Linear Free Energy Relationships in the Intrinsic and GTPase Activating Protein-Stimulated Guanosine 5′-Triphosphate Hydrolysis of p21^{ras}†

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ABSTRACT: Controlling the hydrolysis rate of GTP bound to guanine nucleotide binding proteins is crucial for the right timing of many biological processes. Theoretical, structural, and functional studies have demonstrated that in p21^{ras} the substrate of the reaction, GTP itself, plays a central role by acting as the base catalyst. This substrate-assisted reaction mechanism was analyzed with the help of linear free energy relationships (LFERs). Here we present experimental data that further support the proposed mechanism. We extend the LFER analysis to a wide range of oncogenic as well as nontransforming Ras mutants. It is illustrated that almost all Ras variants follow the observed LFER and thus also the same reaction path. Further, the reduced GTPase reaction rate that characterizes the oncogenic effect of many of the p21 mutants found in human tumors seems to be a consequence of a slightly reduced pK_a of the γ-phosphate group of bound GTP. Factors causing a pK_a deviation of just 0.5 unit are enough to slow the intrinsic GTPase reaction rate significantly, and the system may exhibit as a consequence of this an oncogenic potential. Interestingly, we also found oncogenic mutations that do not follow the regular LFER. This suggests that the oncogenic effect of distinct Ras mutants has a different physical origin. The results presented might aid in the design of drugs aimed at reactivating the GTPase reaction of many oncogenic p21^{ras} mutants. We also analyzed the stimulated GTPase reaction of p21^{ras} by the GTPase activating protein (GAP) and the GTPase reaction of Rap1A, a Ras-related GTP binding protein, with similar approaches. The corresponding results indicate that the GAP-stimulated GTPase as well as the Rap1A-catalyzed reaction seem to follow the same substrate-assisted reaction mechanism. However, the correlation coefficient for the GAP-catalyzed reaction is different from the corresponding coefficient for the intrinsic reaction. While the intrinsic reaction exhibits a Brønsted slope of $\beta = 2.1$, the corresponding value for the GAP-activated reaction is $\beta = 4.9$.

Binding and hydrolysis of GTP by specific proteins are central in regulating a great variety of different cellular processes such as cell growth and differentiation, visual signal transduction, protein synthesis, or vesicular trafficking (Bourne et al., 1990, 1991; Boguski & McCormick, 1993). In the past decade, it has been shown that many of the proteins involved in these processes, the so-called guanine nucleotide binding proteins, share common sequence elements and have a highly homologous structure (Boguski & McCormick, 1993). GTP-binding proteins, like for example p21^{ras}, a protein that plays a central role in signal transduction pathways controlling cell proliferation, can be considered as signal switch molecules that cycle between the GTP-bound ON-state and the GDP-bound OFF-state (Bourne et al., 1991). Guanine nucleotide binding proteins are usually switched on by the action of activated exchange factors that catalyze the exchange of protein-bound GDP by GTP (Bourne et al., 1990). In the GTP-bound on conformation, these GTP binding proteins interact specifically with an appropriate effector molecule and thus transmit the corresponding signal to the next downstream component in the signaling cascade (Wiesmüller & Wittinghofer, 1995).

All known GTP-binding proteins exhibit a GTPase activity that recycles the protein back to its inactive GDP-bound form. The rate of this reaction is crucial for the corresponding timing of the regulated process; the longer a guanine nucleotide binding protein remains in its active GTP-bound state, the longer it will transmit and also amplify a certain signal. Hence, the rate of GTP hydrolysis is of great importance for the right timing of many processes in a cell. It is well-known that mutations that slow the reaction rate biologically activate these signaling proteins. For example, mutations in p21^{ras} at position 12, 13, or 61 drastically reduce the GTPase and lead to an oncogenic Ras protein that permanently produces a cell growth signal.

Very often, the GTPase rate itself is regulated by accessory proteins. The relatively slow intrinsic reaction rate of p21^{ras} for instance can be accelerated by up to 5 orders of magnitude in the presence of highly specific GTPase activating proteins (GAPs)[1] (Gideon et al., 1992). GAPs are presumably negative regulators which accelerate the return of the off state and thus help to terminate the signal.

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[1] Abbreviations: LFER, linear free energy relationship; GAP, GTPase activating protein.

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Nevertheless, it has been demonstrated recently that the intrinsic GTPase reaction of p21^{ras} itself might well be of physiological relevance. Herrmann and co-workers (1995) have shown that GAP cannot bind to the GTP form of p21^{ras} and stimulate the GTPase reaction as long as the Ras-binding domain (RBD) of Raf kinase is bound to this protein. While the intrinsic reaction seems to be responsible for the breakdown of the p21(GTP)−Raf kinase complex, GAP down-regulates the amount of free p21(GTP) that has been activated by receptor tyrosine kinases (Herrmann et al., 1995; Henkemeyer et al., 1995). Thus, elucidating the intrinsic as well as the GAP-stimulated GTPase mechanism is of great importance for understanding signal transduction and tumor formation on a molecular level. Further progress in this direction is essential for finding a way to manipulate the reaction rate of oncogenic mutants by external means.

