Neutron Crystal Structure of RAS GTPase Puts in Question the Protonation State of the GTP γ-Phosphate

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Background: The GTP nucleotide is thought to be fully deprotonated when bound to RAS.
Results: The neutron crystal structure of RAS bound to the GTP analogue GppNHp shows a protonated γ-phosphate.
Conclusion: The active site of RAS significantly increases the pKα of the nucleotide.
Significance: This work provides insight to the GTP hydrolysis mechanism, with implications to the superfamily of small GTPases.

RAS GTPase is a prototype for nucleotide-binding proteins that function by cycling between GTP and GDP, with hydrogen atoms playing an important role in the GTP hydrolysis mechanism. It is one of the most well studied proteins in the superfamily of small GTPases, which has representatives in a wide range of cellular functions. These proteins share a GTP-binding pocket with highly conserved motifs that promote hydrolysis to GDP. The neutron crystal structure of RAS presented here strongly supports a protonated γ-phosphate at physiological pH. This counters the notion that the phosphate groups of GTP are fully deprotonated at the start of the hydrolysis reaction, which has colored the interpretation of experimental and computational data in studies of the hydrolysis mechanism. The neutron crystal structure presented here puts in question our understanding of the pre-catalytic state associated with the hydrolysis reaction central to the function of RAS and other GTPases.

RAS GTPase is at the hub of numerous signaling pathways that regulate cell proliferation, differentiation, survival, and apoptosis (1) and is mutated in about 20% of all human cancers (2). RAS is regulated by guanine nucleotide exchange factors that promote binding of GTP to turn the signal on and GTPase-activating proteins (GAPs) that enhance hydrolysis of GTP to GDP to turn the signal off (3) as a result of large conformational changes in switch I (residues 30–40) and switch II (residues 60–76) (4, 5). In addition, it has been proposed that ligand binding at an allosteric site orders switch II to activate intrinsic hydrolysis in the presence of the effector RAF (6, 7), supporting early indications that intrinsic hydrolysis plays a biologically relevant role in the regulation of RAS (8–10). Whether the focus is intrinsic or GAP-catalyzed hydrolysis, understanding the role of RAS in catalysis is of paramount importance as we seek in-depth knowledge of how this protein works, with broad implications for the GTPase superfamily (11).

The catalytic domain of RAS is composed of a six-stranded β-sheet flanked by five α-helices and 10 connecting loops (Fig. 1A). It contains a guanine nucleotide-binding pocket with elements that are highly conserved in all GTPases (Fig. 1B) (12, 13) and that are essential in creating an environment for the GTP in the ground state in which its charge distribution and bond lengths are shifted toward those expected in the transition state of the reaction (14, 15). The conserved G-domain elements that interact intimately with the nucleotide or are involved in positioning directly interacting residues include the P-loop residues 10–17 with sequence GXXXXGK(S/T) (X residues vary, but their main chain atoms interact consistently across the superfamily with the α- and β-phosphates of GTP), residue Thr-35 in switch I and residues 57–60 of the DXXG motif, which are essential for interaction with the phosphate groups or the Mg2+ ion, Phe-28, residues 116–119 of the NKXD motif, and residues 143–147 of the EXSAK motif that are critical for binding and specificity toward the guanosine moiety.

In the context of this active site, the mechanism through which RAS catalyzes GTP hydrolysis has been a subject of debate for over 20 years, and despite the publication of several proposed mechanisms, the details remain unresolved, in part due to an incomplete picture of the active site that lacks hydrogen atom positions in the pre-catalytic state (6, 16–21). The reaction involves the nucleophilic attack of a water molecule on the γ-phosphate of GTP, with the GDP leaving group remaining bound to the enzyme and inorganic phosphate released into the solvent. It proceeds via a concerted mechanism with dissociative character and a metaphosphate-like intermediate for both intrinsic and GAP-catalyzed hydrolysis (22, 23). Given the highly conserved guanine nucleotide-binding motifs described above and the common dissociative character of the reaction in the absence and presence of GAPs, there are likely to be common characteristics of the pre-catalytic state in both reactions, and indeed associated with GTP hydrolysis across the super-
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FIGURE 1. Conserved motifs in the nucleotide-binding pocket of RAS bound to GppNHp. A, overall structure of RAS (yellow) showing its architectural features and the nucleotide-binding pocket near switch I and switch II. The nucleotide-binding motifs are colored as described below. B, nucleotide-binding pocket highlighting the motifs conserved in the superfamily of GTPases. In both A and B the P-loop or GXXXXGKS (S/T) motif is in mauve (residues 10–17 in RAS, GAGGVGKS), the DXDG motif is in purple (residues 57–60 in RAS, DTAG), the NKID motif is in teal (residues 116–119 in RAS,NKCD), and the EXSAK motif is in gray (residues 143–147 in RAS, ETSAK). Conserved switch I residues Phe-28 and Thr-35 are in light gray. The side chain H-bonding interactions within 3.2 Å as well as interactions with the Mg$^{2+}$ ion are shown by black dashed lines.

family, with specific variation in the details for each one. Hydrogen positions and protonation states, resulting from charge rearrangements and effects on the $pK_a$ value of individual ionizable groups due to binding RAS, are expected to be common features in these various situations.

