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Conserved Motifs in Different Classes of GTPases Dictate their Specific Modes of Catalysis

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GTP Hydrolysis • Ras GTPase • EF-Tu • Empirical Valence Bond • Computational Arrhenius Plots

The GTPase superfamily of enzymes that hydrolyze GTP have a number of conserved sequence regions (the so-called “G-motifs”), and several of the subfamilies also require catalytic activation by specific GTPase activating proteins. In the translational GTPases involved in protein synthesis, this activating function is instead accomplished by their interaction with the ribosome. Despite these similarities, there are distinct differences regarding some of the amino acid residues making up the GTPase active sites. This raises the question of whether the catalytic mechanisms of different types of GTPases are identical or not. We report herein extensive computer simulations of both the intrinsic GTP hydrolysis reaction of Ras, and the considerably faster reaction activated by the interaction with RasGAP. The results of these calculations are compared to earlier simulations of GTP hydrolysis by EF-Tu on the ribosome, and show that the favored reaction pathways are strongly dependent on the composition of the active site. By computing Arrhenius plots for the temperature dependence of the calculated free energy profiles, we further show that different mechanistic pathways are associated with distinct differences in activation entropies and enthalpies. The activation parameters are in good agreement with experimental data and we conclude that calculations of Arrhenius plots from computer simulations can be very useful for dissecting the energetics of enzyme catalysis.

INTRODUCTION

The GTPase superfamily encompasses enzymes that bind and hydrolyze GTP to GDP and inorganic phosphate. Among its members is the Ras family of small GTPases involved in signal transduction, as well as the translational GTPases (trGTPases) that are essential components of the protein synthesis machinery.^{1,2} The catalytic site of these enzymes is located in their structurally conserved G-domain, which is common to all GTPases and contains the switch regions that change conformation depending on whether GTP or GDP is bound.^{1,2} Both the Ras type GTPases and the trGTPases have only low intrinsic enzymatic activity and require interaction partners for their activation, which typically increases the rate of GTP hydrolysis^{3,4} by a factor of 1000. In the Ras family these interaction partners are the GTPase-activating proteins (GAPs), while the trGTPases are activated by interaction with the ribosome. Here, their G-domains have a common binding mode and activation is caused by interaction with the sarcin-ricin loop (SRL) of the large ribosomal subunit.⁵⁻⁷

Since the G-domain of all these GTPases has a number of highly conserved motifs (G1-G4),^{1,2} and since all of them are activated in a seemingly similar manner, it may be reasonable to expect that they also employ a common reaction mechanism in catalysis.⁸ However, despite the strong conservation of G1-G4 there are distinct differences, where particularly the amino acid residue following the invariant glycine in G3 (switch II) is of mechanistic importance as it is located near the γ -phosphate group of GTP. For example, in the Ras, Rho and Ran enzymes this residue is a glutamine, in Rap it is a threonine and in Sar a histidine.⁹ A recent crystal structure of the Rap-RapGAP complex, however, showed that Thr61 in switch II is not in contact with the γ -phosphate, but that Asn290 from

a neighboring helix instead adopts a position similar to Gln61 in switch II of Ras.¹⁰ In the trGTPases the corresponding G3 residue is a histidine which is part of a universally conserved PGH (Pro-Gly-His) motif.^{1,2,11} There are also distinct differences with regard to the key GAP residues inserted into the active site upon activation. For example, RasGAP, RhoGAP and RabGAP insert an arginine sidechain, Arg789 in RasGAP (**Figure 1a**), while RanGAP and RapGAP lack this residue.⁹ Moreover, in the trGTPases there is no corresponding residue inserted, but interaction with the ribosome SRL instead causes a conformational change of the invariant histidine residue (His84 in EF-Tu) so that it moves into contact with the γ -phosphate (**Figure 1b**).^{5-7,12}

