GTPase activating proteins (GAPs) down-regulate Ras-like proteins by stimulating their GTP hydrolysis, and a malfunction of this reaction leads to disease formation. In most cases, the molecular mechanism of activation involves stabilization of a catalytic Gln and insertion of a catalytic Arg into the active site by GAP. Rap1 neither possesses a Gln nor does its cognate RapGAP employ an Arg. Recently it was proposed that RapGAP provides a catalytic Asn, which substitutes for the Gln found in all other Ras-like proteins (Daumke, O., Weyand, M., Chakrabarti, P. P., Vetter, I. R., and Wittinghofer, A. (2004) Nature 429, 197–201). Here, RapGAP-mediated activation has been investigated by time-resolved Fourier transform infrared spectroscopy. Although the intrinsic hydrolysis reactions of Rap and Ras are very similar, the GAP-catalyzed reaction shows unique features. RapGAP binding induces a GTP$^*$ conformation in which the three phosphate groups are oriented such that they are vibrationally coupled to each other, in contrast to what was seen in the intrinsic and the Ras-RasGAP reactions. However, the charge shift toward β-phosphate observed with RasGAP was also observed for RapGAP. A GDP-P$_i$ intermediate accumulates in the GAP-catalyzed reaction, because the release of P$_i$ is eight times slower than the cleavage reaction, and significant GTP synthesis from GDP-P$_i$ was observed. Partial steps of the cleavage reaction are correlated with structural changes of protein side groups and backbone. Thus, the Rap-RapGAP catalytic machinery compensates for the absence of a cis-Gln by a trans-Asn and for the catalytic Arg by inducing a different GTP conformation that is more prone to be attacked by a water molecule.

Ras-like GTP-binding proteins are ubiquitously expressed, evolutionarily conserved molecular switches (2–4) that cycle between active GTP-bound “ON” and inactive GDP-bound “OFF” states. In the active state these proteins interact with a cascade of downstream effectors and couple extracellular signals to various cellular responses. The cycling between the active and inactive form is regulated by specific guanine nucleotide exchange factors and GTPase-activating proteins (GAPs). Guanine nucleotide exchange factors accelerate nucleotide dissociation by orders of magnitude and thereby induce activation, whereas GAPs accelerate the otherwise slow intrinsic hydrolysis of GTP by similar factors and thus terminate the signal.

Rap1 is the closest relative of Ras and shares >50% sequence identity. It attracted much attention as an attenuator of Ras-mediated signaling, because Rap1 was originally found to antagonize K-Ras-mediated transformation and growth factor-induced, Ras-mediated mitogen-activated protein kinase activation (5, 6). However, Rap1 is activated in parallel to Ras by receptor tyrosine kinase activators (reviewed in Ref. 7) and is now generally believed to function independently of Ras. Rap1 is activated by a variety of extracellular signals via a diverse set of guanine nucleotide exchange factors. It is proposed to function in numerous biological processes such as modulation of growth and differentiation, integrin-mediated cell adhesion, and morphogenesis.

In humans, four isoforms, Rap1A, Rap1B, Rap2A, and Rap2B, exist with Rap1A and Rap1B sharing >90% sequence identity. The hallmark of Rap is the absence of the catalytic guanine residue (Gln-61 in Ras), which is conserved and crucial for the GTPase reaction in other Ras-like proteins, and its replacement by a threonine. The general mechanism of activation of the GTPase reaction as seen in Ras, Rho, and Rab GAP catalysis is based on positioning of the nucleophilic water molecule by the crucial glutamine and neutralization of the developing negative charge by an arginine (4). Interestingly, Rap1GAP, the GTPase-activating protein for Rap1, has no sequence homology to RasGAP and does not employ an arginine for catalysis. This is again different from the Ran-RanGAP system, where Ran bears a Gln but RanGAP operates without an arginine finger (8). Furthermore, Rap1GAP can also down-regulate the G12V mutant of Rap1, which is not possible for Ras, Ran, or Rho (9). Although the intrinsic hydrolysis rate of Rap is ~10-fold slower than that of Ras because of the Gln-61$\rightarrow$Thr substitution (10), the rate of the GAP-catalyzed reaction is 5–10 s$^{-1}$, very similar to what has been found for other GAP-catalyzed reactions (9).