In this work, we use linear free energy relationships (LFERs) to examine the mechanism of the intrinsic as well as the GAP-stimulated reaction of Ras proteins. LFERs are among the most fundamental concepts in physical organic chemistry and have been applied extensively to phosphohydrolysis reactions in aqueous solution (Ba-Saif et al., 1989). Such relationships have been used to correlate reaction rates with the corresponding equilibrium constants. They imply that changes in activation free energies ($\Delta\Delta G^{\#}$) due to various perturbations correlate linearly with the concomitant change in free energy difference ($\Delta\Delta G°$) between the reactants, the products, and relevant intermediates. The validity of such relationships for chemical processes in solutions has been the subject of many studies [e.g. Marcus (1964), Albery (1980), Kreevoy and Kotchevar (1990), Hwang et al. (1988), and Warshel (1991)]. Experimental studies (Fersht et al., 1986; Toney & Kirsch, 1989) as well as theoretical studies (Warshel, 1984; Yadav et al., 1991; Warshel et al., 1994) indicate that such relationships are also valid in proteins and can be used as an effective tool to correlate the catalytic power of enzymes with their structure.

Very often, Brønsted plots and other types of LFERs yield valuable information on the mechanism of chemical reactions in solutions and thus can be used as a powerful tool to test mechanistic proposals. However, the application of a LFER to enzymatic reactions is less straightforward. For example, Fersht and co-workers (1986) correlated the reaction rates of a set of mutants to the corresponding equilibrium free energy, $\Delta G°$, while Tony and Kirsch (1988) related the enzymatic rate to the pK_a of different substrates that is measured in the absence of the enzyme. Both approaches are valid, although the first one is more likely to provide a unique correlation [for discussion, see Warshel et al. (1994)]. These studies show that there are different ways to group and correlate thermodynamic and kinetic properties in order to obtain valuable information about enzyme-catalyzed reactions.

Recently, we have demonstrated that the GTPase of p21^{ras} follows such a LFER (Schweins et al., 1995). Together with theoretical and structural studies, this has indicated that the substrate of the reaction, GTP itself, plays a central role in the reaction scheme by acting as the "base catalyst" [see Schweins et al. (1994, 1995)]. On the basis of these studies, it was proposed that GTP hydrolysis in p21^{ras} is initiated by the abstraction of a proton from the catalytic water molecule by the γ-phosphate of protein-bound GTP (Figure 1). The

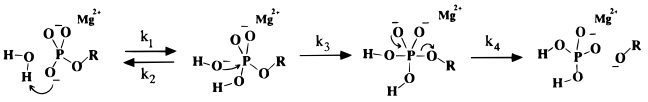

FIGURE 1: Proposed mechanism for p21^{ras}. In this mechanism, the substrate GTP itself acts as a base for its own hydrolysis. The proton transfer from the nucleophilic water to the phosphate activates the nucleophile and decreases the negative charge on the γ-phosphate group.

generated nucleophilic hydroxide ion subsequently attacks the protonated γ-phosphate in an S_N2-like mode and thus creates a trigonal bipyramidal transition or intermediate state, respectively. A stereochemical analysis has indicated that GTP hydrolysis in p21^{ras} results indeed in an in-line attack of the nucleophile (Feuerstein et al., 1986). With GDP as the leaving group, the pentacovalent intermediate finally breaks down into the reaction products phosphate and GDP (Figure 1).

The results presented in this and in the subsequent paper illustrate that almost all Ras mutants examined so far as well as Rap1A proteins seem to follow the same mechanistic reaction path. The reduced GTPase reaction rate that characterizes the oncogenic effect of many of the p21 mutants found in human tumors seems to be a consequence of a slightly reduced pK_a of the γ-phosphate group of bound GTP.

METHODS

Protein Purification. p21^{ras} proteins were purified from *Escherichia coli* as described previously (Tucker et al., 1986). The final purity was >95%, as judged from sodium dodecyl sulfate−polyacrylamide gel electrophoresis. The protein concentration was determined with the Bradford assay using bovine serum albumin as standard. The amount of protein-bound nucleotide was analyzed with C18 reverse phase HPLC column and quantitated with a calibrated detector and integrator, respectively. GAP was purified from Sf9 insect cells and GAP-334 from *E. coli* as described before (Gideon et al., 1992).