There has been considerable discussion of the mechanism of GTPase catalysis, mostly focusing on the Ras-RasGAP system, and although opinions of the detailed transition state (TS) structure may differ, most data speak in favor of a substrate-assisted hydrolysis mechanism.^{3,8,13-15} What this means in practice is that no bases are involved in the reaction other than the terminal phosphate group itself. The question is rather whether bond breaking to the leaving group lags behind bond formation to the water nucleophile, or not, and whether proton transfer to the γ -phosphate is early or late. In fact, as will be shown here, the discussion of the whether the TS is loose or tight (referring to the nucleophile and leaving group bond lengths to P) is of secondary importance compared to the degree of charge separation in the TS. That is, although the two features are of course connected it is the actual charge distribution in the TS that reflects the mode of catalysis, as is this case with most enzymes.¹⁶

Figure 1. Comparison of the active sites of the Ras-RasGAP complex¹³ (a) and the translational GTPases (EF-Tu and EF-G) in their activated conformation⁶ (b). The former complex is solved with GDP•AlF₃ and the latter with GTPCP, as transition state analogs. Water molecules are shown as red spheres and magnesium ions as green spheres.

We have recently reported extensive computer simulations of GTP hydrolysis on the ribosome by EF-Tu,^{12,17,18} which is the fastest of all known GTPases and the prototype for those involved in translation. These calculations encompassed analysis of a number of different possible mechanisms, including associative and dissociative variants with early and late proton transfer to the phosphate group, as well as general base mechanisms involving the neutral form of His84.¹⁷ The conclusion from these simulations was that the EF-Tu complex on the ribosome strongly favors early protonation of GTP and attack by hydroxide ion on the γ -phosphate, and that this mechanism is enforced by the positively charged His84 and the conformation of the universally conserved PGH motif. Subsequent atomic mutagenesis experiments, that removed the negative charge on the SRL phosphate group which interacts with the protonated histidine (Figure 1b), provide additional strong support for this mechanism.¹⁹ Moreover, we recently evaluated the temperature dependence of the predicted EF-Tu mechanism, by calculations of Arrhenius plots directly from computer simulations, and could establish that this mechanism is associated with a very large and favorable (positive) activation entropy.¹⁸ The predicted $T\Delta S^\ddagger$ term at 25 °C of +7 kcal/mol was also found to be in perfect agreement with that measured for k_{cat} by Ehrenberg and coworkers,²⁰ suggesting that the chemistry may indeed be rate-limiting for GTP hydrolysis

rather than GTPase activation. In comparison, the corresponding $T\Delta S^\ddagger$ for uncatalyzed GTP hydrolysis in water was found to be near zero for both associative and dissociative mechanisms that involve neutral water attack, also in good agreement with experimental data.^{21,22} Our calculations further showed that hydroxide attack on the protonated γ -phosphate has an intrinsically positive activation entropy, since this reaction was predicted to yield $T\Delta S^\ddagger = +6$ kcal/mol for the uncatalyzed process in water. Again, this value is in excellent agreement with that reported for hydrolysis of methylphosphate under highly alkaline conditions, where OH⁻ attack on the monoanion dominates the reaction.²³

In order to shed further light on the relationship between the catalytic mechanisms of GTPases and the structure of their active site and, particularly, the identity of their G3 motifs and insertion of GAP residues, it is of considerable interest to quantitatively compare the trGTPases with the Ras family. Here, we report molecular dynamics (MD) free energy simulations of the catalytic reaction of Ras-RasGAP, utilizing our earlier parameterized empirical valence bond (EVB) models used for the EF-Tu reaction on the ribosome. We again examine different possible variants of the reaction mechanism and also report calculations of the temperature dependence for the Ras-RasGAP reaction. The results clearly show that the two GTPases operate by distinctly different mechanisms and that while EF-Tu has a significant catalytic contribution from entropic effects, this is not the case for Ras-RasGAP where catalysis is dominated by reduction of the activation enthalpy.