Presently, the structure of Rap2 is known along with several nucleotide analogues (11) and that of Rap1 along with the Ras binding domain of the postulated effector Raf kinase (12), all of which show a high degree of similarity with the corresponding structures of Ras, especially in the GTP-binding site (Fig. 1a).
The three-dimensional structure of Rap1GAP itself and biochemical experiments suggested that the mechanism of the Rap1GAP-catalyzed GTPase reaction is completely different and employs a catalytic Asn inserted into the active site of Rap1 (1). This prompted us to investigate the reaction by FTIR and compare it to the Ras-RasGAP reaction.

Time-resolved Fourier transform infrared (trFTIR) difference spectroscopy has been successfully applied to elucidate the molecular reaction mechanisms of proteins (13). By performing differences between spectra taken at the educt state and during the reaction, the absorbance changes of a few residues involved in the reaction are selected from the quiescent background absorption of the whole protein. Spectra are taken with a millisecond time resolution in the rapid scan mode (14). Earlier work on Ras emphasized several important aspects in the RasGAP-catalyzed GTPase reaction of Ras as follows. (i) Upon Ras binding, negative charge is drawn toward the non-bridging \(\beta\)-oxygen of GTP (15), which is increased by GAP (16). (ii) A GDP intermediate accumulates during the reaction, and P release becomes rate-limiting; and (iii) the GTPase reaction appears to be reversible (16).

The FTIR measurements were performed by using the fast scan technique (14) where the GTPase reaction was initiated by UV laser flashes as described (16, 27). This converted caged-GTP (P'-1-(2-nitro)phenylethyl GTP) to GTP and, subsequently, the IR absorbance changes were monitored. The very slow intrinsic reaction of Rap was difficult to monitor because of baseline drifts. Hence, a catalytic asparagine residue was added to the reactive photolysis by-product 2-nitrosoacetophenone. For the measurements in \(\text{H}_2\text{O}\), the sample was treated with 10 \(\mu\text{l}\) of \(\text{H}_2\text{O}\) and evaporated four times.

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EXPERIMENTAL PROCEDURES

Materials—The truncated form (amino acids 1–167) of Rap1B (which will be called Rap from this point on) was prepared from Escherichia coli strain BL21-DE3 using the pGEX-expression system through purification on a Q-Sepharose column and subsequent gel filtration as described (25). Rap1GAPv, the catalytically active fragment (amino acids 75–416) of Rap1GAP (simply RapGAP hereafter) was purified from E. coli strain BL21-DE3 using the pGEX-expression system as described (9). A detailed description and more detailed literature for \(\text{H}_2\text{O}\) labeling of GAP and GDP are given in Ref. 28. For mass spectrometric experiments, trilithium salts of GTP (>97% purity; Sigma) and \(\text{H}_2\text{O}\) (>95% enrichment; Isotec) were used.

FTIR Spectroscopic Measurements—The sample solutions were prepared essentially as described (16, 26) with a slight modification for the GAP-catalyzed case in that RapGAP was taken directly from a stock concentration of 25 mg/ml in 20 mM HEPES (pH 7.6), 100 mM NaCl, and 5 mM dithiothreitol without further desalting and rebuffering. This turned out to be crucial for the optimal reactivity of RapGAP. The exchange to caged-GTP (P'-1-(2-nitro)phenylethyl guanosine 5'-O-triphosphate) was ~65% complete, with 35% of Rap remaining in the GDP-bound state. For the GAP-catalyzed reaction, RapGAP was used in 1.6 molar excess over the Rapcaged nucleotide. All of the 1% GAP-catalyzed ("intrinsic") reactions were done at 283 K, and the GAP-catalyzed reactions were done at 255 K. Dithiothreitol, which was used to scavenge the reactive photolysis by-product 2-nitrosoacetophenone. For the measurements in \(\text{H}_2\text{O}\), the sample was treated with 10 \(\mu\text{l}\) of \(\text{H}_2\text{O}\) and evaporated four times.

