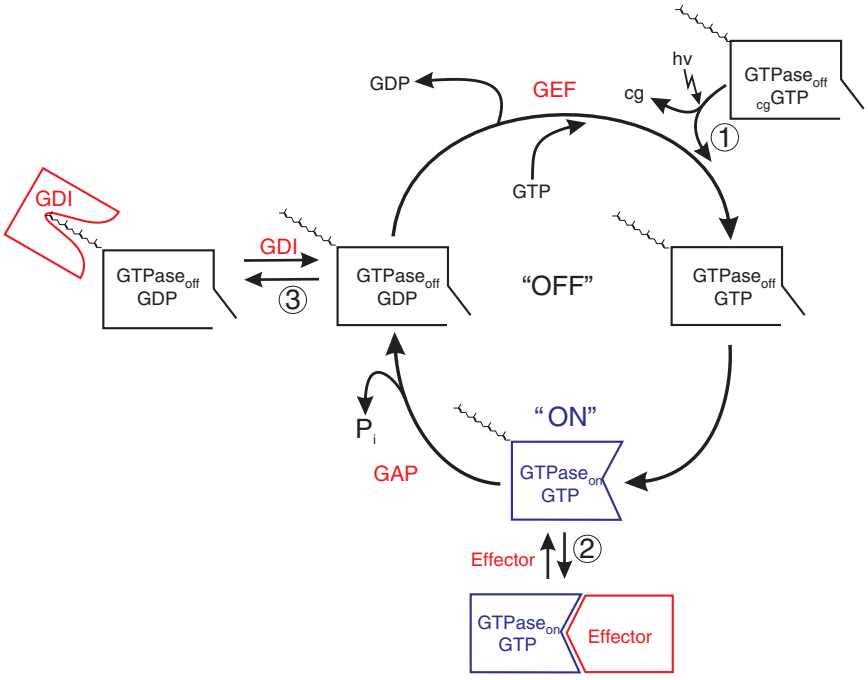

Figure 1 The GTPase cycles between the 'off' state (shown in black) and the 'on' state (shown in blue).
GEF and GAP proteins enhance the molecular switching. ① FTIR spectroscopy investigations of the GTPase reaction require a caged (cg)-GTP that releases GTP upon laser exposure. ATR-FTIR spectroscopy allows for the investigation of ② GTPase effectors or ③ GTPase GDI interactions.

cell growth (Cox and Der, 2010). Rho primarily regulates the cytoskeleton (Sit and Manser, 2011). Rab (Stenmark, 2009) and Arf (Khan and Ménétrey, 2013) are important for vesicular transport and Ran (Jamali et al., 2011) for nuclear transport. Given their critical functions, point mutations cause serious cellular dysfunction and result in several diseases, including cancer in case of Ras (Cox and Der, 2010). For this reason, Ras is the best studied GTPase and the main focus of our FTIR studies.

FTIR of proteins

Time-resolved FTIR difference spectroscopy has been established as a powerful tool for protein studies. Currently, there are over 2000 publications each year using FTIR for protein investigations. The FTIR difference spectroscopy approach was introduced by studies on rhodopsin by stabilizing an intermediate at low temperatures (Siebert et al., 1983). The implementation of room temperature time-resolved FTIR studies, and most important the first clear-cut band assignment by site-directed mutagenesis in bacteriorhodopsin (bR), allowed deduction of a detailed molecular reaction mechanism for the first time for a membrane protein. The bR pump mechanism was elucidated along with detailed protonation changes in the Schiff base, Asp85 and Asp 96 (Gerwert, 1988; Gerwert et al., 1989, 1990). The approach was adapted for chromophore-less GTPase proteins using photolabile caged compounds to initiate the GTP hydrolysis (Cepus et al., 1998). Protein FTIR studies have been extensively reviewed previously (Gerwert, 1993; Barth and Zscherp, 2002; Kötting and Gerwert, 2005a; Siebert and Hildebrandt, 2008; Hering and Haris, 2009; Ataka et al., 2010). The absorbance spectrum of a protein is dominated by the amide bond absorptions (Figure 2A). The amide I band (C=O stretching) can be used to determine the protein secondary structure due to changes in hydrogen bonding (Dannenberg, 2006) and peptide bond torsional angles (Ham and Cho, 2003) leading to band shifts. The secondary structure motif contribution can be estimated by decomposition of the overall amide I band (Güldenhaupt et al., 2008; Goormaghtigh et al., 2009).

In order to gain information on the protein reaction mechanism, difference spectroscopy was employed. Figure 2B shows the difference spectrum between Ras·GDP and Ras·GTP. Of the several thousand vibrational modes present in a protein, most do not change during the reaction and are cancelled out. As a result, the few absorbance

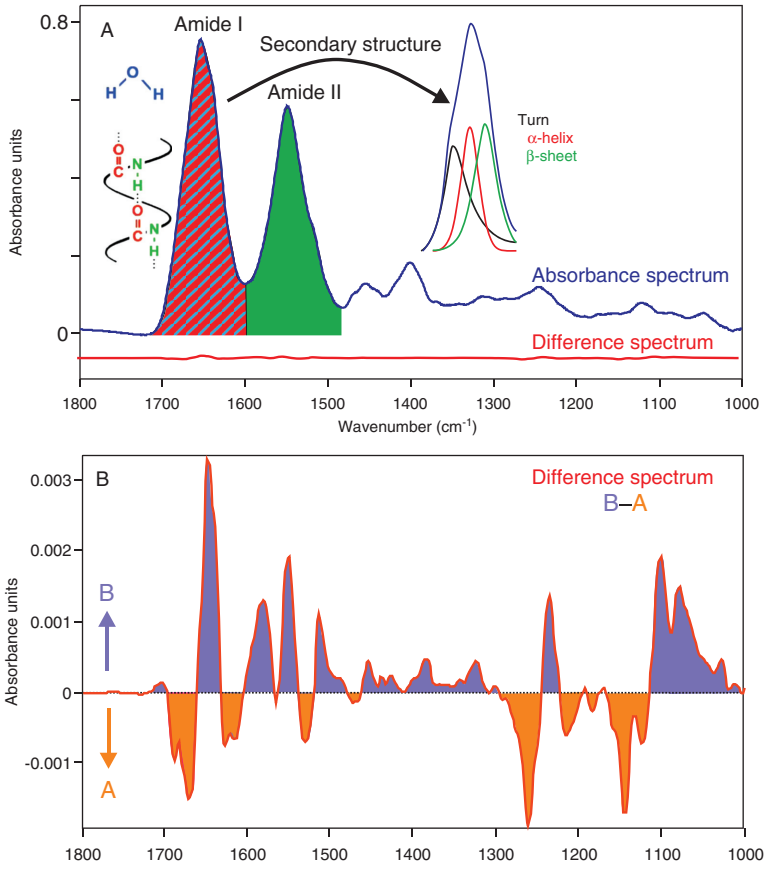

Figure 2 IR-absorbance and IR-difference spectra of proteins.
(A) The IR-absorbance spectrum of the Ras protein (shown in blue). Secondary structure analysis can be performed by decomposing the amide I band. An FTIR difference spectrum of the Ras GTPase reaction is shown in red. Most of the bands do not change during the reaction and are cancelled out. A differently scaled version of the same spectrum is shown in (B). Here, individual functional group absorptions are resolved. Downward facing bands belong to state A (Ras·GTP), and upward facing bands are from state B (Ras·GDP). Adapted from Kötting and Gerwert (2005b).