MATERIALS AND METHODS

MD simulations. Molecular dynamics simulations were performed as reported earlier^{12,17,18} with the program Q,²⁴ utilizing the OPLS-AA force field (Macromodel 9.1, Schrödinger LCC, New York).²⁵ In brief, spherical simulation systems (40 Å in diameter) centered on the γ phosphorous atom of GTP were used after solvation by a TIP3P water droplet of the same size. In the enzyme simulations, protein atoms outside the simulation sphere were tightly restrained to their initial coordinates and excluded from non-bonded interactions. Water molecules close to the sphere boundary were subjected to radial and polarization restraints according to the SCAAS model.^{24,26} Initial coordinates were taken from the crystal structure of the Ras-RasGAP complex¹³ (PDB code 1WQ1). MD simulations were carried out with a 1fs time step and a 10 Å cutoff was employed for direct non-bonded interactions, with electrostatic interactions beyond the cutoff treated by the local reaction field multipole expansion method.²⁷ No cutoff was applied to non-bonded interactions involving the central part of the system that is treated by the EVB model (*i.e.* the triphosphate moiety of GTP and the hydrolytic water molecule). The reference simulations of different Mg²⁺•GTP⁺ hydrolysis mechanisms in pure water have been reported previously,^{17,18} and used solvent spheres of the same size (40 Å in diameter).

Computational Arrhenius plots and EVB models. The Mg²⁺•GTP⁺ hydrolysis reaction mechanisms considered here were described by the EVB method^{28,29} with parameterization of the different models as described earlier.¹⁷ The uncatalyzed associative and dissociative reaction pathways in solution were thus both calibrated to reproduce the experimentally derived^{17,21,22} activation free energy of 27 kcal/mol, as described in detail in ref. 17. It should be noted in this context that recent high-level quantum mechanical calculations, including solvation effects, have consistently shown very similar activation barriers for associative and dissociative pathways.³⁰⁻

³³ These typically differ by at most 1-2 kcal/mol, while the discrimination found here in Ras and Ras-RasGAP is at least 4 kcal/mol (see below). Hence, the conservative calibration of both barriers to the same experimental value, used here, does not affect any of our main conclusions. Calculations of reaction free energy profiles utilized the free energy perturbation (FEP) umbrella sampling method to drive the system between the different EVB states.^{17,34} Each such simulation consisted of an MD equilibration phase with step-wise heating from 1K to 300K while gradually releasing restraints on heavy solute atoms, followed by 800 ps of unrestrained equilibration at the given temperature. The calculation of each free energy profile then involved 1.1 ns of MD sampling, which comprised 21

intermediate FEP windows. For the initial screening of different reaction pathways¹⁷ four independent simulations were used with different randomized initial velocities reassigned during equilibration, according to the Maxwell distribution. Arrhenius plots were then computed both for the Ras-RasGAP and intrinsic Ras GTP hydrolysis reactions. This involved calculation of 40 and 30 independent free energy profiles for Ras-RasGAP and Ras, respectively, at each of the five temperatures 290, 295, 300, 305 and 310 K. Averaging of these free energy calculations for each temperature yielded reaction and activation free energies with a standard error of the mean (s.e.m.) of less than 0.3 kcal/mol, which is a sufficiently good precision for obtaining reliable Arrhenius plots.³⁴

Figure 2. Summary of calculated free energy profiles (in kcal/mol) for the different mechanistic reaction pathways examined for GTP hydrolysis in the Ras-RasGAP complex, utilizing the EVB models from ref. 17. Reaction paths (a) and (b) correspond to associative mechanisms with a transient pentacoordinated intermediate with early and late proton transfer to the γ -phosphate, respectively. Pathway (c) denotes a fully concerted reaction represented by a two-state EVB model,¹⁷ while pathway (d) represents a dissociative mechanism with a loose transition state and a transient meta-phosphate-like intermediate.¹⁷

RESULTS AND DISCUSSION

Catalytic mechanism. For a direct mechanistic comparison between Ras-RasGAP and EF-Tu we first evaluated the energetics of proton transfer from the catalytic water molecule to the γ -phosphate of GTP. While this was found to be an almost isoenergetic process in EF-Tu (~ 2 kcal/mol uphill),¹⁸ it turns out to be highly disfavored in the Ras-RasGAP complex. In this case the cost of proton transfer to phosphate, relative to the corresponding reference reaction in solution, is calculated to be $\Delta\Delta G_{PT} = 10.2 \pm 0.3$ kcal/mol. With our earlier estimate from pK_a values of the free energy cost of proton transfer in water, $\Delta G_{PT}^{wat} = 12.2$ kcal/mol,^{12,17} this would bring the resulting state with protonated GTP and hydroxide ion to over 20 kcal/mol above the reactant state. Since the experimental