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The absorbance changes \(\Delta A\) in the infrared spectrum are analyzed with sums of \(n\) exponentials with apparent rate constants \(k_i\) and amplitudes \(a_i\). In the analysis, shown in Equation 2.

\[
\Delta A(v, t) = \sum_{i=1}^{n} a_i e^{-k_i t} + a_0(v) \quad \text{(Eq. 1)}
\]

The charge redistribution toward the \(\beta\)-phosphate of GTP is probably due to the positively charged Lys-16 and Mg\(^{2+}\), which are equi-
distant from the nonbridging \(\beta\) and \(\gamma\)-oxygen (20). This leads to a redistribution of excess charge from \(\gamma\) to \(\beta\)-phosphate, as shown by QM/MM calculations.\(^2\) In addition, the P-loop backbone N-H groups point toward the nonbridging (S\(_P\))\(-\beta\)-oxygen and might assist in this charge shift as proposed (15, 16). In the presence of GAP, there is an even larger charge shift toward the (S\(_P\))\(-\beta\)-oxygen of GTP, and it was assumed that this is due to the presence of the additional positive charge of the arginine finger (16). Because this is the only effect on the GTP and GDP vibrations due to GAP binding, it was proposed to be crucial for the catalysis. The question arises as to whether Rap1GAP without an arginine finger still shifts charge toward the \(\beta\)-phosphate and whether the features described for the Ras-RasGAP system are generally applicable to other GTP-binding proteins and their respective GAPs. Here, the Rap-RapGAP system appeared to be particularly interesting because of the absence of both a Gln in the GAPase and an Arg residue from the GAP that are considered to be crucial in the case of the Ras (21), Rho (22, 23), and Rab GTPases (24).

\(^{2}\) M. Klähn, J. Schlitter, and K. Gerwert, unpublished results.
the weighted sum of squared differences (f) between the fit with n rate
constants (k_i) and data points at n measured wavelengths (v_i) and n
integration points (t_i) is minimized.

Evaluation of Data Quality—Each spectrum presented in this
manuscript corresponding to unlabeled as well as to different 18O-
labeled nucleotides is the average of at least 3-5 reproducible meas-
urements. The baseline stability of the 1% GAP-catalyzed reaction is
discussed elsewhere. The reproducibility and baseline stability of the
spectral data for the GAP-catalyzed reaction. Two representative amplitude spectra of the unlabeled GTP corre-
responding to the third rate (see “RapGAP-catalyzed Reaction” under “Results”) are superimposed in Fig. 2. Trace A.
In principle, if the GTP vibrations were coupled, labeling
may appear because of baseline shifts and small variations
between different sample preparations. The double difference
spectra shown in Fig. 1c and 1d are highlighted. The
Assignments of GTP-γ at 1143 cm$^{-1}$ with a shoulder band of GTP-γ at 1157 cm$^{-1}$, GTP-β at 1215 cm$^{-1}$, GDP-α at 1260 cm$^{-1}$, the
GDP-β band at 1100 and 1136 cm$^{-1}$, and GDP-α at 1236 cm$^{-1}$
(Supplemental Fig. 2, trace A) are in excellent agreement with
previous assignments on Ras (26) and are summarized in Fig. 3.
The results show that the structural arrangement and charge distribution of GTP and GDP are very similar for Ras and Rap,
and the intrinsic hydrolysis reaction agrees well for both
proteins.