Kinetic Analysis. Purified Ras protein was converted to its GTP-bound form by incubating the protein solution with a 100-fold excess of GTP in the presence of 5 mM EDTA. After 1 h, free nucleotide was exchanged by gel filtration (PD-10, Pharmacia) (John et al., 1989). p21−GTPγS was generated by incubating p21−GDP with a 1.5-fold excess of GTPγS in the presence of 200 mM $(NH_4)_2SO_4$, 10 u of alkaline phosphatase, and 10 u of phosphodiesterase (Boehringer Mannheim). The progress of nucleotide exchange was monitored with HPLC. Intrinsic reaction rates of Ras and Rap1A proteins were determined with HPLC by measuring the concentration of protein-bound GTP and GDP as described (Schweins et al., 1995). GTPase rates were determined in 40 mM Hepes and 2 mM $MgCl_2$ at pH 7.4 and 37 °C for the intrinsic and at 25 °C for the GAP-stimulated reaction. The final GAP concentration in the assay was 4.1 nM. The reaction was started by raising the temperature to 37 °C (intrinsic reaction) or by adding GAP. For the faster GAP-catalyzed reaction, 40 μL aliquots were taken, frozen in liquid nitrogen, and analyzed with HPLC to determine the GTP/GDP ratio on a calibrated C18 reverse phase HPLC column.

Intrinsic rates were obtained from the change of the GTP/(GTP + GDP) ratio with time to single-exponential curves.

In the case of the GAP-stimulated reaction, we applied either the classical Michaelis−Menten analysis or the integrated Michaelis−Menten equation in a nonlinear regression fit according to the procedure described by Duggleby and Clarke (1991) to determine K_m and k_{cat} of the reaction. When the progress curve for an enzyme-catalyzed reaction follows the integrated Michaelis−Menten equation, the maximum velocity and the Michaelis constant can be determined from a single experiment. This type of data processing is possible for stable enzymes catalyzing an irreversible reaction with a single substrate and for which none of the reaction products is inhibitory, which is the case for the GAP-stimulated GTPase of p21. The intrinsic reaction as a function of temperature was determined by using the standard GTPase assay and a calibrated thermoelement.

pK_a Determination of p21^{ras}-Bound GTP. The pK_a of p21^{ras}-bound GTP was determined either directly by ^{31}P-NMR or indirectly by analyzing the pH profile of the hydrolysis reaction. ^{31}P-NMR spectra were run with p21^{ras} complexed to Mg−GTP in 40 mM Hepes, 10 mM $MgCl_2$, and 0.1 mM DTE at 5 °C, using a Bruker AMX-500 spectrometer operating at 202 MHz. The spectra were referenced to 85% phosphoric acid contained within a glass sphere which was immersed in the sample. The pH was varied by addition of 0.1 N HCl. The temperature was kept constant at 5 °C. Typically, 1024 scans were averaged for one spectrum. The signal-to-noise was improved by exponential multiplication of the FID before Fourier transformation to an addition line broadening of 3 Hz. From the change in chemical shift as a function of pH, we determined the corresponding pK_a for 14 Ras and Rap1A proteins. However, for faster mutants (rates of >0.028 min^{-1}), we had to use an indirect approach to determine the pK_a since the half-life of p21−GTP was too short to have reasonable amounts to which to apply ^{31}P-NMR spectroscopy. The pK_a of GTP bound to seven proteins was determined by analyzing the pH profile of the hydrolysis reaction. For three Ras proteins, we used both methods independently and were able to show that the different methods yield pK_a values that are almost identical. For example, for Ras wild type protein, we measured with ^{31}P-NMR spectroscopy a pK_a of 2.95, while the corresponding pH profile yields a pK_a of 2.9.

RESULTS AND DISCUSSION

The original proposal for the GTP as a base mechanism came from theoretical analysis of the energetics of these and other mechanisms in solutions and proteins (Schweins et al., 1994). This included indirect analysis of experiments in solutions and calculation of the energy difference of moving the relevant reacting system from water to the protein site. An additional direct experimental support is provided by the observation of a LFER between the logarithm of the overall GTPase reaction rate of p21^{ras} and the pK_a of the γ-phosphate of the protein-bound GTP. The corresponding Brønsted plot establishes the existence of a linear correlation between these two parameters. The higher the pK_a of the γ-phosphate and the stronger its proton abstraction potential, the faster the reaction rate at neutral pH. This relationship provides a very strong indication that the γ-phosphate of the GTP itself is the base of the reaction.