activation free energy in Ras-RasGAP is only about 16 kcal/mol, derived from the reported catalytic rates of ~ 20 s⁻¹,^{3,35} we can immediately conclude that the hydroxide mechanism can be ruled out in this case. As noted earlier, the presence of both the negatively charged Asp21 and the positively charged His84 in EF-Tu favors charge migration to the right in **Figure 1b**, and causes stabilization of the hydroxide ion state. In contrast, Ras-RasGAP lacks both of these charges and instead has the positively charged arginine finger (Arg789) from GAP at roughly the same position as Asp21 in EF-Tu (**Figure 1a**). Hence, the electrostatic situation in the active is essentially reversed and this is what causes the large destabilizing effect on the hydroxide mechanism.

While the early proton transfer mechanism leading to a protonated γ -phosphate and hydroxide ion is clearly not viable in Ras, the MD/EVB simulations instead predict that both variants of the associative pathway,¹⁷ where proton transfer to one of the non-bridging oxygens is either concerted with or lags behind P-O bond formation, are strongly stabilized by the enzyme. This is in agreement with recent studies by Warshel and coworkers,⁸ which identified a relatively flat plateau on which proton transfer takes place for such pathways. Our simulations predict these associative variants to have activation free energies of 17.1 and 18.2 kcal/mol, where the early proton transfer mechanism is favored by about 1 kcal/mol over the one with later proton transfer (**Figure 2a,b**). The calculated free energy barriers are also in good agreement with the observed $\Delta G_{cat}^\ddagger$ of 16 kcal/mol.^{3,35}

Figure 3. Comparison of representative MD structures in the TS region for the Ras-RasGAP reaction (**a**) and the GTP hydrolysis reaction of isolated Ras (**b**). In Ras-RasGAP, Gln61 is sterically sequestered by the interaction with RasGAP and its hydrogen bonding network is indicated with dashed lines. Without RasGAP the active site becomes largely solvent exposed. Magnesium ions are shown as green spheres.

Furthermore, the simulations predict both the fully concerted phosphoryl transfer mechanism and the dissociative pathway to have higher activation free energies of 19.8 and 20.9 kcal/mol, respectively (**Figure 2c,d**). The fully concerted variant is represented by a two-state EVB model with no transient phosphorane intermediate, while the dissociative pathway involves a transient intermediate with partial metaphosphate character.¹⁷ The latter activation barriers are in fact similar to those obtained for EF-Tu with the neutral form of His84.¹⁷ It may further be noted that even though the EVB models corresponds to direct proton transfer from the nucleophile to one of the nonbridging γ -phosphate oxygens, the MD structures at the associative TS plateau often show a second water molecule bridging between the nucleophile and the

phosphate group (**Figure 3a**). This indicates that proton transfer also may occur via the two-water pathway examined by Warshel and coworkers,⁸ which was found to yield similar energetics to the one-water mechanism³⁶ (similarly to the corresponding reaction in aqueous solution).³³ An associative mechanism in Ras-RasGAP is also implicated from time-resolved FTIR studies that show a transition state with protonated γ -phosphate.³⁷

Effect of Gln61 and RasGAP. In order to further probe the catalytic mechanism of Ras the catalytic mechanism of Ras we carried out simulations of the Ras-RasGAP reaction with the partial charges of the Gln61 sidechain turned off, as done in refs.14 and 38. In this case the non-polar sidechain relaxes to a different rotamer and makes no specific interaction with either the γ -phosphate group or the catalytic water molecule, and the substitution increases the activation free energy by 4 kcal/mol. This result is in reasonable agreement with earlier simulations^{14,38} and supports the notion that it is not only the polarity of Gln61 that is important for the catalytic effect, but also its steric shape. That is, the effect of the likewise nonpolar Gln61Leu mutation is experimentally found to be significantly larger than that predicted for a nonpolar glutamine sidechain.³⁸