RapGAP-catalyzed Reaction—While a single apparent rate
constant can describe the intrinsic hydrolysis reaction, at least
two are required to describe the GAP-catalyzed hydrolysis re-
action. The IR absorbance changes for the GAP-catalyzed re-
action of Rap are shown in Fig. 4 as a function of wave number
and time (logarithmic), where the marker frequencies of GTP-β
at 1128 cm$^{-1}$, the intermediate at 1172 cm$^{-1}$, GDP-β at 1104 cm$^{-1}$,
and the released P$_i$ at 1075 cm$^{-1}$ are highlighted. The absorbance changes relax to zero because the final spectrum is
used as reference. After photolysis (described by $k_1 = 5.7 \pm 2$
s$^{-1}$), the GTPase reaction takes place in the two steps as shown in Scheme 1,
than RasGAP by inducing a different GTP conformation. Delocalized vibrations are observed for unbound GTP in solution. A different orientation of the three phosphate groups relative to each other can cause different coupling. It is not yet clear which relative orientations of GTP cause such delocalization. Further studies are required to clarify this phenomenon. Nevertheless, the main contributing vibration to the normal mode can still be assigned as described in the following sections.

As in the case of Ras (16), two intermediate bands are identified. These bands appear at 1170 and 1085 cm⁻¹ for the Rap-RapGAP system. The band at 1170 cm⁻¹ is shifted to 1158 cm⁻¹ because of the γ⁻¹⁸O label [βγ⁻¹⁸Oγ⁻¹⁸O] (Fig. 5a, trace A, red line), which is seen in the double difference spectra (Fig. 5b, trace A, red line) as a difference band at 1172/1159 cm⁻¹. The second band of the intermediate shifts from 1090 to 1072 cm⁻¹ because of [βγ⁻¹⁸Oγ⁻¹⁸O] labeling. The shifts indicate that the bands at 1170 and 1090 cm⁻¹ are either GTP-γ or cleaved P₃. To distinguish between the two possibilities, the reaction was performed in H₂¹⁸O, which can only lead to a shift if cleavage has taken place (29). As for the γ-label, the intermediate band at 1170 cm⁻¹ (Fig. 5a, trace B) indeed loses intensity and another band appears at 1155 cm⁻¹, creating the 1172/1156 cm⁻¹ peaks in the double difference spectrum (Fig. 5b, trace B). Thus, we conclude that cleavage of the β-γ bond has already taken place in the intermediate B. A shift of the second intermediate band is not yet resolved with H₂¹⁸O, most likely because its shifts are not above the noise level.

γ-Labeling not only affects the γ vibration but also several other band shifts because of coupling of the phosphate groups. The γ⁻¹⁸O labeling (Fig. 5, a, trace A and b, trace A) also affects the GTP-α vibrations, as seen from the downshift in the double difference spectra at 1262, 1256/1240, 1231 cm⁻¹, which represents a GTP-α vibration. The same shift is seen in the H₂¹⁸O measurement at 1256/1231 cm⁻¹ in the double difference spectra (Fig. 5b, trace B) with lower intensity. The effect of the γ⁻¹⁸O label on the GTP-α band at 1262, 1256/1240, 1231 cm⁻¹ is attributed to the long-range coupled α-γ vibration in GTP. The GTP-γ bands seen in the photolysis spectra at 1144 and 1156 cm⁻¹ (similar to the intrinsic spectra in Fig. 1b; data not shown) are not observed as negative bands in the k₂̂ amplitude spectrum. GTP vibrations are uncoupled in the photolysis and become highly coupled in the GTP state reflected by the k₂̂ hydrolysis amplitude spectrum, which is thus denoted GTP*.

Because the complete photolysis of the caged compound takes ~100 ms, the reaction to GTP* is not time-resolved and must be faster than 100 ms. However, comparison of the photolysis and hydrolysis amplitude spectra resolves the different GTP conformations.