Obviously, linear free energy relationships cannot reproduce the experimental data when the active site changes in a drastic way or when the rate-limiting step changes. However, when LFERs are observed, it indicates that the mechanism of the reaction is unchanged despite small changes in the reaction conditions. That is, one can use a LFER not only to obtain new insights into a reaction mechanism for a particular protein but also to examine whether certain mutants or even different proteins follow the same mechanistic reaction route. In order to test if different Ras mutants have functional similarities, we extended the original LFER found for p21^{ras} to a wide range of oncogenic as well as nontransforming p21^{ras} mutants and plotted the logarithm of the reaction rate as function of the pK_a of protein-bound GTP (Figures 2 and 3).

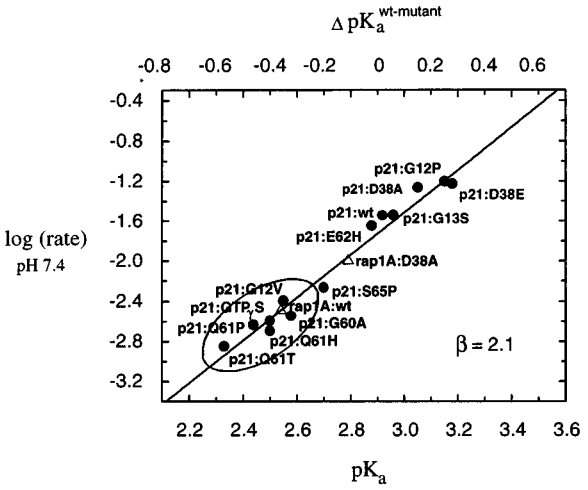

FIGURE 2: LFER plot of the intrinsic GTPase reaction rate (in min^{-1}) as a function of the apparent pK_a of the terminal GTP phosphate. Apparent pK_a's were determined by ^{31}P-NMR as described before (Schweins et al., 1995) or by measuring the pH dependence of the GTPase reaction over the relevant pH range and fitting the data to the Henderson−Hasselbalch equation using up to 30 data points. Many of the mutants that fall into the highlighted region exhibit an oncogenic potential (Q61T, Q61H, Q61P, and G12V). While Ras proteins are represented by filled circles, Rap1A proteins (rap1a:wt and rap1a:D38A) are shown by triangles. The observed LFER relationship can be described by the Brønsted formula $\log(k_{cat}) = \beta\Delta pK_a^{(wt-mutant)} + c$, where $\Delta pK_a^{(wt-mutant)}$ is the difference in pK_a of bound GTP between wild type and mutant protein. As shown in the subsequent work, this formula is closely related to a modified Marcus relationship. Note that the observed Brønsted correlation coefficient β is 2.1 for the set of mutants displayed in this figure.

This Brønsted plot convincingly demonstrates that a clear correlation between log(k) and the pK_a of the γ-phosphate exists; 13 p21^{ras} and 2 Rap1A proteins follow the observed correlation and as a consequence of this very likely also share the same reaction mechanism (Figure 2). This is true not only for most of the examined oncogenic mutants in position 12 (G12V) and 61 (Q61T, Q61H, and Q61P) but also for the effector mutants in position 38 (D38A and D38E) and the nucleotide mutant p21−GTPγS. The difference in rate compared to that of the wild type protein can solely be explained by a different pK_a of the terminal phosphate of the bound substrate GTP. It is interesting to note that many proteins with low pK_a's (marked by a square in Figure 2) show oncogenic properties and that all "fast" and nontransforming mutants have a pK_a of more than 2.9. It seems that a pK_a change of just 0.5 unit is enough to slow the intrinsic GTPase reaction rate significantly and to induce oncogenic potential on p21^{ras}.

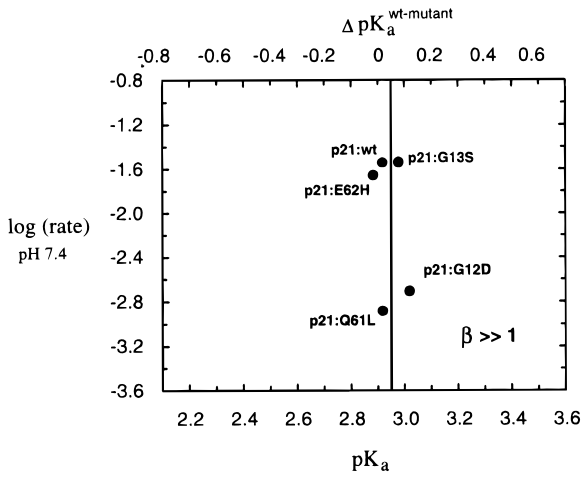

FIGURE 3: Plot of the rate against pK_a for some Ras proteins, including the oncogenic mutants Q61L and G12D. The Brønsted slope of LFER changes from $\beta = 2.1$ in Figure 2 to $\beta \gg 1$. This result suggests that Q61L and G12D affect the reaction mechanism in a different way than other mutations.