Finally we examined the catalytic effect of the interaction with RasGAP, which is the key to activating the GTPase. By removing RasGAP from the simulation system and evaluating reaction free energy profiles for the isolated Ras enzyme we obtain an activation barrier of 21.5 kcal/mol, which is in very good agreement with the experimentally derived barrier of 22 kcal/mol.^{3,21} Importantly, however, the dissociative mechanism (further promoted by the interaction of the catalytic metal center with the leaving group) now becomes strongly favored and the associative pathway yields no catalytic effect at all. The primary reason for this change of mechanism when RasGAP is not present is that, in the RasGAP complex, Arg789, Arg903 and Leu902 from the GAP create a rather well-defined cavity for Gln61 from Ras. A hydrogen bond between the carbonyl group of the arginine finger (Arg789) and the Gln61 sidechain nitrogen also helps to keep Gln61 in place in the Ras-RasGAP complex (**Figure 3a**). Apparently it is this strict positioning of Gln61 together with the positive charge and conformation of Arg789 that are mainly responsible for promoting an associative mechanism. This makes the RasGAP complex somewhat similar to EF-Tu, where the protonated active site histidine not only plays the same role as Gln61 of Ras in positioning the nucleophile, but also provides additional charge stabilization to promote hydroxide attack on a protonated phosphate in an associative substrate-assisted mechanism.¹² Hence the earlier conclusion that the locking of Ras into a catalytic conformation by RasGAP is essential for the catalytic effect¹⁵ is supported by the present calculations. RasGAP shields the active site from solvent and when it is not present both the γ -phosphate and Gln61 become solvent exposed (**Figure 3b**), and the Gln61 sidechain is considerably more mobile. This very different active site situation is thus predicted to instead promote a dissociative mechanism.

The catalytic role of Gln61 was further examined by restraining this sidechain to its position in the Ras-RasGAP complex, in simulations of the isolated Ras reaction. These calculations show that 2.1 kcal/mol out of the total 4.4 kcal/mol stabilization of the free energy barrier, caused by RasGAP, is recovered just by locking Gln61 into its catalytic conformation. We further examined the direct electrostatic contribution of the Arg789 sidechain in Ras-RasGAP by annihilating its Coulomb interactions in the free energy calcula-

tions, while retaining the native MD trajectories with these interactions present. This yielded only a small destabilizing effect of 0.4 kcal/mol, indicating that direct TS stabilization by the Arg789 charge is relatively small (this should not be confused with the effect of mutating Arg789 to, e.g., alanine, in which case solvent can enter and the situation becomes similar to the isolated Ras case). The above result is not so strange in view of the relatively similar charge distributions in the reactant and transition states. However, the Arg789 charge still appears important in dictating the favored mechanism.

Computational Arrhenius plots. To further compare the GTPase mechanisms of Ras with that of EF-Tu we carried out extensive MD/EVB simulations of the temperature dependence of activation free energies as reported earlier.^{18,34} This allows extraction of thermodynamic activation parameters

with good precision and these can be directly compared to available experimental data. We have recently reported this type of calculations for the GTPase reaction of EF-Tu on the ribosome and for uncatalyzed GTP hydrolysis in water.¹⁸ The uncatalyzed water reaction was modeled both by associative or dissociative pathways, and the resulting Arrhenius plots for both alternatives yielded near-zero activation entropies, in excellent agreement with experiments (**Figure 4a,b**).^{21,22} Hence, the values of $T\Delta S^\ddagger$ (at 298 K) for hydrolysis of $\text{Mg}^{2+}\cdot\text{GTP}^+$ in water were predicted to be -1.0 kcal/mol and $+1.2$ for the associative and dissociative mechanisms, respectively, which can be compared to the experimentally derived value²¹ of -0.8 kcal/mol (the corresponding value for ATP hydrolysis has been reported as -1.9 kcal/mol).²²

Figure 4. Calculated Arrhenius plots relating the activation free energy ($\Delta G^\ddagger$) to inverse temperature for uncatalyzed hydrolysis of $\text{Mg}^{2+}\cdot\text{GTP}^+$ in water, for the two limiting mechanistic cases corresponding to associative (**a**) and dissociative (**b**) reaction pathways (taken from ref. 18). Panels (**c**) and (**d**) show the corresponding computed Arrhenius plots for the favored mechanisms in isolated Ras and the Ras-RasGAP complex, respectively. The temperature range is 290-310 K in all cases.

