Review

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Mechanistic insights into Ras-catalyzed GTP hydrolysis: conformational dynamics, catalytic mechanisms, and emerging therapeutic strategies

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Abstract: Ras is a key regulator of signal transduction in cells. Ras malfunction is associated with a huge variety of oncological diseases. It is turned off by hydrolysis of bound GTP, which is accelerated by GTPase-activating proteins (GAPs). This minireview discusses the mechanism of Ras-catalyzed GTP hydrolysis, focusing on conformational dynamics and catalytic mechanisms. We discuss structural changes and the role of key residues such as Thr35, Gly60, Tyr32, Gln61, Gly12, and Gly13. Biophysical techniques such as X-ray crystallography, time-resolved FTIR spectroscopy, and hybrid quantum mechanics/molecular mechanics calculations have revealed the detailed reaction mechanisms, including the entry of the arginine finger and the rate-limiting step of inorganic phosphate release. Recent studies on the hydrolysis mechanism favor a solvent-assisted pathway. In addition, we summarize recent advances in Ras-targeting drugs.

Keywords: GTPase; Ras; FTIR-spectroscopy; X-ray crystallography; QM/MM calculations

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1 Introduction

GTPases function as molecular switches, regulating diverse cellular processes (Brunsveld et al. 2006; Cherfils and Zeghouf 2013). Their signaling state is determined by surface modifications induced by the γ-phosphate group of GTP (Gasper and Wittinghofer 2019). All GTPases share a conserved G-domain comprising five α-helices and a six-stranded β-sheet, and are classified into heterotrimeric (Sprang 2016), small (Mishra and Lambright 2016), dynamin (Daumke and Praefcke 2016), and translational GTPase families (Maracci and Rodnina 2016). Small GTPases are further divided into Ras (cell growth), Rho (cytoskeletal regulation), Rab and Arf (vesicular transport), and Ran (nuclear transport) subfamilies (Mishra and Lambright 2016; Yin et al. 2023).

Here we will concentrate on Ras, a key regulator in signal transduction (Cox and Der 2010; Simanshu et al. 2017; Wittinghofer and Vetter 2011), and how the GTPase reaction is catalyzed by Ras and the Ras-GAP complex. We discuss conformational changes during hydrolysis, the exact molecular mechanism of GTP hydrolysis and its kinetics obtained by X-ray crystallography, time-resolved Fourier transformed infrared (FTIR) spectroscopy, quantum mechanics/molecular mechanics (QM/MM) calculations and other methods. Finally, we will give a short overview about recent advances of drugs directly interfering with Ras.

Ras is activated by its guanine nucleotide exchange factor (GEF) SOS, which promotes GDP release and GTP binding by reducing Ras's affinity for nucleotide and Mg^{2+} (Bos et al. 2007). In its GTP-bound state, Ras interacts with effectors, primarily activating the MAPK pathway. GTP hydrolysis, accelerated by GAPs, returns Ras to its inactive GDP-bound form, thus regulating signal transduction. GTP hydrolysis, which terminates signaling, is slow in solution ($t_{1/2} \approx 200$ days at 25 °C) but is accelerated by GTPases (e.g., Ras, $t_{1/2} \approx 20$ min) (Kötting and Gerwert 2004). GTPase-activating proteins (GAPs) further enhance hydrolysis (e.g., GAP-Ras, $t_{1/2} \approx 50$ ms) (Kötting et al. 2008).

2 The structure of Ras and the role of selected amino acids

From a structural point of view, four regions are most prominent within Ras (Figure 1A): the P-loop (residues 10–17) (Saraste et al. 1990); the two switch regions (residues 30–47 and 60–76, respectively) (Wittinghofer and Nassar 1996) and the hypervariable region (HVR, residues 167-terminus) (García-España and Philips 2023). While the G domain (residues 1–166) is almost conserved within the Ras isoforms, the HVR differs. This leads to different post-translational modifications. For example, H-Ras has one farnesyl and two palmitoyl groups, N-Ras has one farnesyl and one palmitoyl group, and K-Ras 4B has a polybasic domain and one farnesyl group. These differences lead to different membrane targeting. Many studies originally done with H-Ras are repeated with the biomedically most relevant K-Ras leading to very similar results. However, the frequency of the oncogenic mutations are different among the isoforms (Johnson et al. 2017) and some manuscript discuss differences due to allosteric interactions (Volmar et al. 2022).