Note also that Rap1A and the corresponding Rap1A mutant D38A follow the LFER displayed in Figure 2. This result strongly suggests that the GTPase mechanism of Rap1A and p21^{ras} are identical. We also tried to examine if other GTP-binding proteins follow the same LFER and as result of this also the same reaction mechanism. Preliminary results have shown that the pK_a of GTP bound to the small GTP-binding protein Rab7, a protein that is involved in membrane trafficking, as well as the corresponding intrinsic GTPase rate are significantly lower than those for p21^{ras} (T. Schweins and M. Geyer, unpublished data). It might well be possible that Rab7 follows the LFER displayed in Figure 2. However, all other Ras-related GTP-binding proteins that we examined (as for example p24^{ran} or EF-Tu) denature irreversibly when titrated to a low pH. Thus, it is not feasible to determine the corresponding pK_a of bound GTP. Nevertheless, because of the high structural similarities, it is reasonable to assume that most if not all GTP-binding proteins that share a similar structure also follow a similar reaction path. For those where a structure of the triphosphate form has been determined by X-ray crystallography, a nucleophilic water has been found in an identical position in the active site (Pai et al., 1991; Berchtold et al., 1993; Kjeldgaard et al., 1993; Sondek et al., 1994).

Interestingly, we also found deviations from the LFER plot; Q61L and G12D, two prominent oncogenic mutants that are both found in a large number of human tumors, do not follow this relationship. The GTPase rate is significantly lower than that for wild type Ras, and yet the pK_a of bound GTP is very similar. If p21−Q61L and p21−G12D are grouped in a second set of Ras proteins, one can see from the corresponding Brønsted plot (Figure 3) that these proteins follow a different LFER. The corresponding Brønsted slope that determines the correlation coefficient β changes from $\beta = 2.1$ for the first set of mutations (displayed in Figure 2) to $\beta \gg 1$. This result indicates that p21−Q61L as well as p21−G12D destabilize the transition state without having an influence on the pK_a of the bound GTP. It is obvious from this that the nature of the effect on the rate is different for the two sets of proteins displayed in Figures 2 and 3. This suggests that the transforming properties of oncogenic Ras mutants that belong to different sets of mutants (e.g. G12V in set I and Q61L in set II) have a different physical origin. In the extreme case, the different classes of mutants might even follow a different reaction path.

For many biochemical (chemical) reactions, one finds two sets of mutations (molecules) that display different LFERs. In fact, for tyrosyl-tRNA synthetase, Fersht and co-workers found a similar behavior (Fersht et al., 1986). While a large group of mutants displays a LFER with a slope of less than 1, a second set exhibits a slope of $\gg 1$. For such systems, it is justified to classify different mutants to different groups (Fersht et al., 1986; Warshel et al., 1994). This does not necessarily mean that the two sets of mutations follow a different reaction path. It is conceivable that all mutants regardless of their group follow the same reaction mechanism and have a similar reaction potential surface. However, if a set of mutants falls in a LFER plot on the same line, it is very likely that these mutations affect the reaction (and thus the reaction potential surface) in the same manner (i.e. by stabilizing or destabilizing the same specific resonance structures).

A second set of mutations of the same system that fall on a line with a different slope very often follow the same mechanism. However, this set might affect the reaction in a different way (for example by stabilization or destabilization of a different intermediate). As long as a mutant displays a residual enzymatic activity as in the case of p21^{ras} (where the reaction in the mutant enzyme is still orders of magnitude faster than the corresponding solution reaction), it is questionable whether such a mutation leads to a completely different mechanism. Instead, it is more likely that a single point mutation will lead to a small change of the wild type reaction potential surface than to an entirely different potential surface. For a detailed analysis, we refer to the subsequent paper (Schweins et al., 1996).

In this respect, it is interesting to note that the G12D mutant is the only mutant analyzed so far that crystallizes in a space group different from that of the wild type. The most drastic structural changes relative to Ras wild type occur around the active site (Franken et al., 1993). These changes are probably due to the interaction of the aspartic side chain in position 12 with Tyr32, Gln61, and the γ-phosphate. Asp12 might also conflict with the catalytic water that is not clearly defined in the crystal structure of this mutant. Thus, in this light, it is not surprising that G12D does not follow the regular LFER displayed in Figure 2. However, the odd behavior of this mutant cannot be clarified only by analyzing the static ground-state crystal structure of the GppNHp complex, and it remains an open question as to whether the intrinsic reaction mechanism of this G12D is fundamentally the same or different compared to that of wild type Ras.