The computed Arrhenius plot for the favored dissociative mechanism of isolated Ras without RasGAP is shown in **Figure 4c**. This yields values for the thermodynamic parameters at 298 K of $\Delta H^\ddagger = 17.4$ and $T\Delta S^\ddagger = -4.0$ kcal/mol. The experimental values reported from FTIR spectroscopy, in the same temperature range as our computer simulations (290-310 K), are $\Delta H^\ddagger = 19.8$ and $T\Delta S^\ddagger = -2.3$ kcal/mol (at 298 K) for the intrinsic GTPase reaction of Ras.²¹ For comparison, the intrinsic reaction of the Rheb GTPase yielded similar values of $\Delta H^\ddagger = 20.9$ and $T\Delta S^\ddagger = -2.9$ kcal/mol (at 298 K), as determined by a real-time NMR assay.³⁹ Hence, we can conclude that the agreement between simulation and experiment, both for the uncatalyzed reaction and the intrinsic Ras activity, is quite remarkable. The reason for why the activation entropy becomes more negative for the dissociative mechanism in Ras than in water appears to be, at least partly, due to the fact that

Gln61 is distinctly more mobile in the reactant state than the transition state. For the much faster Ras-RasGAP reaction the corresponding computed Arrhenius plot for the favored concerted associative mechanism (**Figure 4d**) yields $\Delta H^\ddagger = 13.4$ and $T\Delta S^\ddagger = -3.7$ kcal/mol (at 298 K). Hence, the calculations predict that the catalytic effect of Ras-RasGAP is essentially entirely of enthalpy nature, with $\Delta\Delta H^\ddagger \sim -14$ kcal/mol compared to the uncatalyzed reaction (**Figure 4a,b**). In this case FTIR experiments have also been reported but these measurements were carried out at extremely low temperature (255-265 K, with antifreeze agent) and a narrow temperature range of only 10 degrees.⁴⁰ A very high activation enthalpy and large positive activation entropy was reported⁴⁰ which, however, overpredicts the rate at room temperature by more than a factor of 100 compared to what is actually measured at 298 K.^{3,35} If the rates at the two highest temperatures (262 and 265 K)⁴⁰

are used together with that actually measured at 298 K (20 s⁻¹),^{3,35} one instead obtains values of $\Delta H^\ddagger = 12.3$ and $T\Delta S^\ddagger = -3.3$ kcal/mol (at 298 K). These values are in very reasonable agreement with our computationally derived activation parameters for Ras-RasGAP and again support the conclusion that the catalytic effect is enthalpic in nature. This is also what was found experimentally for the related Rheb reaction, activated by the TSC2-GAP, which showed an enthalpy lowering of $\Delta\Delta H^\ddagger \sim -15$ kcal/mol and a large negative activation entropy.³⁹

CONCLUSIONS

The present computer simulations are based on our earlier reported EVB models for different types of detailed reaction mechanisms in solution.¹⁷ It is notable that with identical parameterizations of these EVB models three different types of GTP hydrolysis mechanisms are obtained for the reaction of EF-Tu on the ribosome, the Ras-RasGAP catalyzed process and the intrinsic Ras reaction. This supports earlier findings that with a relatively flat transition state region for the uncatalyzed reaction,^{8,33,41} enzymes that catalyze GTP hydrolysis may stabilize different kinds of transition states, thereby modulating the reaction path.⁴² We have further characterized the different enzyme reactions, as well as the solution reaction, by calculating Arrhenius plots for the temperature dependence of the activation free energies. This allows the determination of activation enthalpies and entropies directly from MD/EVB computer simulations, which can then be compared to experimental measurements to shed further light on reaction mechanisms.