2.1 The role of Thr35 and Gly60

Below we will discuss the key residues within the G domain. The switch mechanism was named “loaded spring mechanism” by Fred Wittinghofer (Vetter and Wittinghofer 2001). The springs are the interactions of Thr35 and Gly60 with the gamma phosphate (Figure 1B). Consequently, these interactions are lost after the cleavage of the γ-phosphate in the hydrolysis reaction and in the absence of these interactions (Patel et al. 2023) the switch regions reach its less ordered “off” state. Only in the “on” state downstream effectors can bind to the fixed conformation of switch I (Spoerner et al. 2001). Many mutants shift the equilibrium from the “on” state (also called state 2 in the literature) to the “off” state (also called state 1 in the literature), e.g. G12V, Y32F, Y32W, Y32R, P34R, T35S, T35A, D38A, D38E, Y40C, A59T (Kötting et al. 2007; Spoerner et al. 2004). Transitions between these two states have been thoroughly studied by theoretical methods (Hu et al. 2024; Matsunaga et al. 2017).

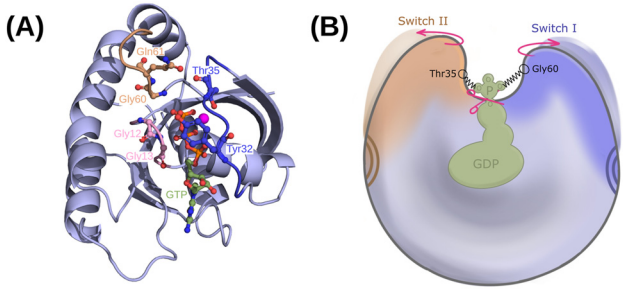

Figure 1: The Ras protein structure and its changes during the hydrolysis reaction. (A) 100K X-ray crystallography structure of Ras with bound GTP (PDB-ID 1QRA) (Scheidig et al. 1999) highlighting switch I (orange), switch II (blue) and P-Loop (pink) including all key residues discussed in this publication (balls and sticks representation). (B) Only the switch I and II regions change their conformation during the hydrolysis reaction due to the cleavage of the γ-phosphate group and their tight interactions with this group.

2.2 The role of Tyr32

Tyr32 is a prominent residue within switch I that has been extensively discussed in the literature. Some literature describes Tyr32 as an important residue for hydrolysis (Fink et al. 2024) in accordance with time-resolved crystallography indicating that Tyr32 orientation towards γ-phosphate is a step preceding intrinsic hydrolysis (Lin et al. 2025). However, the intrinsic hydrolysis rate of Ras Y32A is similar to wild type (Rudack et al. 2015) and γ-phosphate bond breaking in the GAP catalyzed reaction is even faster for Y32A compared to wild type (Li et al. 2018), ruling out a direct mechanistic role during hydrolysis. Interestingly a tyrosine in the same position has an anticatalytic function in other GTPases such as RheB (Mazhab-Jafari et al. 2012) and Ran (Brucker et al. 2010). In Ran but not in Ras, the tyrosine occupies the optimal position of the nucleophilic water molecule (Rudack et al. 2015).

2.3 The role of Gln61

Gln61 is one of the most important residues for hydrolysis, and mutations of this residue are frequently associated with cancer. Its role is usually described as the positioning of the nucleophilic water molecule (Pai et al. 1990). Some theoretical investigations even found an imine isomerization within its side chain as an important chemical step within the mechanism (Polyakov and Nemukhin 2023) (see below).

2.4 The role of Gly12 and Gly13

Two other prominent oncogenic mutants are Gly12 and Gly13. As the reason for oncogenicity steric hinderance is usually given, as any mutant has a larger side chain compart to glycine (Krengel et al. 1990). However, biophysical data shows very similar ground state characteristics and the

main reason is probably an influence on the difficult to measure transition state (Scheffzek et al. 1997).