The crystal structure of Q61L is less informative since the loop L4 (that region that includes residue 61) is poorly resolved (Krengel et al., 1990). The X-ray structure (Pai et al., 1990) as well as the NMR structure (Kraulis et al., 1994) of p21−GppNHp indicate that loop L4 is very flexible and mobile. Also, the affinity of GTP toward Ras is not changed significantly when Gln61 is exchanged with Leu (Feig & Cooper, 1988). Thus, it is reasonable to assume that Gln61 does not interact strongly with the GTP in the ground state of the catalytic reaction. However, very recently, Mittal et al. (1996) have found evidence that Gln61 may interact directly with the proposed pentacovalent transition state of the GTP hydrolysis, at least in the presence of GAP. It was

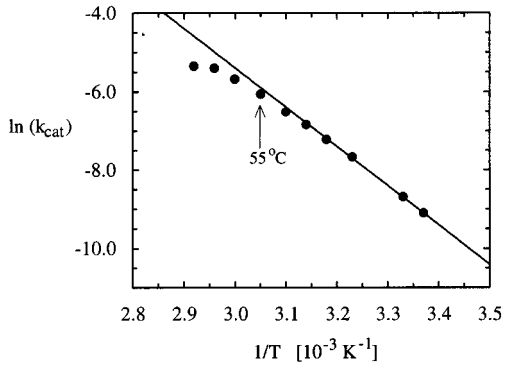

FIGURE 4: Arrhenius plot of the intrinsic GTPase reaction (37 °C). For the linear curve fit, we only considered data points measured at temperatures of less than 55 °C. From the analysis of this data with the Arrhenius equation, we get estimates for the activation energy E_a of 19.9 kcal/mol, the activation entropy $\Delta S^{\#}$ of −0.8 cal/mol, the activation enthalpy $\Delta H^{\#}$ of 19.3 kcal/mol, and the free energy of activation $\Delta G^{\#}$ of 19.5 kcal/mol.

also shown that this interaction is reduced significantly when Gln61 is replaced by Leu. Thus, Gln61 seems to mainly stabilize the transition state (at least in the presence of GAP) without affecting the stability of the relevant ground state of the reaction. Such a behavior would lead, as outlined in the following paper, to a Brønsted slope β that is very large ($\beta \gg 1$) and is in agreement with the results displayed in Figure 3.

Another approach that can help in elucidating important features of a reaction mechanism in enzymes is the evaluation of the temperature dependence of the reaction rate. Deviations from an Arrhenius plot can be due to proton tunneling (Cha et al., 1989) or to sequential steps in a reaction mechanism that do show a similar rate (Wilson & Cabib, 1956). In Figure 4, the temperature dependence of the intrinsic GTPase is shown in an Arrhenius plot. For temperatures up to 50 °C, the reaction of p21^{ras} does follow the Arrhenius relationship. Above this temperature, one can observe a deviation from the linear Arrhenius line. However, this behavior is probably just due to denaturation of the protein. In fact, it has been shown previously with CD spectroscopy that the melting temperature of p21−GTP is approximately 55 °C (Reinstein et al., 1991). The activation free energy that can be determined from the slope of the straight line is 19.5 kcal/mol. This value is similar to our estimate [$\Delta G^{\#} \sim 22.4$ kcal/mol; see Langen et al. (1992) and Schweins et al. (1994)] using the general preexponential factor of $k_B T/h \approx 6 \times 10^{12}$ s^{-1}, which seems to be appropriate for most adiabatic reactions in condensed phases [e.g. Warshel (1991)].

While the intrinsic GTPase reaction seems to be responsible for the breakdown of the p21(GTP)−Raf kinase complex, the GTPase activating protein, GAP, downregulates the amount of uncomplexed p21(GTP) in the cell. Thus, understanding the intrinsic as well as the GAP-stimulated GTPase mechanism is of great importance for understanding signal transduction on an atomic level. On the other hand, it is of general interest to understand how a certain protein can affect the reaction rate of another enzyme.

In principle, two extreme classes of mechanisms for GAP activation of the p21^{ras} GTPase reaction are conceivable. In one of these, the basic mechanism is the same in p21^{ras} and in the p21^{ras}−GAP complex. The role of GAP in the activation of the GTPase reaction could be for example the stabilization of a particular conformation of p21^{ras} and a resulting change of electrostatic properties in the active site (Muegge et al., 1996). As a result of this, crucial interactions between p21^{ras} and the corresponding transition state of the reaction might increase. In the other type of mechanism, the involvement of one or more side chains of the GAP molecule in the catalytic reaction is imaginable. This would imply that the mechanism of GTP hydrolysis by GAP-activated p21^{ras} might be fundamentally different than in p21^{ras} alone.