All proposed mechanisms to date assume that the GTP phosphate groups are fully deprotonated on the enzyme (total charge of $-4$), as they are expected to be in solution at physiological pH ($7.0–7.2$) in the presence of magnesium (24). Detecting a possible change in $pK_a$ of any of the GTP phosphate groups on the enzyme is a challenge due to the fact that hydrogen atoms in biological systems are difficult to visualize even in high resolution x-ray crystal structures. To date, chemical intuition and indirect methods have been used to infer the presence and action of hydrogen atoms in the RAS active site. For example, the $pK_a$ value for the $\gamma$-phosphate of GTP bound to RAS has been determined to be close to 3 from a study of the reaction rate as a function of pH (21). The authors showed that they could detect NMR chemical shifts for the three phosphate groups of GTP at around pH 3, consistent with a change in the GTP environment, presumably due to the change in protonation state of the $\gamma$-phosphate. Results of this same set of experiments with oncogenic mutants indicated a clear linear free energy relationship of the reaction rate as a function of the $pK_a$ as determined either by NMR or by measuring the pH dependence of the reaction rate over a large pH range (21, 25, 26).

These studies assume that the changes in reaction rates or chemical shifts with decreasing pH are directly due to protonation of the terminal phosphate group of GTP. However, there are several aspartic and glutamic acid residues near the active site, with two aspartic acid residues in the conserved guanine nucleotide-binding pocket (Asp-57 and Asp-119), that if protonated could have a significant effect on both the reaction rate and chemical shifts of the GTP phosphate groups.

The assumption that the $\gamma$-phosphate in RAS-GTP is doubly deprotonated at physiological pH also had support from FTIR spectroscopy, where the vibrational bands associated with the $\gamma$-phosphate are determined by isotopic difference spectra (27). The shift in the FTIR spectra in going from GTP in solution to GTP on RAS is small, and this was taken as support for a dianionic state for the $\gamma$-phosphate on GTP-bound RAS at pH 7.5 (24). Once this assumption was accepted, a deprotonated GTP became the starting point for quantum mechanical/classical mechanical (QM/MM) calculations and was used in the interpretation of subsequent FTIR spectra of GTP on RAS. Intriguingly, QM/MM based on the deprotonated state predicts correctly all but one of the RAS-induced shifts in the FTIR spectra associated with the $\gamma$-phosphate group of the nucleotide (14).

NMR spectroscopy has been used to obtain the $pK_a$ values of the $\alpha$, $\beta$, and $\gamma$-phosphates of both GTP and its non-hydropolyzable analogue GppNHp (28, 29). Although the results were straightforward for the nucleotides in the absence and presence of Mg$^{2+}$ in solution, the authors point out that the estimated $pK_a$ value for the nucleotide when bound to RAS is accompanied by large errors (28).

Neutron crystallography is a method that does allow direct visualization of protonation states in protein-active sites (30). It differs from x-ray crystallography in that neutrons interact with atomic nuclei rather than with electrons, giving rise to strong signals associated with electron-poor hydrogen or deuterium atoms, with deuterium showing up particularly well in the positive neutron density maps (31). Here, we present the neutron crystal structure of RAS bound to GppNHp and Mg$^{2+}$ with well defined deuterium positions, and we show that the $\gamma$-phosphate is protonated at pH 8.4, a relatively high pH that compensates for the fact that GppNHp-Mg$^{2+}$ has a higher $pK_a$ value than GTP-Mg$^{2+}$ in solution (28). This result puts in question the protonation state of the $\gamma$-phosphate of GTP, warranting a re-evaluation of the pre-catalytic state of GTP hydrolysis on RAS with focus on a protonated $\gamma$-phosphate (total GTP charge of $-3$) at physiological pH. This has the potential to change long-lasting assumptions and to transform the context of our understanding of GTP hydrolysis on RAS. Given the highly conserved nature of the nucleotide-binding pocket, understanding the consequences of GTP binding to the G-domain of RAS has broad implications for the entire GTPase superfamily, which includes proteins that together have a global impact on cellular function (3).
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Experimental Procedures

Protein Expression, Purification, and Crystallization—Hydrogenated HRAS, residues 1–166 (referred to here as RAS), was expressed in Escherichia coli and purified, and the bound nucleotide was exchanged for GppNHp using previously reported methods (6, 32). Purified hydrogenated RAS-GppNHp was concentrated to 15–18 mg/ml in 20 mM HEPES (pH 7.5), 50 mM NaCl, 5 mM MgCl₂, and 1 mM DTT, flash-frozen in 50-µl aliquots, and stored for later use at −80 °C.