What is perhaps most remarkable is that GTP hydrolysis by EF-Tu, which is the fastest known GTPase,⁴³ is characterized by an unusually large positive activation entropy ($T\Delta S^\ddagger = +7$ kcal/mol at 25°C). It is also noteworthy that both computer simulations¹⁸ and experiments^{20,44} give the same value for this quantity. We have earlier shown that this positive activation entropy is characteristic of a mechanism with early proton transfer to the γ -phosphate followed by nucleophilic attack.¹⁸ That is, both the calculations and experiments²³ show that it is OH⁻ attack that gives rise to the positive entropy contribution, which can be attributed to the charge becoming more delocalized over the substrate in the TS.¹⁸ Hence, there is an intimate connection between high catalytic rate of EF-Tu and the specific mechanism that it employs, where the universally conserved PGH motif, unique to the translational GTPases, is the key structural feature.

The present MD/EVB simulations predict that in Ras and the Ras-RasGAP complex the catalytic mechanisms become distinctly different from that in EF-Tu. This change can be attributed to the replacement of His84 in EF-Tu by Gln61 in Ras, which removes the critical positive charge on the sidechain at this position in the active site (**Figure 1**). Hence, in Ras-RasGAP the favored reaction mechanism is predicted by the simulations to be an associative water attack on the γ -phosphate with concerted proton transfer. This agrees with the predicted effects of having either a neutral His84 sidechain, or mutating it to glutamine, in EF-Tu, which in both cases resulted in a concerted water attack mechanism.¹⁷ Our results for Ras-RasGAP also essentially agree with the analysis of Warshel and coworkers.^{8,14,38} Moreover, the calculations predict that in absence of the activating RasGAP partner the favored reaction pathway becomes dissociative. This effect could be attributed to, not only the removal of the positive arginine finger charge, but also to the solvent shielding and

steric effects of RasGAP which lock Gln61 into a favorable position. We note that the calculated activation free energies for both Ras-RasGAP and Ras are also in good agreement with experimental data.^{3,35}

In contrast to the EF-Tu reaction on the ribosome, our computed Arrhenius plots show that the two Ras reactions are characterized by significantly more negative activation entropies, which are closer to that observed for the uncatalyzed GTP hydrolysis reaction in water.^{21,22} Hence, in both Ras-RasGAP and isolated Ras the catalytic effect is mainly due to reduction of the activation enthalpy, while in EF-Tu there is a very large favorable entropy contribution that is associated with the hydroxide mechanism. It is apparent that while there are distinct similarities among the Ras family and translational GTPases, e.g., in the GTP-binding G1 and G4 motifs,^{1,2,11} the evolutionary strategies leading to specific catalytic mechanisms are clearly different in the two families. Thus, the conserved PGH motif in the trGTPases (in G3), together with the conserved aspartate in G1 and the so-called hydrophobic gate composed of two bulky valine/isoleucine residues⁵⁻⁷ (belonging to G1 and G2), leads to a specific mechanism upon activation by the sarcin-ricin loop. This can best be described as a switch, both in terms of conformation and pK_a of the catalytic histidine (His84 in EF-Tu), which becomes positively charged and moves through the hydrophobic gate into the active site upon making contact with the SRL.^{5-7,12} The fact that the catalytic mechanisms in the Ras family and the trGTPases depend on residues not only in the G3 motif, but also in G1 and G2, as well as on the activating partner (RasGAP versus the SRL), explains why single His $\leftrightarrow$ Gln mutations in the G3 motif destroy activity. That is, both the Q61H mutation in Ras and the H84Q mutation in EF-Tu are essentially inactive.^{3,4}

It further appears that the hydroxide mechanism employed by EF-Tu may actually be a requirement for reaching the very high rates of GTP hydrolysis exceeding 500 s⁻¹.^{20,43-45} We have shown that this high catalytic rate is made possible by the uniquely large positive activation entropy.¹⁹ For other GTPases such as the Ras family, which presumably are not subject to the same strong selection pressure for speed, more modest reaction rates (~ 20 s⁻¹) are probably sufficient. Consequently, we find that Ras-RasGAP employs a reaction mechanism more similar to the uncatalyzed one in solution and does not work by large entropic effects.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

GAP, GTPase activating protein; trGTPases, translational GTPases; SRL, sarcin ricin loop; MD, molecular dynamics; FEP, free energy perturbation; EVB, empirical valence bond; TS, transition state.

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