bands that change during the reaction become apparent. The individual functional group absorptions can be elucidated and provide atomic scale information. In order to investigate a reaction by time-resolved FTIR spectroscopy, the reaction must be initiated by a fast, short trigger event. The sample must be equilibrated within the spectrometer to detect the small signal differences within a large background. Temperature and light path differences induced by sample handling could cause a background change larger than the difference signal. Figure 3 shows a typical FTIR setup, based on a Bruker instrument and developed by the Ruhr University Bochum Department of Biophysics. The spectrometer is placed on a vibrationally isolated table in an air-conditioned lab (±0.5°C). Best results are obtained using a vacuum spectrometer, avoiding CO_2 and water vapor disturbances. In the simplest case, a highly concentrated protein sample in a buffer solution is positioned in a vacuum tight cuvette with two CaF_2 windows in a sample chamber (Figure 3B). In the case of proteins with chromophores, the reaction can be initiated directly by a laser flash. For small GTPases, a photolabile nucleotide, caged GTP, is bound to the GTPase. Small GTPases bind caged GTP with high affinity (6×10^8 M^{-1} for Ras), but the caged GTP is not hydrolyzed (Schlichting et al., 1989). As water has a very strong infrared absorbance, approximately 10-µm path lengths are used. This makes high protein concentration a necessity. For a single sample, approximately 100 µg of GTPase is required. A UV laser flash (e.g., 308 nm from a XeCl-excimer laser) initiates photo dissociation of the caged group, leaving GTP within the binding pocket of the GTPase. Caged GTP in the form of the P3-1-(2-nitrophenyl)ethyl ester (Barth et al., 1997; Cepus et al., 1998) is commercially available. Under some conditions, the photolysis of this compound is slow, necessitating the use of P3-para-hydroxyphenacyl ester (Klán et al., 2013).

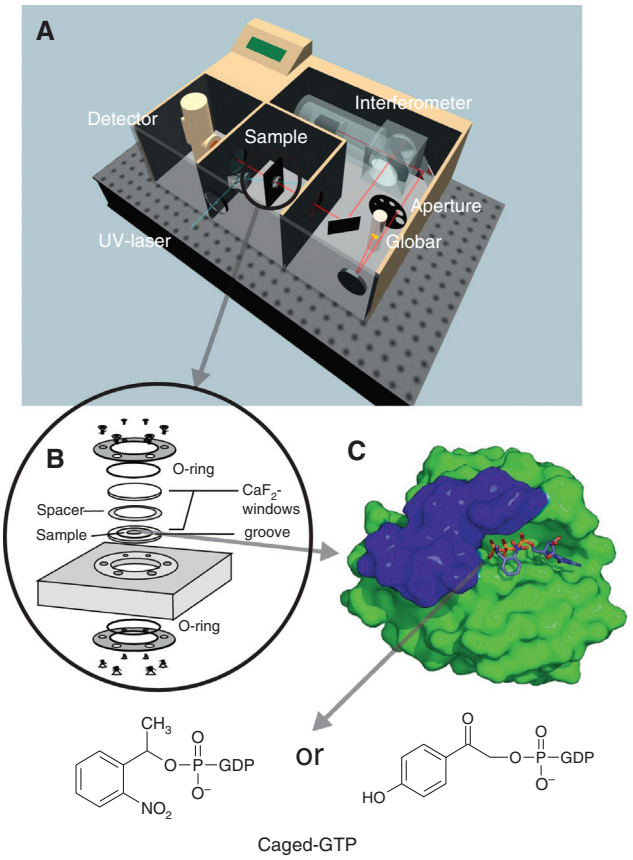

Figure 3 The experimental setup for time-resolved FTIR measurements of the GTPase reaction.
(A) The FTIR spectrometer with an additional UV-laser directed on the sample. (B) A transmission cuvette containing highly concentrated GTPase in solution is placed in the sample compartment. (C) A caged nucleotide is bound to the GTPase. The GTP is released using a UV laser flash, allowing the GTPase reaction to be monitored. In the GTPase reaction, the conformation of the switch region of the GTPase (shown in blue) is changing. Adapted from Kötting et al. (2010).

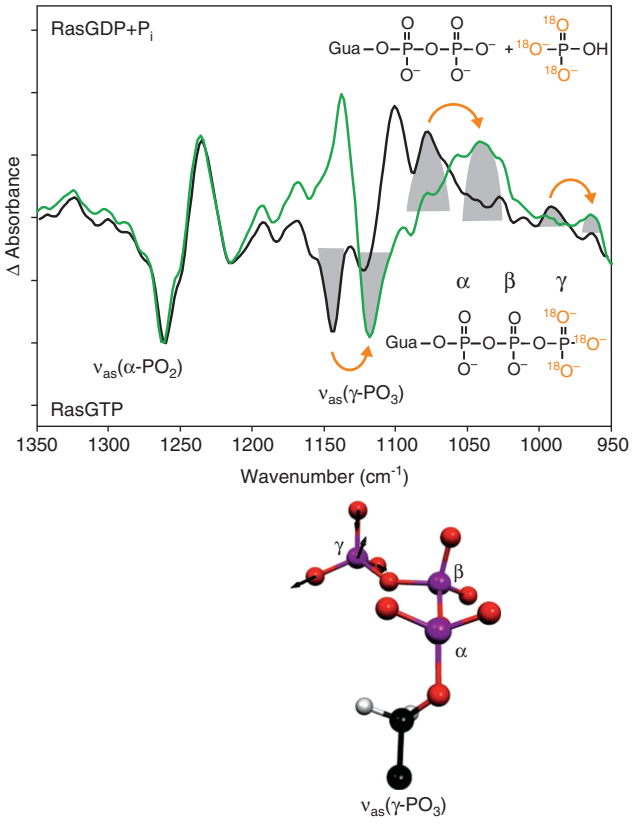

Figure 4 Band assignment by isotopically labeled nucleotide.
The phosphate region of a difference spectrum for the Ras GTPase reaction is shown in black (also see Figure 2).
Downward facing bands belong to Ras·GTP, and upward facing bands are from Ras·GDP+P_i. The same difference spectrum using γ-$^{18}O_3$-labeled GTP is shown in green. The ν_{as}(γ-PO_3)-vibration absorption shifted upon labeling, whereas the other bands such as the ν_{as}(α-PO_2)-vibration absorption remain unchanged. The released P_i is also labeled, inducing shifts of the 1078 and 990 cm^{-1} bands. The calculated ν_{as}(γ-PO_3)-vibration normal mode is shown below the spectrum. Adapted from Gerwert and Kötting (2010).