As stated above and in the subsequent paper, the strongest support for the proposed GTP as a base mechanism for the intrinsic reaction is probably provided by the LFER between the logarithm of the reaction rate of p21^{ras} and the pK_a of proteins bound GTP−γ-phosphate (Figure 2). If the GAP-activated GTPase of p21^{ras} follows the same GTP as a base mechanism, one would expect that the GAP-stimulated reaction also exhibits a LFER of the kind found for the intrinsic GTPase.

Unfortunately, it is not possible to determine the pK_a of GTP−γ-phosphate in the p21^{ras}−GAP complex, since the protein−protein interaction between these proteins is very sensitive to any pH change and also because GAP does not tolerate pH changes. Nevertheless, it is valid to correlate the logarithm of the GAP-stimulated GTPase rate with the pK_a of GTP−γ-phosphate bound to p21^{ras} alone, as long as the mutated residue does not conflict with the physical interaction of these two proteins.[2]

We determined the corresponding parameter for a small set of Ras proteins that can be simulated by GAP and plotted log(k_{cat}) as function of the pK_a of the γ-phosphate (Figure 5). From this Brønsted plot, one can depict that for the GAP-stimulated reaction a linear correlation between these two parameters (very similar to the results that are displayed in Figure 2) exists. The higher the pK_a of the bound nucleotide and the stronger the proton abstraction potential of the γ-phosphate, the faster the reaction of the GAP-stimulated reaction at neutral pH. In fact, from this relationship, one can conclude that the GTP as a base mechanism is probably operating also in the GAP-activated reaction.

In this context, it is very interesting to note that the correlation coefficient β for the GAP-catalyzed reaction is different from the corresponding coefficient for the intrinsic reaction. While the intrinsic reaction exhibits a Brønsted slope of $\beta = 2.1$ (Figure 2), the corresponding value for the GAP-activated reaction is $\beta = 4.9$ (Figure 5). A given pK_a change due to mutation seems to have a larger impact on the reaction rate when GAP is present. A Brønsted slope β that is more than twice as large for the GAP-stimulated reaction relative to the intrinsic reaction can be explained with the stabilization of a more product-like structure of p21^{ras} as a result of GAP binding (for a detailed analysis, see the subsequent paper). As was suggested before, GAP might accelerate the GTPase reaction by providing a positively charged residue that directly interacts with the

[2] Very often, the kinetic properties of enzymatic reactions are correlated to thermodynamic properties of substrates that were determined in aqueous solution and thus in the absence of the enzyme [i.e. Tony and Kirsch (1988)]. This is similar to the LFER displayed in Figure 5 because GAP can be considered the enzyme and p21−GTP the corresponding substrate. The rate constant for the GAP-catalyzed reaction is plotted as function of the "intrinsic" pK_a of GTP bound to p21^{ras}.

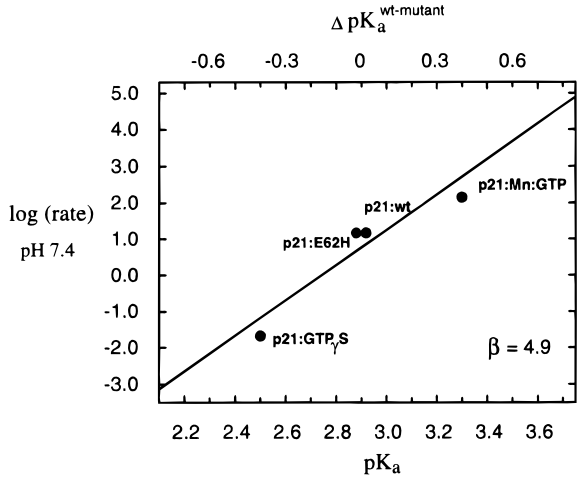

FIGURE 5: LFER plot of the logarithm of the GAP-catalyzed GTPase reaction rate (in s^{-1}) as function of the apparent pK_a of the GTP bound to p21^{ras}. Note that the Brønsted slope has changed from $\beta = 2.1$ for the intrinsic reaction (Figure 2) to $\beta = 4.9$ for the GAP-catalyzed reaction. It was not possible to determine the pK_a of GTP bound to the p21^{ras}−GAP complex because GAP is very sensitive to changes in pK_a. Thus, we plotted the logarithm of the GAP-catalyzed reaction rate as function of bound GTP in Ras alone (see footnote 2).

Table 1

	intrinsic GTPase	GAP-catalyzed GTPase		
	k_{cat} (min^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})
p21−Mg^{2+} H_2O	0.028	19	8.9	2.1
p21−Mg^{2+}−GTP				
p21−Mg^{2+}−GTPγS	0.0025	0.02	14.2	0.0014

negatively charged γ-phosphate (Schweins et al., 1993).