Similarly to the RasGAP-catalyzed situation (16), the binding of RapGAP also induces a large downshift of a second β-vibration from 1140 to 1128 cm⁻¹ (Fig. 3). The frequency downshift of the second β-vibration is even larger than for GAP-Ras (1140 cm⁻¹; Fig. 3). The two stereo-specific β-labels, the (S)ₜ-β⁻¹⁸O (Fig. 5, a, trace C and b, trace C) and the (R)ₜ-β⁻¹⁸O (Fig. 5, a, trace D and b, trace D) show the same shifts. The GTP-β bands shift from 1210 to 1200 cm⁻¹ and from 1330 to 1191 cm⁻¹. Thus, the bands at 1210 and 1128 cm⁻¹ (Fig. 5a, traces C and D) can be attributed primarily to a GTP-β vibration. The bands have higher intensity for (S)ₜ-β⁻¹⁸O than for (R)ₜ-β⁻¹⁸O. The difference between the (R)ₜ and (S)ₜ labels

**Fig. 3. Summary of the band assignments of GTP for the intrinsic and GAP-catalyzed hydrolysis reactions of Rap, in comparison to Ras, as indicated.** The GDP vibrations agree in all the cases between Ras and Rap and are not shown. Notable is the downshift of the β vibration due to RasGAP and RapGAP binding as the largest effect on the GTP vibration.

**Fig. 4.** The absorbance changes in the infrared between 1800 and 950 cm⁻¹ for the Rap-GAP-catalyzed reaction of Rap is shown as a function of wave number and time (logarithmic). In the fitted curves of the global multi-exponential kinetic analysis, the disappearance of GTP-β at 1128 cm⁻¹, the accumulation of an intermediate at 1172 cm⁻¹, and the appearance of the products GDP and P₃ at 1104 and 1075 cm⁻¹, respectively, are highlighted.

where the apparent rate constants k₂̂ and k₂̂' (at 258 K) describe the appearance of an intermediate (A → B) and the decay to GDP and P₃ (B → C) by the slowest rate, respectively.

A to B Transition—The amplitude spectrum of k₂̂ represents the transition of the GTP to the intermediate, is shown in Fig. 5a (black lines). The band assignments in the k₂̂ amplitude spectra of the Rap-GAP-catalyzed reaction are, in general, more complicated than either the intrinsic reactions of Ras and Rap or the Ras-RasGAP reaction, because the GTP vibrations now become highly coupled. This shows that RapGAP activates phosphoryl transfer by a different mechanism
FIG. 5. Spectral signatures of the intermediate. a, the amplitude spectra (black lines) of the reaction described by \( k_2^* \), the A to B transition (appearance of intermediate), where the negative bands correspond to GDP and positive bands correspond to intermediate. The spectra of labeled GTP are shown in colored lines using \( [\beta,\gamma^{18}O]^{32}P^{32}A_1 \) (trace A), \( H_2^{18}O \) (trace B), \( (S_p)_{[\beta^{18}O]} \) (trace C), and \( (R_p)_{[\beta^{18}O]} \) (trace D). b, the double difference between the amplitude spectra of labeled and unlabeled GTP in panel a, with the original and the shifted frequency in black and in color, respectively.

is much smaller as compared with Ras and is most likely due to scrambling of the isotope label due to rotation of GDP-\( \beta \) after cleavage and before reformation of GTP.

The \( \beta^{18}O \) labels also shift the intermediate band from 1172 to 1154 cm\(^{-1} \), just as the \( \gamma^{18}O \) label does vice versa for the \( \beta \) vibration at 1130 cm\(^{-1} \). This is seen in the double difference spectra as a difference band at 1132/1120 cm\(^{-1} \) (Fig. 5b, trace A). In addition, the \( (S_p)_{[\beta^{18}O]} \) and \( (R_p)_{[\beta^{18}O]} \) labels shift the second intermediate band from 1100 to 1074 cm\(^{-1} \). It seems that the second intermediate band absorbs at 1100 cm\(^{-1} \). In the case of \( \gamma \)-labeling, because of an additional positive band it seems to be shifted from its original position at 1100 cm\(^{-1} \) down to 1090 cm\(^{-1} \) (Fig. 5b, traces A, C, and D, respectively). In the third rate, the intermediate band is seen at 1090 cm\(^{-1} \) for the \( \gamma \)-labeling (Fig. 7b, trace A).