Another requirement to gain insight into reaction mechanisms is the assignment of the absorbance bands to specific functional groups within the protein. These assignments can be performed using site-directed mutagenesis (Gerwert et al., 1989), isotopically labeling specific protein sites (Engelhard et al., 1985), or introducing site-specific prosthetic groups (Gerwert and Siebert, 1986). As most mutations in the interesting region are too invasive, GTPase band assignments were primarily made using isotopic labeling (Warscheid et al., 2008). Chemical synthesis of GTP with site-specific ^{18}O-labeled phosphate allows us to assign all phosphate stretching modes. Figure 4 shows the PO stretching mode assignment of the GTP γ-phosphate. GTP band shifts observed during GTPase binding and GAP protein interactions reveal the catalytic action of the protein on the substrate. These are small changes, but they can be decoded by combining experimental and theoretical FTIR spectroscopy (discussed in more detail below). Isotopic labels can be incorporated during protein expression by using modified M9 media supplemented with a stable isotope-labeled amino acid (Warscheid et al., 2008). This method is only group specific, with all the amino acids of a specific type labeled. However, as the experiment is based on the difference spectra, and there is often only one labeled amino acid within the reactive region, a clear assignment is feasible. In some cases, coupling isotopic labeling with site-directed mutagenesis is necessary. Figure 5 shows an example of a protein group assignment (Thr 35) for Ras

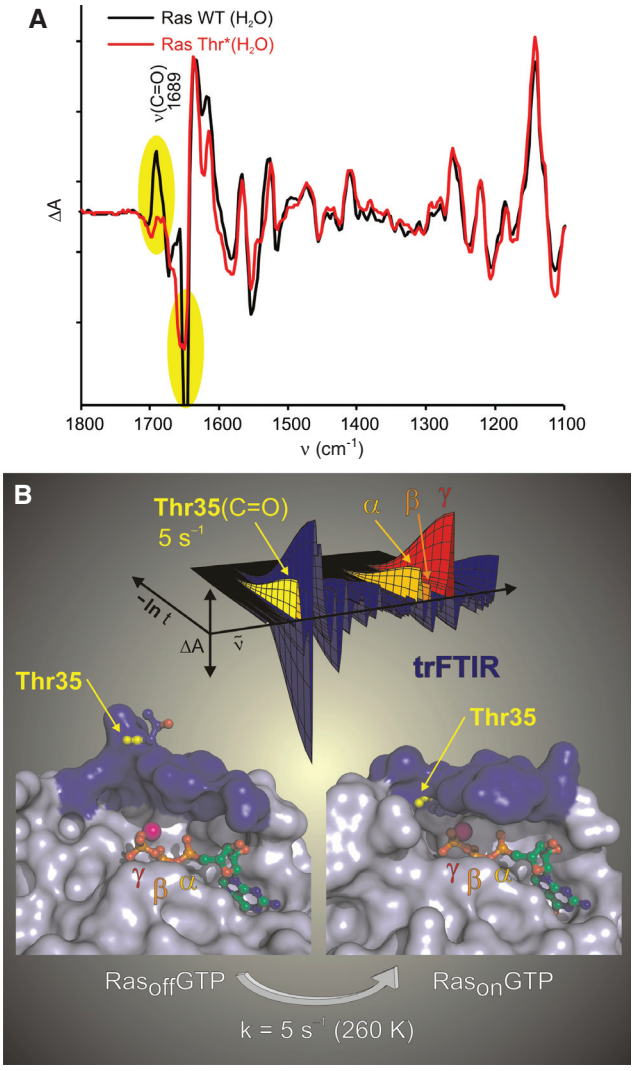

Figure 5 Band assignment by isotopically labeled protein. (A) An FTIR difference spectrum of the Ras 'off' to 'on' conformational change. Downward facing bands belong to the 'off' state and upward facing bands are from the 'on' state. The same difference spectrum using $^{13}C^{15}N$-Thr-labeled Ras is shown in red. The 1689 cm^{-1} band shifted upon labeling and can be assigned to the carbonyl stretching vibration of unlabeled Thr35 in the Ras 'on' state. (B) A time-resolved FTIR spectrum of the Ras conformational change. The Thr35 carbonyl group is shown in yellow. In the 'off' state, the group is in a hydrophilic environment, whereas it moves to a more hydrophobic environment in the 'on' state. This induces an unusual blueshift of the 1689 cm^{-1} band. The conformational change can also be seen with the phosphate band changes, indicated in the time resolved FTIR spectra. Adapted from Kötting et al. (2007).

(Kötting et al., 2007). In the 'on' state, the Thr35 backbone carbonyl group is in an unusual hydrophobic environment, leading to a vibrational blueshift to 1689 cm^{-1}. Even though all the Thr residues are $^{13}C^{15}N$ labeled, only the Thr35 band is observed, whereas the remaining Thr bands remain unchanged and do not appear in the difference spectrum.