In order to find out if the basic reaction mechanism for isolated p21^{ras} and for the p21^{ras}−GAP complex are identical, one can examine if different features that are known to perturb the rate of the intrinsic reaction also affect the velocity of the GAP-stimulated GTPase. It is known that the intrinsic rate is slowed considerably when the Ras-bound GTP is exchanged with GTP analog GTPγS, where one of the γ-phosphate oxygens is exchanged with sulfur (Rensland et al., 1991). From Table 1, one can depict also that the GAP-stimulated GTPase is reduced significantly, when GTPγS instead of GTP is bound to p21^{ras}. In the subsequent paper, it is shown that the exchange of the solvent H_2O with D_2O affects the intrinsic and GAP-stimulated GTPase reaction in the same manner.

In summary, so far, it has not been possible to observe any fundamental differences in chemistry between the intrinsic and the GAP-stimulated GTPase of p21^{ras}.

CONCLUSION

This work has shown that 13 Ras and 2 Rap1A proteins follow the LFER displayed in Figure 2. From this result, we can conclude that these proteins exhibit the same GTP as a base reaction mechanism that was proposed before for wild type Ras. The difference in GTPase rate seems to be a consequence of a pK_a change of the terminal GTP phosphate due to the corresponding mutation. The higher the pK_a of the γ-phosphate and the stronger its proton abstraction potential, the faster the reaction rate at neutral pH. Interestingly, most of the common known oncogenic mutants of p21^{ras} follow this relationship. This suggests that they follow the same reaction mechanism as wild type Ras. A pK_a deviation of less than 0.5 unit seems to be enough to slow the intrinsic GTPase reaction rate in oncogenic mutants. This effect might be indirectly responsible for the oncogenic potential that these mutants display.

Furthermore, it is shown that Rap1a proteins follow the same type of LFER as p21^{ras} does. Thus, we conclude that Rap1A proteins very likely also follow the same proposed GTP as a base mechanism. This supports the notion that many if not all members of the superfamily of guanine nucleotide binding proteins not only are closely structurally related to each other but also share a common reaction mechanism. In fact, structural studies with transducin (Sondek et al., 1994) and myosin (Fisher et al., 1995) reveal that the active sites of these proteins resemble that of p21^{ras}, and it was suggested that the GTP as a base mechanism is also operative in these proteins. It would not be a surprise if other enzymes or even ribozymes that exhibit ATPase, GTPase, or phosphatase activity follow a similar reaction route. Our results show that the reduced GTPase rate of the nucleotide analog GTPγS by p21^{ras} seems to be the consequence of a reduced pK_a of its terminal phosphate group. It is known that many other ATP and GTP hydrolyzing and synthesizing enzymes like myosin or transducin are slowed by ATPγS and GTPγS, respectively. It is tempting to speculate that this observation may be explained with a corresponding pK_a reduction that slows the overall reaction rate in a related GTP as a base reaction mechanism.

The data presented here indicate that the mechanism of GTP hydrolysis by GAP-activated p21^{ras} is probably closely related to that in p21^{ras} alone. Up to this point, it has not been possible to observe any fundamental differences in chemistry between these systems. All perturbations that lead to a different intrinsic rate also lead to a comparable change of the GAP-stimulated reaction rate. The LFER result of Figure 5 shows that the rate of the GAP-stimulated GTPase of p21^{ras} correlates with the basicity of bound GTP in Ras alone. This indicates that the GAP-stimulated reaction might also follow the GTP as a base mechanism. The role of GAP in the activation of the GTPase reaction could be the stabilization of a particular conformation in the active site of p21^{ras} (i.e. a more product-like state). GAP might also lead to a change of the electrostatic properties in the active site of p21^{ras}. The active site of p21^{ras} is directly located at the protein surface. It is conceivable that the binding of GAP to p21 dehydrates this region and decreases the dielectric properties in the active site. Mechanistically important interactions between p21^{ras} and the corresponding transition state of the reaction might increase as a result of GAP binding. It remains to be seen if GAP is able to "catalyze" or induce a conformational change in p21^{ras} that precedes the transition state and thus leads to a more active form of p21^{ras} (Muegge et al., 1996). A clearer picture of the GAP-activated mechanism might emerge, when the three-dimensional structure of a p21−GAP complex is solved.

It is tempting to propose that the results presented in this work may be used to design drugs that reactivate the GTPase reaction of these oncogenic p21^{ras} mutants. Our result suggests that any small compound that binds to p21^{ras} and slightly increases the pK_a of GTP should also speed the reaction and hence reduce the oncogenic potential of these

mutant Ras proteins. It is not essential that such a compound would bind in the vicinity of the γ-phosphate in the active site of $p21^{ras}$. For example, the carboxylate group of Asp38 is about 10 Å away from the γ-phosphorus, and yet as shown in Figure 2, the effect on mutating this residue to Glu or Ala leads to a significant increase in the pK_a of GTP.

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