The \( \alpha^{18}O \) label also affects both intermediate vibrations (data in Supplemental Fig. 2 in the on-line version of this article). Because \( \alpha \)- and \( \beta \)-labeling shifts the intermediate bands, they should usually represent \( \gamma \)-phospho vibrations covalently bound to a GDP counterpart. Because the band at 1172 cm\(^{-1} \) is shifted by \( H_2^{18}O \), cleavage has taken place. Bond breakage is required for incorporation of the \( 18O \) label from \( H_2^{18}O \) into GTP. On the other hand, coupling between GTP vibrations would either require covalent attachment of the \( \gamma \)-phosphate or, alternatively, be induced by dipolar interaction between non-covalently bound states (30). For this to occur, \( P_i \) has to be less than a few Ångstroms from \( \beta \)-GDP. Thus, the intermediate state represents a protein-bound GDP-\( P_i \) as in Ras-RasGAP, but with an altered geometry.

Kinetic Scheme—Based on the IR results the following kinetic scheme, called here Scheme 2,

\[
\begin{align*}
\text{GAP} & \xrightarrow{\text{GAP}} \text{RapGTP}^* \xrightarrow{k_{2}} \text{RapGTP}^* \xrightarrow{k_{3}} \text{RapGDP}^* + P_i \xrightarrow{k_5} \text{RapGDP} + P_i + \text{GAP} \\
\text{RapGTP}^* & \xrightarrow{\text{GAP}} \text{RapGTP}^* \xrightarrow{k_{2}} \text{RapGTP}^* \xrightarrow{k_{3}} \text{RapGDP}^* + P_i \xrightarrow{k_5} \text{RapGDP} + P_i + \text{GAP} \\
\end{align*}
\]

is deduced. In this scheme, \( k_2, k_3, k_5, \) and \( k_{-3} \) are microscopic rate constants. The nature of the intermediate in the Rap-RapGAP reaction is similar to that in the GAP-catalyzed reaction of Ras. However, the dipolar coupling between \( P_i \) and GDP is not observed in the RasGAP-catalyzed reaction. Therefore, \( P_i \) seems to be a shorter distance away from GDP in the Rap-RapGAP than in the Ras-RasGAP intermediate complex.

Reversibility of the GTPase reaction and \( 18O \) exchange—Using \( H_2^{18}O \), we observe a large amount of label incorporation into GTP\(^* \), indicating that the reverse reaction, i.e. synthesis of GTP\(^* \) from \( P_i \) and \( H_2^{18}O \), is considerably more pronounced than what has been reported for the Ras-GAP system by Allin et al. (16) and in agreement with recent quenched flow data for the Ras-NF1 reaction where a ratio of 88 between the forward and backward chemical step has been found (19). This observation is further indication that in the Rap-RapGAP complex, \( P_i \) might be closer to GDP than in Ras-RasGAP. The \( P_i \) release reaction seems sufficiently slow, such that cleaved \( \gamma \)-phosphate and GDP remain bound to the protein long enough and in close contact to allow an \( 18O \) water-mediated phosphate-water oxygen exchange to take place. This backward reaction has long been known for ATPases such as myosin (31).

The back reaction can also be conveniently demonstrated by analyzing IR samples by mass spectrometry after running the reaction in \( H_2^{18}O \) (Fig. 6a). In addition to \( 18O_{-}P_i \) (m/z 97), one (m/z 99), two (m/z 101), three (m/z 103), and even four (m/z 105) \( 18O \) atoms are incorporated into \( P_i \). Qualitatively, we can see that after the cleavage of protein-bound GTP by nucleophilic attack of \( H_2^{18}O \), a bound \( P_i \), bearing one \( 18O \) can reform GTP.

Replication of this process leads to progressive accumulation of \( 18O \) into released \( P_i \), as shown in the scheme of Fig. 6. Because of the fast exchange of the sample with atmospheric water, the \( 18O \) label is diluted with \( 16O \). Therefore, the mass spectrometric data cannot be used for quantitative analysis of \( 18O \) incorporation in \( P_i \). The overall equilibrium for GTP hydrolysis, due to the large free energy of hydrolysis (\( \Delta G \)), is almost exclusively on the product side.