Ras reaction mechanism

Once a band is assigned, the time-resolved behavior of the corresponding residue can be elucidated. For example, the Thr35 absorbance band was used as a marker band for the Ras 'on' state. This absorption shows up when the switch is in the 'on' state and disappears when switched off. Figure 6 shows Thr35's behavior during the GAP-catalyzed GTPase reaction of Ras. A global kinetic analysis can be performed to analyze all the absorbance changes simultaneously (Hessling et al., 1993). The apparent rate constants and their corresponding amplitude spectra are revealed. Various marker bands, such as Thr35, the Arg finger, and isotopically labeled phosphate, can now be followed over the course of the reaction. For Ras, the global fit analysis revealed that the absorbance changes can be described by three exponential functions corresponding to three apparent rate constants (Kötting et al., 2006, 2008, 2012). After Ras_{off}·cgGTP photolysis, Ras_{off}·GTP was formed. The first rate constant (k_1) showed the conformational change toward the 'on' state through the Thr35 marker band evolution. During the second step, described by the second rate constant (k_2), no Thr 35 marker band change was observed, and the switch remained in the 'on' state. Afterwards, Ras switched off, producing the final rate constant (k_3). The marker band kinetic data was combined to deduce the order of events in the reaction mechanism. For example, the Arg finger marker band is in a hydrophilic environment initially (green plot in Figure 6B) and showed no change with k_1, but decreased with k_2, indicating that the Arg finger moved into the hydrophobic environment of the GTP-binding pocket (Kötting et al., 2008). Already with the same apparent rate, protein-bound phosphate, a product of GTP hydrolysis, appeared (purple plot in Figure 6B). This implies that the bond breakage was much faster than the Arg finger movement. As soon as the arginine completes the active site, bond breakage occurs instantaneously. During the final step, the phosphate leaves the protein (purple marker band decreases), the Arg finger leaves the binding pocket for the hydrophilic environment (green marker band increases), and the switch regions shift into the 'off' conformation (blue marker band decreases). This P_i release from the protein into the bulk solvent has the slowest rate (k_3). Therefore, in Ras, P_i release is the rate-limiting step of the overall reaction rather than GTP bond cleavage.

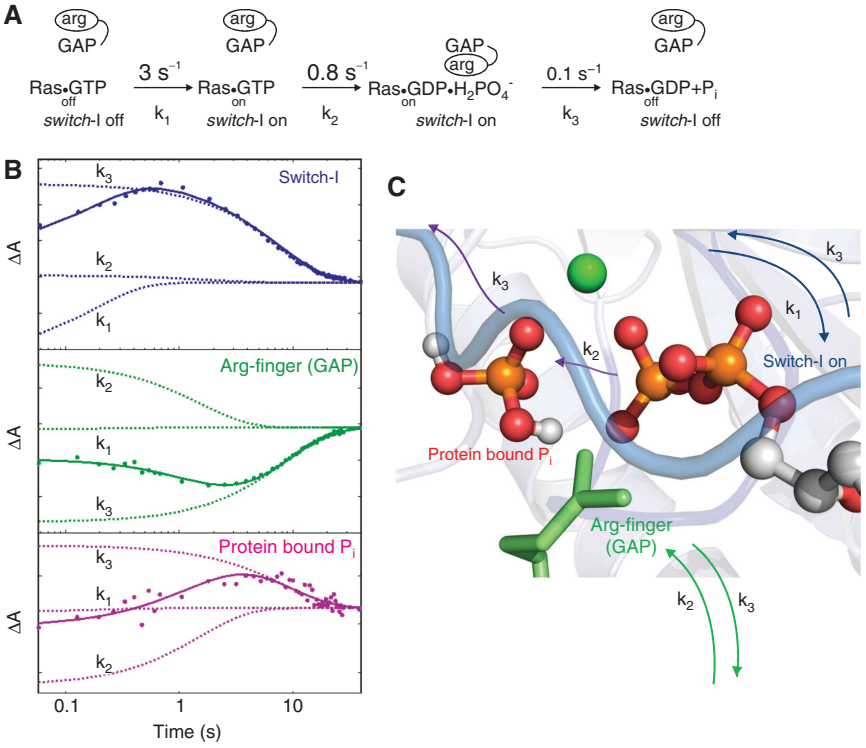

Figure 6 The Ras·RasGAP GTPase reaction.
(A) The overall reaction mechanism can be described by three apparent rate constants; the shift to the 'on' conformation, the shift of the Arg finger with immediate hydrolysis of GTP, and the release of P_i into the bulk solvent. (B) Important events are monitored by the marker band kinetics. The switch I marker band is the carbonyl absorption of Thr35 (blue – also shown in Figure 5). The Arg finger marker band (green) and the protein-bound phosphate band (purple) were also assigned. The contributions to each apparent rate constant are shown with dotted lines. (C) A structural model of the protein bound intermediate. Switch I is shown in blue and the GAP Arg finger in green. Adapted from Kötting and Gerwert (2013).

Other small GTPase reaction mechanisms

Besides the Ras protein, we investigated many other GTPases using time-resolved FTIR spectroscopy (Figure 7). For Rap, another member of the Ras subfamily, the kinetic scheme is similar with a protein-bound phosphate intermediate (Chakrabarti et al., 2007). Interestingly, the other small GTPases demonstrate different mechanisms. For Rho, Ran (Brucker et al., 2010), and Rab (Gavriljuk et al., 2012) the bond cleavage is the rate-limiting step. In these cases, the GTP marker band decreased, and the bulk solvent P_i band increased at the same rate. No protein-bound P_i was resolved. It seems likely that the additional Arg finger positive charge would slow down the release of the negatively charged P_i. However, RhoGAP has an Arg finger, and no protein-bound P_i intermediates were resolved. Additionally, RapGAP lacks an Arg finger, but a P_i intermediate was observed.

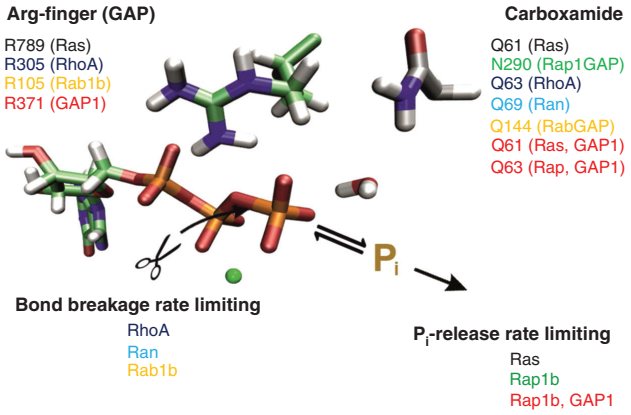

Figure 7 The similarities and differences among various GTPase·GAP complexes.
Some GAPs provide an Arg finger, whereas some do not. For some complexes, bond breakage is the rate-limiting step, and in others, it is P_i release. In all the complexes, a carboxamide plays an important role. This carboxamide can be provided by the GTPase or the GAP. Adapted from Kötting and Gerwert (2013).