3 GTP hydrolysis by Ras

GTP hydrolysis is intrinsically slow due to a high-energy transition state, requiring an in-line nucleophilic attack by water on the phosphorous atom of γ-GTP, that is shielded by negatively charged oxygen atoms. The GTPase active site contains specific amino acids that stabilize charges, coordinate Mg^{2+} between β- and γ-GTP and position the attacking water molecule. Notably, a P-loop lysine and the arginine "finger" alter β- and γ-GTP charge distribution (Gasper and Wittinghofer 2019).

Time-resolved FTIR spectroscopy and X-ray crystallography have revealed Ras and Ras-GAP complex reaction mechanisms (Kötting et al. 2008; Pai et al. 1990; Scheffzek et al. 1997). During GTP hydrolysis, three rate constants were identified in the Ras-GAP complex via time-resolved FTIR difference spectroscopy (Figure 2). Difference spectra between the initial GTP state and reaction intermediates were calculated to isolate signals from specific protein and substrate groups in the active site, effectively removing background absorbance from non-contributing protein regions and solvent.

In FTIR experiments, Ras is loaded with caged GTP (cgGTP), which is photolyzed by a brief laser flash to initiate the GTPase reaction synchronously (Kötting et al. 2007). The initial conformational switch from "off" to "on" is detected by changes in the switch-I marker band (rate constant k_1). Subsequently, the arginine finger enters the catalytic site (k_2), facilitating GTP hydrolysis and formation of protein-bound $H_2PO_4^-$ (Kötting et al. 2006, 2008). In the final step (k_3), inorganic phosphate is released, switch-I reverts to the "off" state, and the arginine finger exits the binding pocket. These findings demonstrated that, unlike with transition state analogs, the arginine finger is not located within the GTP binding pocket in the ground state (Scheffzek et al. 1997), confirming X-ray structural models of Rho-RhoGAP complexes (Rittinger et al. 1997). The entry of the arginine finger (k_2) directly precedes bond cleavage, and Pi release is the rate-limiting step in Ras-catalyzed GTP hydrolysis.

Integration of FTIR data with QM/MM simulations has elucidated the structural and electronic properties of Ras-bound GTP (Rudack et al. 2012a). The close agreement between experimental and simulated IR spectra at the catalytic center validates the simulation approach (Rudack et al. 2012b), enabling detailed mechanistic insights. Comparison of GTP in solution, Ras-bound, and Ras–GAP-bound states reveals that protein binding induces key changes in GTP, including a shift in charge distribution – specifically, increased positive charge on the γ-phosphorus – and an increased β–γ phosphorus distance (Figure 3). The phosphate groups adopt an eclipsed conformation, introducing strain and driving GTP toward the transition state. These protein-induced alterations lower the activation barrier for hydrolysis, with the intrinsic binding energy of GTP compensating for the energetic cost of these conformational changes (Kötting and Gerwert 2004; Kötting et al. 2008). The