B to C Transition—Fig. 7a shows the amplitude spectrum for \( k_7^* \), which describes product release. The former positive bands of the intermediate (e.g. 1170 cm\(^{-1} \), black line) appear as negative bands as it disappears with \( k_7^* \), whereas the product peaks of the GDP and \( P_i \) appear as position bands. The 4-fold \( \gamma \)-label [\( \beta,\gamma^{18}O/\gamma^{18}O \)] shifts the intermediate band from 1170 to 1157 cm\(^{-1} \) (Fig. 7a, trace A, red line), which is seen in the double difference as a difference band at 1170/1155 cm\(^{-1} \) (Fig. 7b, trace A, red line). The GDP band is shifted from 1100 to 1091 cm\(^{-1} \), which appears as a band at 1111/1090 cm\(^{-1} \) in the double difference spectrum (Fig. 7b, trace A, red line), and the
Released phosphate is identified as 1075/1050, 992/962 cm\(^{-1}\) in the double difference spectrum. These shifts are also seen for the intrinsic reaction for Rap (Supplemental Fig. 1, trace A, available in the on-line version of this article) and Ras (26). The most notable feature of the B to C transition is the similarity between the double difference spectra obtained with \([\beta\gamma^{18}\text{O}]\gamma^{18}\text{O}_3\) GTP and \(\text{H}_2^{18}\text{O}\) due, to large extent, by reverse reaction.

Furthermore, the \((S_p^\beta)\) and \((R_p^\beta)\) labels show exactly the same shifts and intensities. The double difference spectra (Fig. 7b, traces C and D) show the expected effect on the \(P_i\) intermediate band at 1172/1155 cm\(^{-1}\) and on the protein-bound GDP product at 1137/1116 and 1107/1090 cm\(^{-1}\). The difference bands observed at 1137/1116 and 1107/1090 cm\(^{-1}\) are in good agreement with the band assignment of GDP as seen for the intrinsic reactions of Ras (26) and Rap (Supplemental Fig. 1) and for the GAP-catalyzed reaction of Ras (16). The \(\beta\)-GDP vibrations are not affected by RapGAP as for RasGAP.

**Time Course of the Reaction**—Based on the band assignments, the kinetics of selected groups are shown in Fig. 8. The global fit procedure adopted in this manuscript provides apparent rate constants. This allows us to analyze the kinetics in a minimal, model independent way. The apparent rate constants of Scheme 1 describe the two main reaction steps of the GTPase reaction. These apparent rate constants direct elementary reactions only if reverse reactions can be neglected. However, as shown in Scheme 2, four microscopic rate constants are needed to describe the elementary steps, but only two apparent rate constants are experimentally accessible. Because the apparent rate constants differ by an order of magnitude (Scheme 1), they describe in a first approximation the elementary reactions.

Modeling of the experimentally underdetermined reaction with estimated microscopic rate constants that are able to describe the absorbance changes show that the back reaction leads to prolongation of the GTP decay. Actually, the GTP decay at 1271 cm\(^{-1}\) is described not only by one apparent rate constant \(k_2\), but it is prolonged by \(k_3\). The disappearance of the GTP band at 1271 cm\(^{-1}\) has been chosen as marker band to monitor the disappearance of GTP, because the usual marker band for GTP, the GTP at 1155 cm\(^{-1}\), is masked by the appearing intermediate band. Because the \(\gamma\) vibration is coupled to the \(\gamma\) vibration, its absorbance change is the same as for the \(\gamma\) vibration. It is clearly seen that the marker GTP band primarily disappears.
the Rap-RapGAP reaction must be due to a different structural rearrangement. One possible explanation could be that instead of the positively charged guanidinium group of Arg, H-bonding of the polar carboximide group of Asn induces a charge shift toward β-phosphate. An Asn residue, Asn-233 in Dictyostelium discoideum myosin II, is found in the transition state analog structure of myosin, where it contacts the β-phosphate and vanadate (33–35). However, the position of this asparagine is not conserved in other transition state structures of myosin (36), and mutational analysis indicates that this Asn is involved in the binding rather than the hydrolysis of ATP (37); thus, a different role is suggested for this residue in myosin, where one or two serine residues are believed to be involved in catalysis (33). The absorbance change at 1703 cm⁻¹ observed here seems to indicate a movement of an Asn residue during catalysis, but further studies have to confirm the assignment. However, a possible movement of Asn during catalysis is still consistent with the idea that the Asn of RapGAP substitutes the Gln-61 of Ras (1).