The one essential catalytic moiety found in all GTPase GAP combinations is a carboxamide, either from an Asn or a Gln. Its hypothesized role is to orient a catalytic water molecule in an optimal position for nucleophilic attack (Vetter and Wittinghofer, 2001). It is surprising that this task is exclusively performed by a carboxamide and not by other side chains. It has been speculated that the carboxamide structure is necessary to stabilize the transition state generated by the water attack and has been modeled computationally (Grigorenko et al., 2007). This catalytic carboxamide is often a GTPase residue (e.g., Gln61 in Ras), but in some systems like Rap·RapGAP, the carboxamide is provided by the GAP (Scrima et al., 2008). The Rap GTPase can also be catalyzed by a GAP from the GAP1 family (Kupzig et al., 2006). In the latter case, neither Rap nor GAP1 seems to have a Gln or Asn in the appropriate position. Using FTIR experiments, we demonstrated that a Rap Gln situated two residues away from the usual position is moved by GAP1 into the catalytic position (Sot et al., 2010). This was recently confirmed by an X-ray structure (Wang et al., 2013). In GTPase·GAP mutagenesis studies, it is often difficult to elucidate whether the reduced catalytic rate of a mutant is due to decreased affinity of GTPase and GAP or due to the residue's direct involvement in hydrolysis. Because FTIR experiments are performed at millimolar protein concentrations, the effects of decreased interaction affinity are minimized, and residues directly involved in the catalytic function can be found. For example, FTIR experiments of Rap1GAP Arg388 mutants show that Rap GTPase catalysis was unaffected (Chakrabarti et al., 2007), while other biochemical assays give the impression that this mutant is nearly inactive (Daumke et al., 2004).

Further, we investigated several Rab·RabGAP systems (Gavriljuk et al., 2012). In many cases, we observed relatively weak GAP catalysis. Surprisingly, many of the Rab·RabGAP pairs described in the literature showed acceleration factors by the GAP far below the usual 10^5 factor seen in most GTPase·GAP systems (Frasa et al., 2012). We found that TBC1D20, involved in hepatitic C infection, is a very effective GAP for Rab1b. One special feature of the Rab·RabGAP system investigated was a Gln finger provided by the RabGAP in addition to the Arg finger. This finding for a natural human Rab·RabGAP pair agrees with a mechanism identified in a hybrid yeast/mouse system (Pan et al., 2006). The additional Gln finger seems to be unnecessary as Rab already has an intrinsic Gln. However, this intrinsic GTPase Gln is replaced by the GAP Gln finger, and only then can full GAP activity be reached. Mutation of the intrinsic GTPase Gln does not have a major effect on activity. This implies that Rab Gln mutants are not suitable as 'on' state mutants in cell experiments (Nottingham and Pfeffer, 2014).

The reaction intermediate and catalysis

The structure of Ras protein-bound phosphate was calculated in detail by QM/MM simulations (Figure 8A) (Xia et al., 2012). The calculated hydrogen bonding network showed why Ras was still in the 'on' state. Two hydrogen bonds between P_i and the Thr35 backbone position switch I in an orientation similar to the ground state. Hydrogen bonds between P_i and the GDP along with coulomb interactions between P_i and the Mg^{2+} stabilize the intermediate. Interestingly, the corresponding spectra of the protein-bound intermediates from other GTPases varied significantly (Figure 8B) (Chakrabarti et al., 2007). For Rap, the asymmetric stretching vibration was redshifted. Different intermediate band positions were found for both Ras and Rap catalyzed by the dual GAP1 proteins. Biomolecular dynamics calculations of these states are needed to explain the observed differences.

Further insights into GTPases intermediate variations are provided by the thermodynamic parameters. By

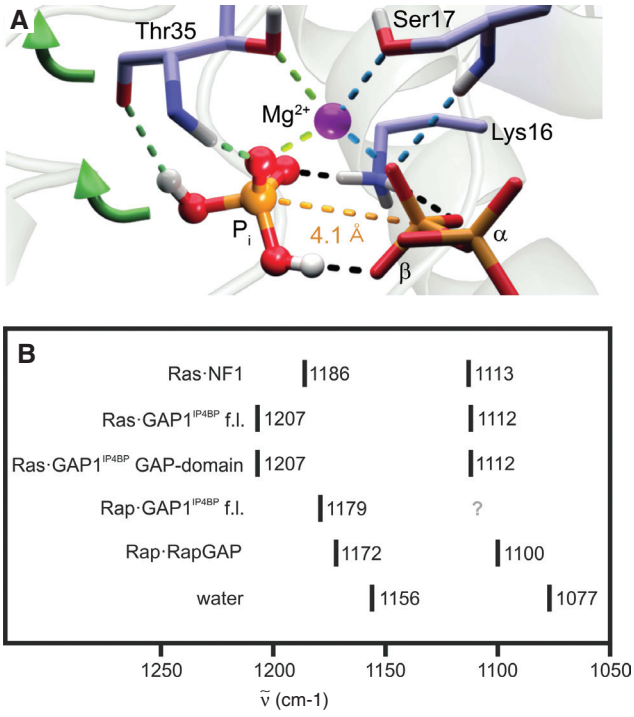

Figure 8 The protein-bound P_i intermediate.
(A) The structure of P_i bound to the Ras·RasGAP complex obtained from a combination of FTIR and biomolecular simulations (Xia et al., 2012). (B) The band positions and thus the structures of the P_i intermediate among different GTPase·GAP complexes vary (Sot et al., 2010).

measuring the reaction rates at different temperatures, the activation enthalpy and entropy for each reaction step can be obtained. While the intrinsic GTPase reaction is normally governed by activation enthalpy (Kötting and Gerwert, 2004), the GAP-catalyzed reaction is dominated by activation entropy (Kötting et al., 2008). However, TCS2-GAP-catalyzed RheB was decisively driven by activation enthalpy (Marshall et al., 2009). These differences are probably due to different rate-limiting steps. For reactions where the bond breakage, itself, is rate limiting, enthalpy should play the major role. Conversely, in processes where the movement of an Arg finger into the binding pocket or the P_i release is the rate-limiting step, entropy is likely to be important. Overall, it seems that the reaction mechanisms among the small GTPases are remarkably different when looked at in detail. These differences may facilitate GAP specificity for its cognate GTPase despite the very similar G-domains that catalyze the same reaction.

Theoretical infrared spectroscopy

The vibrational spectrum is very sensitive with respect to changes in geometry and charge distribution. For example, a 0.001-Å change in bond length corresponds to approximately 1 cm^{-1} vibrational frequency shift in the case of phosphates (Deng et al., 1998). In principle, very small changes can be resolved by a typical FTIR measurement with a 4 cm^{-1} resolution. However, the vibrational mode energy is also influenced by geometry changes other than bond length and coupling with other vibrational modes. Therefore, the geometry cannot be directly deduced from experimental spectra. It has to be decoded through biomolecular simulations such as quantum mechanics/molecular mechanics (QM/MM) algorithms. With quantum chemical calculations, theoretical vibrational spectra can be generated and compared with experimental spectra. The comparison of theoretical with experimental IR spectra is an important measure of QM/MM simulation quality. For example, the force field parameters and the interface between QM and MM is often error prone. The mean band deviation of a calculated spectrum from an experimental one in a state-of-the-art QM calculation is about 30 cm^{-1} (Jacobsen et al., 2013). This corresponds to a bond length accuracy of approximately 0.01 Å. To calculate GTPase spectra, we employed the QM/MM technique. While most of the system was treated by molecular mechanics, the area of interest was calculated using quantum mechanics. This correlates nicely with FTIR difference spectroscopy, where only the reactive region is seen in the difference spectrum. If the theoretical and experimental spectra agree, the calculation results can be analyzed, and the geometry and charge distribution are obtained.