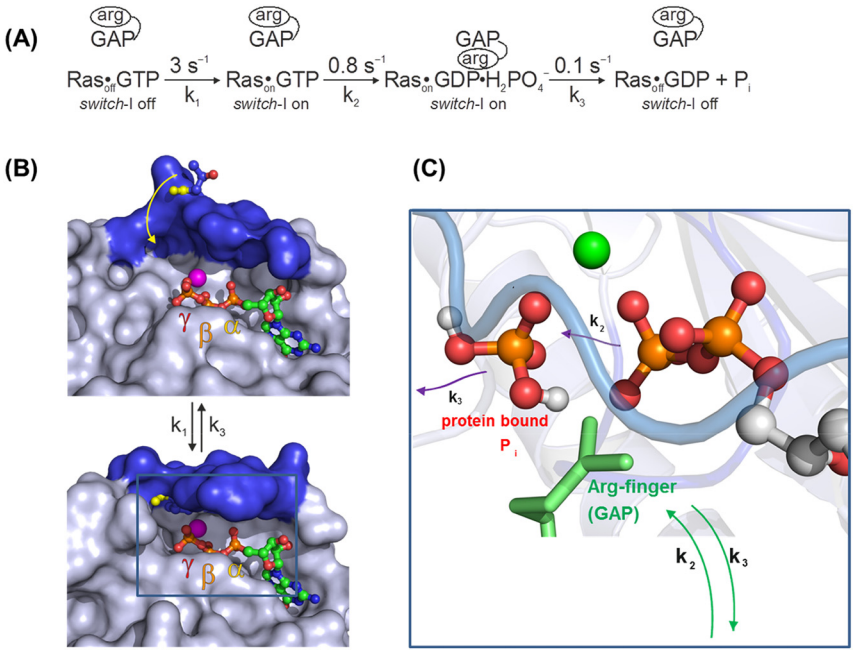

Figure 2: Kinetics of the Ras·Ras-GAP GTPase reaction. (A) Three apparent rate constants describe the reaction mechanism: k_1 describes the shift to the "on" conformation, k_2 describes the movement of the Arg-finger with immediate hydrolysis of GTP, and k_3 describes the release of P_i into the bulk solvent. (B) Structural changes of Ras within k_1, illustrating how the switch-I (dark blue) movement closes the nucleotide binding pocket and form the signal transducing "on" state. With the last apparent rate, k_3, the binding pocket opens again, and Ras returns into the "off" state. (C) With k_2 the Arg-finger (green) of Ras-GAP moves into the binding pocket and the bond connecting the γ- and β-phosphate is cleaved leading to the formation of a protein-bound $H_2PO_4^-$(P_i). Within k_3 which is the rate-limiting step, the $H_2PO_4^-$ is released from the protein into the bulk solvent (Gerwert et al. 2017).

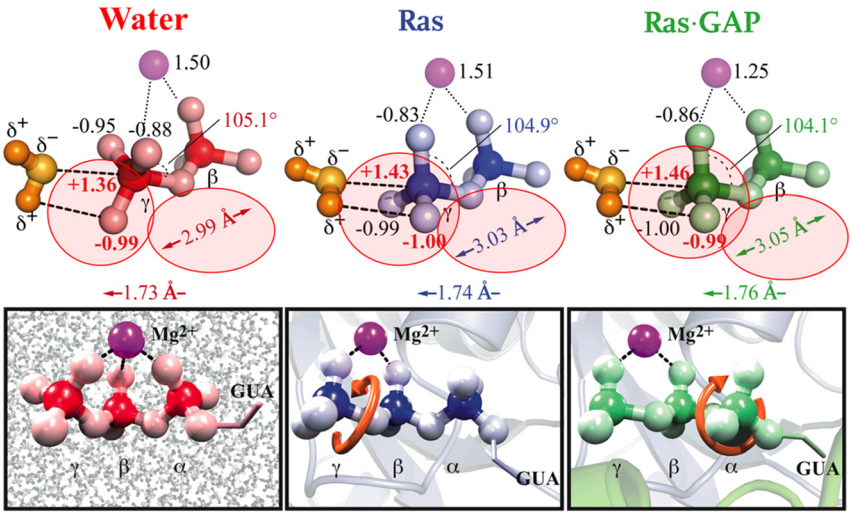

Figure 3: Environment dependent changes of the GTP hydrolysis reaction (Gerwert et al. 2017). Comparison of the detailed structures and charge distributions of GTP free in water (red), bound to Ras (blue), and in the Ras·Ras-GAP complex (green). The changes in the GTP environment lead to structural changes and charge shifts, bringing the conformation of the GTP educt state closer to that of the transition state. The shown results were obtained by combining experimental FTIR spectroscopy and theoretical IR spectroscopy (Gerwert et al. 2017; Rudack et al. 2012b).

combination of FTIR data with QM/MM simulations further enabled to obtain structural insights into the $H_2PO_4^-$-Guanosine diphosphate intermediate state of the Ras-GAP complex (Xia et al. 2012).