Nevertheless, the origin of the charge movement toward β-phosphate is unclear. We might speculate that another positively charged residue is brought close to (S₆₋₁)β-oxygen to induce the charge shift. Interestingly, from the recent structure of RapGAP and related mutational analysis (1, 9), it is clear that there is a cluster of positively charged residues (lysine 285, arginine 286, and histidine 287) in the catalytic helix (α-7) just before the catalytic asparagine 290 (KRHIGND). Mutation of these residues of RapGAP exert a medium to dramatic effect on catalysis. However, because of the invasive nature of these mutants, it is still not clear how they would exert an effect of the charge shift to β-oxygen in FTIR.

The conformation of the P-loop in Ras and Rap is very similar and is, in fact, very similar between all structurally characterized GTP-binding proteins. Although the main chain NH groups of the backbone and, in addition, the lysine side of the P-loop point toward the β- and γ-phosphate oxygens and thus contribute to the charge redistribution of GTP, this is unlikely to be a major contributor to catalysis because the P-loop does not change its structure when comparing educt (GTP), transition state mimic (GDP±AlF₃), and product (GDP) states, at least in the resolution of the presently available X-ray structures (4). However it cannot be excluded that RapGAP binding could induce small conformational changes of the P-loop and thereby reposition Lys-16 such that more negative charge accumulates on β-phosphate.

A clear difference between the GAP-catalyzed reaction of Rap and Ras is that RapGAP binding induces a different GTP* conformation in the educt state, which is characterized by highly coupled vibrations. This has not been seen previously in protein bound GTP. Thus, activation by RapGAP is different from activation by RasGAP. The coupled vibrations indicate a different orientation of the three phosphate groups to each other as compared with GAP-catalyzed hydrolysis of Ras and the intrinsic reactions. We postulate that this unusual conformation of GTP contributes to rapid phosphoryl transfer.

As for the RasGAP-catalyzed reaction, an intermediate is observed in which cleavage of γ-phosphate has taken place, but P₁ is a shorter distance from GDP* as compared with Ras-RasGAP. The GDP* in the intermediate also shows coupled vibrations. In line with the shorter GDP-P₁ distance and the lifetime of the intermediate, we observe a more pronounced back reaction to GTP*. The reversibility of the hydrolysis reaction and the fact that P₁ release is at least partially rate-limiting represent common features not only in GTP but also in ATP hydrolyzing P-loop-containing proteins.
In addition to the phosphate vibrations, several bands characteristic of protein groups are observed in the intermediate, which seems to indicate conformational changes of residues in the active site that are required to catalyze cleavage, and these changes are larger than those observed previously for the Ras-RasGAP reaction. In the product state, protein-bound GDP and released Pi appear at nearly the same positions as compared with the intrinsic reaction of Ras and Rap, indicating that GAP binding has almost no influence on the final GDP and Pi states for both Ras and Rap.

It might be interesting to note that the different activation mechanism shown by RapGAP, which seems to rely on the introduction of an asparagine into the active site, is independent of the presence of a Gln residue, which in Ras is absolutely required for catalysis. We have also shown that the RapGAP-mediated hydrolysis is only weakly reduced in the presence of a mutation of Gly-12. The same mutation in Ras creates an oncogene that can no longer be down-regulated by RasGAP. This supports the notion that it should be feasible to induce a more general strategy for diseases associated with a defective hydrolysis reaction such as neurofibromatosis type I or tuberous sclerosis, where the disease-associated tumor suppressor genes for RasGAP and RheBGAP, a homologue of RapGAP (39), are deleted or mutated.

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