Our methodology was explained in detail previously (Rudack et al., 2012a). Briefly, the QM/MM simulations were based on an X-ray structural model. Model modifications were necessary including exchanging the nucleotide analog with GTP. Next, a classical MD simulation refined the system closer to the real structure. Then, several simulation snapshots during this simulation were further optimized using QM/MM simulations, and several spectra were generated. The statistics were used to estimate the absorbance bandwidth. If, as in our case, experimental and calculated spectra agree, the geometry and charge distribution are obtained with very high accuracy.

Catalytically important structural changes usually occur below typical X-ray structure resolution. For example, a 0.1-Å change in a C-C single bond corresponds to an energy of 5 kJ/mol. While an X-ray structure may not be able to accurately observe this change, they are easily resolved by vibrational spectroscopy. We calculated the hydrolysis of GTP free in solution, bound to Ras, and bound to the Ras·GAP complex in order to understand the catalytic mechanism in detail. As discussed above, GTP hydrolysis in water is very slow with a rate of 5×10^{-8} s^{-1} at 303 K. The rate increases by 4–5 orders of magnitude in Ras and by another 4–5 orders of magnitude in the Ras·GAP complex (Phillips et al., 2003; Kötting and Gerwert, 2004). The most important findings are summarized in Figure 9 (Rudack et al., 2012b). When the free GTP in solution was compared with bound GTP in Ras and Ras·GAP, several observations were made. In the order from GTP in water via GTP in Ras to GTP in Ras·GAP (i) The γ-phosphorus atom charge became more positive facilitating the nucleophilic attack by a water molecule. At the same time, the oxygen atom that is accepting a proton did not change its charge (pink circles in Figure 9). (ii) The distance between the β- and γ-phosphorus atoms increased (pink circles in Figure 9). (iii) The phosphate oxygen atoms moved from a staggered to an eclipsed conformation. This was observed for the β-γ phosphate in Ras and all three phosphates in the Ras·GAP complex (orange arrows in Figure 9). The Ras and GAP act like keys in a toy car, winding up a spring. This drives the GTP conformation and charge distribution toward the transition state, reducing the energetic barrier to hydrolysis, exactly as expected for catalysis.

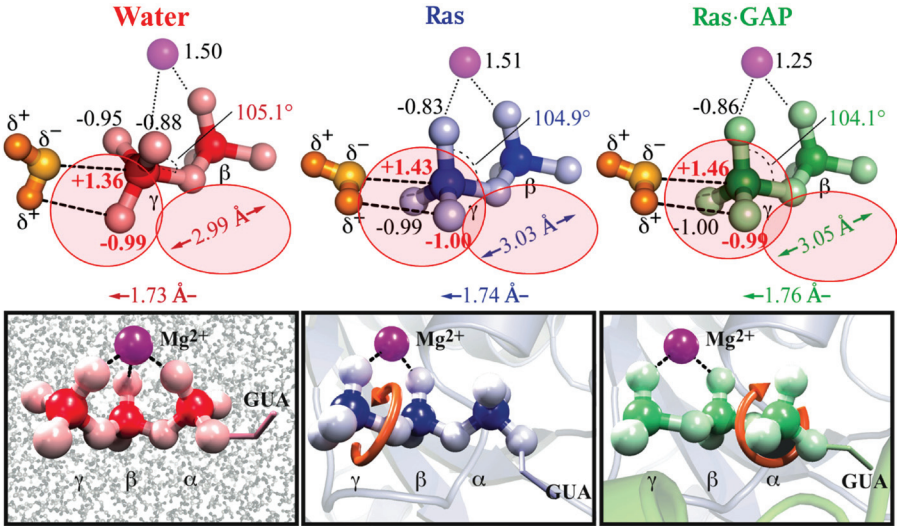

Figure 9 The Ras·RasGAP GTPase catalytic mechanism revealed by FTIR and biomolecular simulations.
The orientations, bond distances, and conformational geometry of pre-hydrolysis GTP are shown for free GTP in water (red), in Ras alone (blue), and in the Ras·RasGAP complex (green). The reaction is catalyzed by charge shifts that favor nucleophilic attack by a water (orange), increased β- and γ-phosphate distance, and GTP conformational strain buildup. Adapted from Rudack et al. (2012b).

GTPase-membrane interactions

Most GTPases are posttranslationally modified with lipid anchors (Gelb, 1997). Membrane tethering and targeting plays an important functional and regulatory role (Henis et al., 2009). Historically, most studies, especially X-ray structures, were performed on anchorless GTPases; however, in recent years, GTPase membrane interactions have come into focus (Gruschus et al., 2014). This includes GDI function (Cherfils and Zeghouf, 2013), lipid anchor attachment and detachment, and membrane targeting dynamics.

FTIR spectroscopic investigations of GTPase membrane interactions are feasible using the ATR technique. In our studies, we used a semisynthetic N-Ras with two lipid anchors (Bader et al., 2000). Compared to the natural N-Ras (Ahearn et al., 2011), only two minor changes were present. First, a maleimido linker was introduced after amino acid 181. This was needed to couple the recombinant protein to a C-terminal synthetic linker. Second, the palmitoyl group was replaced by hexadecyl to prevent hydrolysis. Cell studies demonstrated that the semisynthetic N-Ras is a good substitute for natural N-Ras (Rocks et al., 2005). In the ATR-FTIR experiments, a model membrane was attached to a germanium crystal through hydrophilic interactions. N-Ras then bound to the membrane specifically via its anchor, whereas anchorless N-Ras did not bind (Güldenhaupt et al., 2008). Figure 10A shows a schematic of Ras bound to a solid supported membrane. The immobilized Ras protein can be used for several biophysical experiments. Figure 10B shows the intrinsic GTPase reaction kinetics of membrane-bound Ras compared with Ras in solution, and Figure 10C shows the corresponding difference spectra. Both the rate and the spectra were very similar, and this demonstrated the reproducibility of the difference spectra using either the transmission or ATR technique. The comparison showed that membrane binding produced no major changes in the G domain (Kötting et al., 2012). The only prominent difference spectra deviation was the disappearance of the released phosphate band that is observed in a closed transmission cuvette, but flushed away in a flow-through ATR cell. These results indicated that the main function of the lipid modification is to immobilize Ras. Therefore, reliable results for investigations unrelated to membrane targeting should be obtainable using artificial anchors. We also found that polyhistidine tag Ras anchoring was possible (Pinkerneil et al., 2012; Schartner et al., 2013). In fact, we were able to obtain the same K_d for Ras to its effector, NORE, (Stieglitz et al., 2008) and very similar difference spectra for Ras_{off} and Ras_{on}.