The hydrolysis mechanism itself has also been discussed for a long time. In the beginning there was one main question that Fred Wittinghofer and others always asked: Is the reaction associative or dissociative? (Wittinghofer 2006; Florian and Warshel 1998). Dissociative or associative means that either the bond between β- and γ-phosphate is cleaved first and then the new bond between the attacking water molecule and the cleaved group is formed, or *vice versa*. Later, instead of a fully dissociative or fully associative pathway, concerted pathways (Figure 4) were favored (Klähn et al. 2006) which was confirmed in recent quantum chemical calculations (Calixto et al. 2019). While the crucial role of Gln61 for positioning the nucleophilic water molecule is accepted in the field for a long time (Pai et al. 1990), glutamine tautomerization (Figure 4D) is a mechanism that is also discussed in some theoretical paper (Grigorenko et al. 2019; Pardos et al. 2025). In support of this theory, it can be argued that water positioning should also be possible with other side chains that form equally stable hydrogen bonds with water, but the glutamine is conserved in almost all GTPases. However, the majority of all publications do not favor this mechanism. An extensive computational study comparing the solvent assisted pathway without a general base, a general base catalyzed pathway, and the substrate assisted pathway (Figure 4) showed a good agreement of the calculated free energy with the experimentally measured one only for the solvent assisted pathway without a general base (Calixto et al. 2019). Another recent study brings into play a more important role of the Mg ion, which stabilizes an OH-, allowing the protonation of the γ-phosphate (Yan et al. 2025).

4 Drugs targeting the Ras protein

Despite enormous efforts, Ras has long been "undruggable" (Cox and Der 2025). The first direct Ras inhibitor AMG510 (Sotorasib) was approved by the FDA in 2021 (Skoulidis et al. 2021) more than 30 years after the structure was solved (Pai et al. 1989).

Since drugs that compete for binding to the nucleotide binding site fail due to the picomolar affinity for guanine nucleotides (John et al. 1990) and the flat Ras surface that lacks other binding pockets, the first attempts to target Ras were made by farnesyltransferase inhibitors. However, K-Ras and N-Ras were found to be substrates of geranylgeranyltransferase instead, restoring membrane localization (Brunsveld et al. 2006). Nevertheless, farnesyltransferase inhibitors are now in clinical trials for H-Ras (Witzig et al. 2024). A related approach is to block the prenyl-binding protein PDEδ that is important for the correct localization of K-Ras (Zimmermann et al. 2013).

A breakthrough was the discovery of a druggable pocket next to the switch II by the Shokat group and the covalent binding of an inhibitor to the Cys12 of the K-Ras G12C mutant (Ostrem et al. 2013). This was the starting point for the development of several inhibitors for Ras G12C and later non-covalently bound inhibitors for other oncogenic mutants such as MRTX1133 for G12D (Hallin et al. 2022). A covalent inhibitor for another mutant is G12Si-5, which binds to Ser12 of the G12S mutant by acylation (Zhang et al. 2022a). Other covalently bound small molecules have been found for