Several biophysical experiments can be performed using proteins immobilized on an ATR crystal. From these experiments, an array of additional information can be obtained. This includes protein-protein interaction investigations, similar to surface plasmon resonance (SPR), but with additional detailed chemical information from FTIR spectroscopy. Difference spectroscopy of reaction

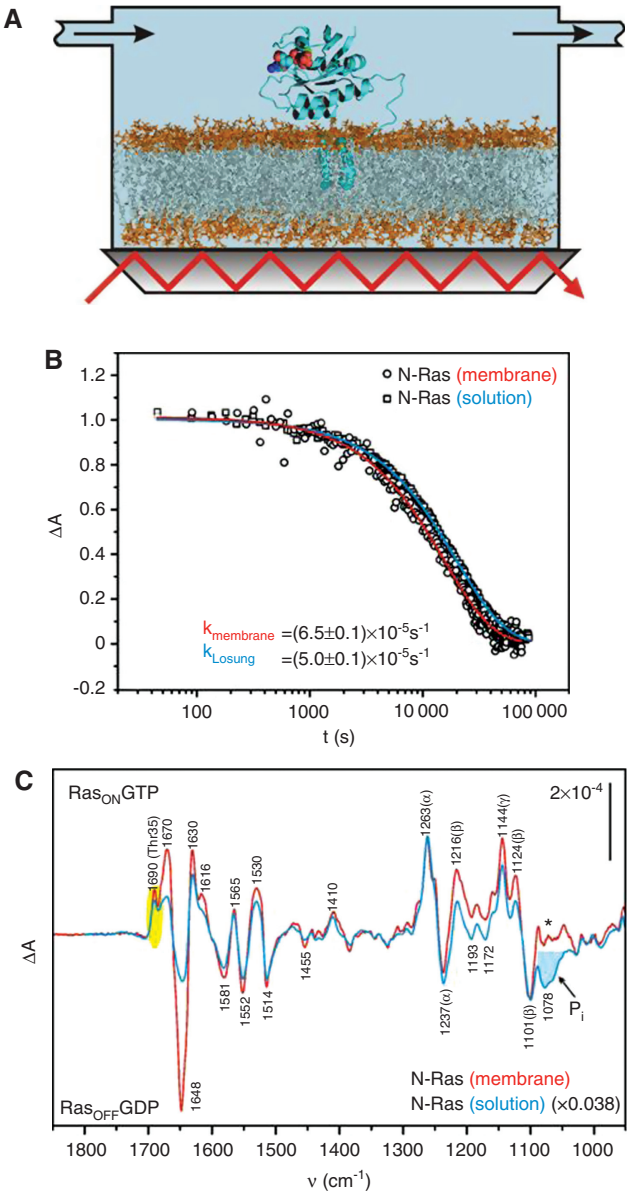

Figure 10 ATR-FTIR investigations of membrane-anchored Ras. (A) A representation of N-Ras immobilized on a POPC model membrane. (B) Kinetics and (C) FTIR difference spectra indicate no significant differences between the GTPase reaction in solution and at the membrane. Adapted from Kötting et al. (2012).

steps such as ligand exchange can be performed without caged compounds, as demonstrated with the GTPase Ras (Figure 10C). Finally, using polarized IR beams, the spatial orientation of the protein relative to the membrane can be deduced. This experiment was performed on the Ras protein. In Ras, the helical protein content was in an upright orientation, perpendicular to the membrane. This was inconsistent with MD simulations of monomers performed in our lab and others (Prakash and Gorfe, 2014). Computation indicated that Ras could only maintain the upright orientation if it was dimeric (Güldenhaupt et al., 2012). This dimer was confirmed by FRET measurements (Figure 11). Recently, an H-Ras dimer was also found by other workers (Lin et al., 2014). Ras dimerization was identified to be a new signal modulation mode, which opens avenues for a new class of drugs that interfere with the dimerization interface (Santos, 2014).

Rab-GDI interactions

Further GTPase membrane interaction studies were performed on Rab GTPases (Gavriljuk et al., 2013). Figure 12 shows the binding of a singly geranylgeranylated Rab GTPase to a negatively charged, solid supported membrane containing a 9:1 molar ratio of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) to 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS). Next, Rab-free buffer was circulated in the system until equilibrium between membrane-bound Rab and Rab in solution was formed.

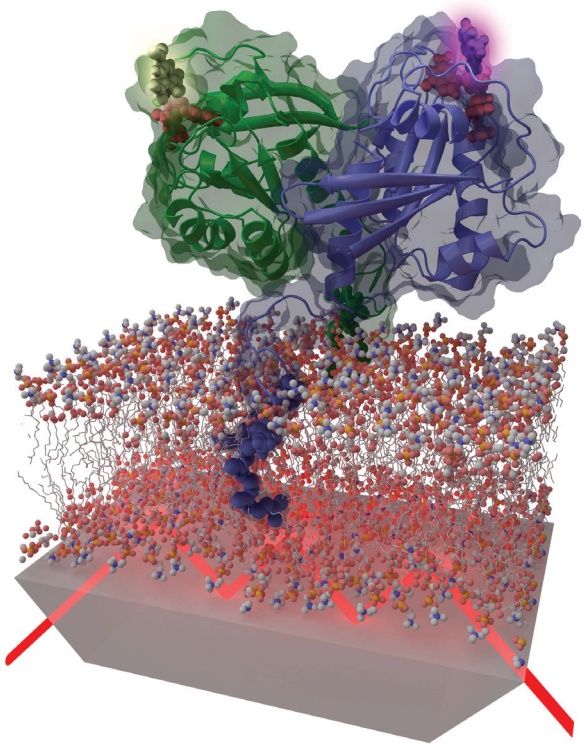

Figure 11 An N-Ras dimer structure on a solid supported POPC model membrane based on ATR-FTIR membrane spatial orientation measurements, MD simulations of membrane-anchored Ras dimers, and FRET measurements.
Adapted from Güldenhaupt et al. (2012).