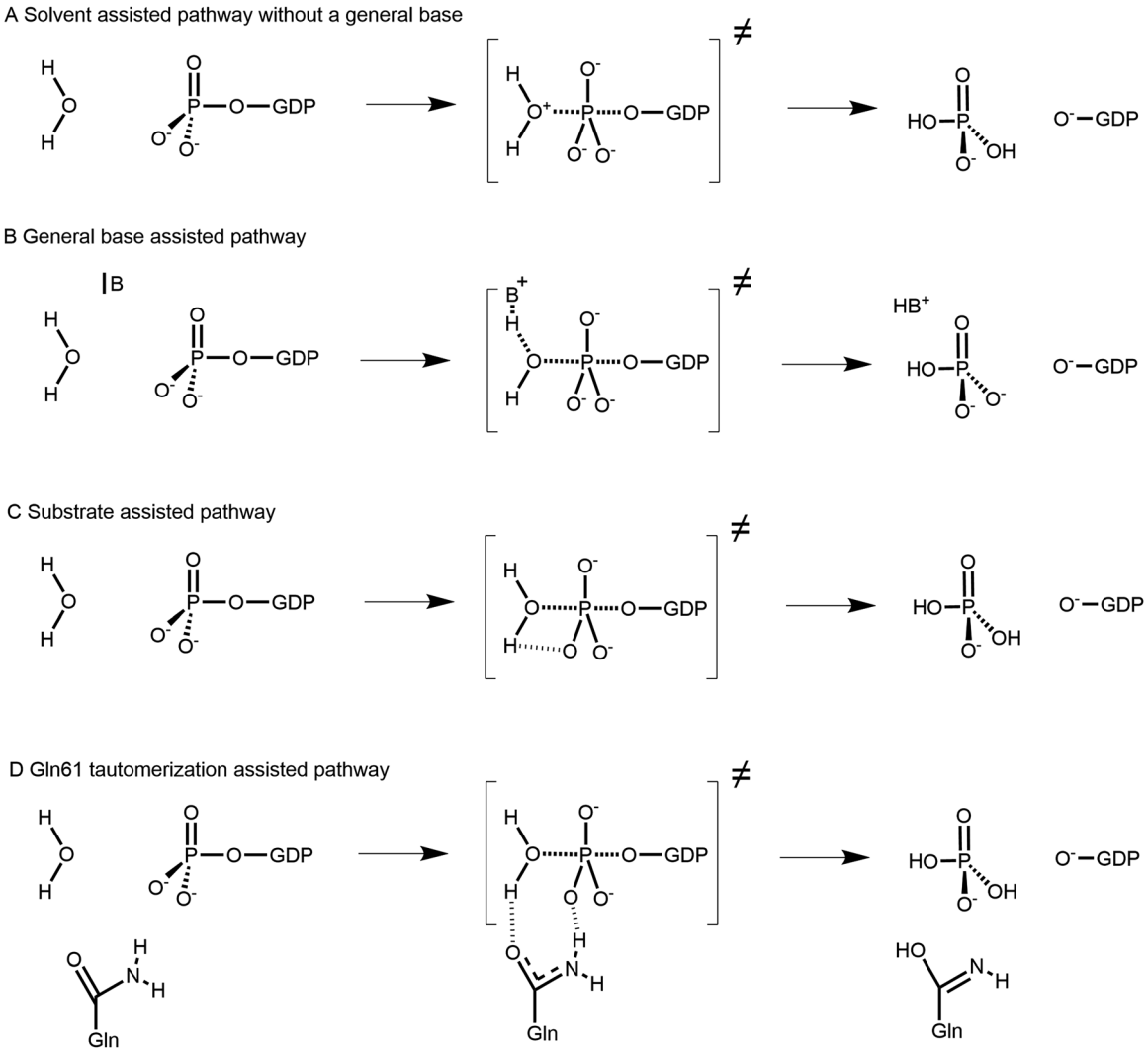

Figure 4: Possible reaction mechanisms for GTP hydrolysis (Calixto et al. 2019; Grigorenko et al. 2019). (A) Solvent assisted pathway with the nucleophilic attack as the first step. (B) General base assisted pathway. (C) Substrate assisted pathway with the substrate as the base. (D) Gln tautomerization assisted pathway with an imide intermediate which will eventually react back to the amine.

G12R (Zhang et al. 2022b). Another promising class of Ras inhibitors are "tricomplex" inhibitors, where the small molecule binds to cyclophilin A and then to Ras (Wasko et al. 2024). In this way, pan-Ras inhibitors can be developed. Further it was found that they can restore GTPase activity (Cuevas-Navarro et al. 2025). In addition, nucleotide-competitive inhibitors have been developed for G12C and G13C mutants that take advantage of covalent binding to cysteine (Goebel et al. 2023; Hunter et al. 2014). Several recent reviews extensively discuss the development of Ras inhibitors and their use in therapy (Cox and Der 2025; Moore et al. 2020; Nussinov and Jang 2024; Pandey et al. 2024; Yin et al. 2023).

In conclusion, the intensive mechanistic and structural insights gained over many years by Fred Wittinghofer and others, have provided a deep understanding of Ras-catalyzed GTP hydrolysis, and opened up new avenues for the development of targeted therapeutic strategies against Ras-associated diseases.

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