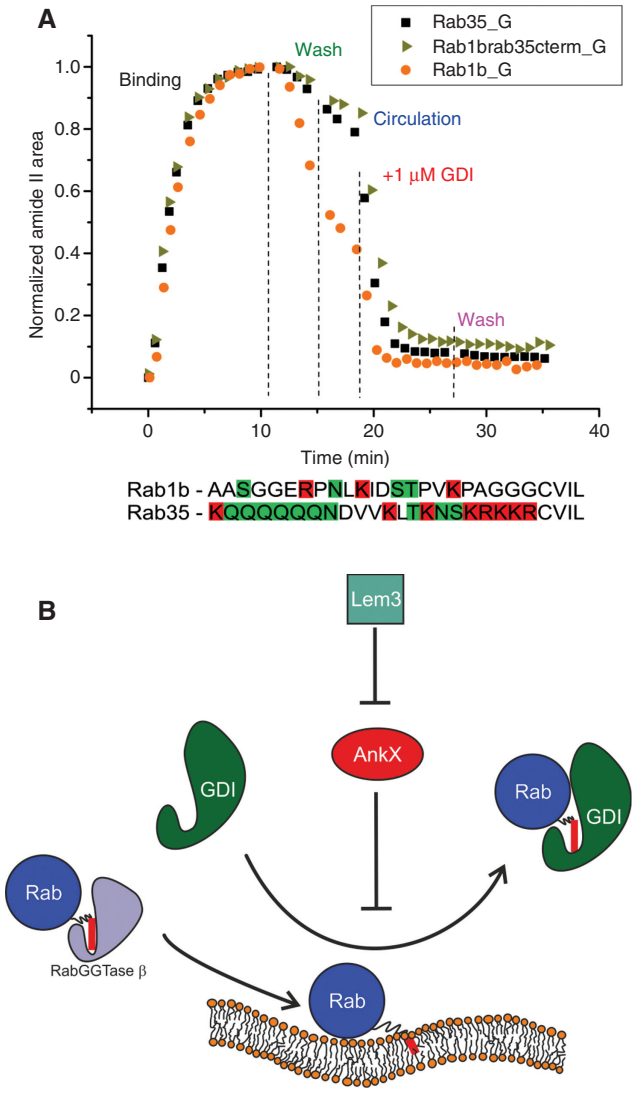

Figure 12 ATR-FTIR investigations of singly geranylgeranylated Rab binding and GDI extraction at the membrane.
(A) The C-terminus sequence determines the membrane extraction rate. This is demonstrated by a chimeric Rab1b with a Rab35 C-terminus that extracts at the same rate as Rab35. (B) The *Legionella pneumophila* pathogens can hijack Rab function. AnkX phosphocholinates Rab preventing membrane extraction. Lem3 restores the natural Rab extraction rate. Adapted from Gavriljuk et al. (2013).

Then, GDI was added into the system. This triggered fast Rab detachment from the membrane. Interestingly, this process was faster than the k_{off} in the absence of GDI; therefore, detachment by GDI is an active process and not just a removal of unbound Rab as would be expected in the case of a simple solubilization factor. Without GDI Rab1b was removed from the membrane much faster than Rab35. This is due to the Rab35 C-terminal sequence, which is more polar and charged compared to Rab1b. This rationale was verified by engineering a chimeric Rab1b with a Rab35 C-terminus (Figure 12A). The chimeric protein behaved like Rab35 with respect to the membrane interaction. Additional experiments showed that GDP-bound Rab is released faster than GTP-bound Rab, in keeping with the known lower affinity of Rab·GTP than Rab·GDP for GDI (Wu et al., 2010).

Rab interactions with pathogens were also studied. Rab phosphocholinated by *Legionella pneumophila* AnkX is not readily extracted from the membrane by GDI. Rab dephosphocholination by Lem3 restores fast membrane extraction.

In all these experiments, it was important to monitor the amide I band shape to directly observe potential secondary structure changes. This ensures that the GTPase maintains its native fold during the experiment. As a further control, GTPase activity can be evaluated in the same way as described above for Ras. We showed that Rab proteins prenylated in the absence of detergent showed broad amide I absorptions indicating denaturation. These proteins are also more stably bound in an unspecific manner. In contrast, prenylation in the presence of detergent, which was subsequently removed, produced successfully immobilized, natively folded Rab. Methods other than FTIR would not have these controls available, which might produce misleading conclusions.

In summary, the complete molecular reaction mechanism of small GTPases and their interactions was studied by time-resolved FTIR in combination with X-ray structural analysis and biomolecular simulation. This approach was extended to membrane-bound small GTPases, which allowed additional membrane extraction and delivery information to be monitored. This provided detailed spatiotemporal insights into the mechanisms and interactions of small GTPases at the membrane.

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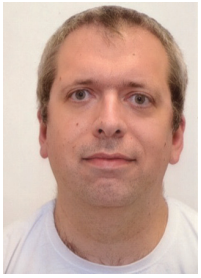

Carsten Kötting obtained a PhD in physical organic chemistry from the chemistry department of Ruhr-University Bochum, Germany in 1999, working on matrix isolation of organic intermediates. Afterwards he joined as a Feodor Lynen fellow the Zewail group at Caltech, were he did femtochemistry of organic intermediates. After two years he returned to Ruhr-University but joined the biophysics institute of Klaus Gerwert. He finished his habilitation in chemical biology working on time resolved FTIR spectroscopy of GTPases in 2009. His current research interests include besides the details of the reaction mechanisms of GTPases, the extension of vibrational spectroscopy to larger systems using Raman microscopy and the development of chemically modified surfaces for protein immobilization on ATR crystals.

Klaus Gerwert is head of the Department of Biophysics at RUB. He is internationally recognized for his contributions in protein-dynamics with very high spatiotemporal resolution using a combination of time-resolved vibrational spectroscopy (FTIR and Raman) and biomolecular simulations (QM/MM). He elaborated the role of protein-bound water molecules in proton-transfer in proteins, especially in microbial rhodopsins like bacteriorhodopsin and channelrhodopsin. Furthermore, he contributed to the detailed understanding of the catalysis of G-proteins by their respective G-activating proteins like Ras and Rab. Recently his approach is extended to marker-free vibrational imaging in cells and tissue for their application in diagnostics. K. Gerwert graduated in biophysical chemistry (Freiburg 1985), was research scientist at the Max-Planck-Institute Dortmund (1986–1989), became Heisenberg-fellow at Scripps, USA, and MPI Dortmund (1990–1993). Since 1993 he is a full professor at the Ruhr-University Bochum (chair of biophysics). Since 2004 spokesman of SFB642 and since 2010 of PURE. Since 2009 Max-Planck fellow at the Max-Planck- CAS Partner-Institute in Shanghai (2009–2013 as director in a dual appointment). He received several awards and is a member of the NRW academy of science.