RAS was crystallized with the sitting drop method in 9-well glass plates set up in a sandwich box from Hampton Research, Inc. (Aliso Viejo, CA). The reservoir solution was prepared by mixing 25 ml of a solution containing 200 mM calcium acetate, 20% w/v PEG 3350, and 0.1% w/v n-octyl-β-D-glucoside, 7.5 ml of the protein stabilization buffer (same as storage buffer above), and 2.5 ml of 50% w/v PEG 3350. Crystallization drops with a total volume of 50 and 100 µl containing a 1:1 protein to reservoir solution ratio were set at 18 °C and left undisturbed for at least 1 month. Large three-dimensional crystals (>1.0 mm in the longest dimension) were subjected to a deuterium exchange protocol to replace exchangeable hydrogen atoms in the crystal for deuterium atoms. The deuterium exchange was performed using vapor diffusion, where the hydrogenated reservoir solution was replaced with an identical solution made with deuterated water and calibrated to a reading of 8.0 on the pH meter glass electrodes, which when corrected for the deuterium effect corresponds to a pD of 8.4 (see conversion details below) (33). The deuterated reservoir solution was replaced with an identical fresh solution every 2 weeks during a period of 2 months. Crystals were harvested into quartz capillaries with a small amount of mother liquor surrounding, but not touching, the crystal on either end. The capillaries were plugged on both ends and sealed using wax for storage and shipment to Oak Ridge National Laboratory. During the shipment, the crystal on which the data were collected for the present structure was immersed in the deuterated buffer in the capillary and thus soaked directly in the buffer for about 1 week before it was remounted properly for data collection.

Determination of pD from Measurements Using the Glass Electrodes on the pH Meter—An accumet XL benchtop pH meter was used for determining the pH or pD of all buffers used in the experiments reported here. The glass electrodes were calibrated for pH measurements in H₂O such that readings in D₂O yielded apparent pH values, pH*, that do not correspond directly to the pD. The conversion to pD was worked out in the 1950s as pD = pH* + 0.4 (34), and refined in the early 1960s to pD = pH* + 0.44 (35). A more recent study that included a large number of ionizable compounds of diverse types concluded that the most accurate conversion factor is pD = pH* + 0.45 and stated that the pKₐ values measured from pH* titration using a glass electrode in deuterated medium can be converted into pKₐ values by the addition of 0.4 (or 0.45) factor (36). We simply used a conversion factor of +0.4. In this study the deuterated buffer in which the RAS crystal was soaked prior to data collection had a glass electrode reading of pH* = 8.0 and thus a pD of 8.4. The pKₐ values obtained for GppNHp-Mg²⁺ and GTP-Mg²⁺ by NMR were reported as 6.3 and 4.7, respectively, as measured directly with the glass electrodes in deuterated medium (28) and thus correspond to the uncorrected pKₐ values, which we converted to the true pKₐ values of 6.7 and 5.1 by the addition of 0.4 as stated above.

Data Collection and Structure Refinement—Quasi-Laue data were collected on the IMAGINE beam line (37) at the High Flux Isotope Reactor at Oak Ridge National Laboratory using a narrow bandpass 3.3–4.5 Å optical configuration (λₘₐₜ = 3.9 Å, dλ/λ = 30%) that delivers 4.6 × 10⁶ n cm⁻² s⁻¹ at the sample position. Data collection was performed at 293 K. Data extended to 1.9 Å resolution with 24 h of exposure. The neutron data were collected from three crystal orientations to help fill the blind region and therefore increase completeness. A total of 18 images were collected with the goniometer arcs adjusted to Rₚ = Rₛ = −20° (nine images collected in φ steps of 7°) and Rₚ = Rₛ = +20° (nine images collected in φ steps of 10°). The capillary was then re-oriented by −80° using a custom-made setup mounted on the goniometer head. An additional five images were collected in this orientation with 14° step intervals. Laue images were measured, indexed, and integrated using the LAUEGEN suite of programs from CCP4 (38, 39); wavelength was normalized to account for the spectral distribution of the quasi-Laue beam using LSSCALE (40) and then scaled and merged using SCALA (41).

An x-ray data set was collected at room temperature on a smaller crystal grown under the same conditions and mounted in a quartz capillary. The resulting structure was used as a starting model for refinement with the neutron data set. The x-ray data were collected on a Rigaku HomeFlux x-ray setup equipped with a MicroMax-007 HF x-ray generator, Osmic VarioMax optics, and an R-AXIS IV+ image plate detector. The diffraction data were indexed, integrated, and scaled using the HKL-3000 software suite (42). The x-ray crystal structure was solved to a resolution of 1.7 Å using phenix.refine in the PHENIX suite of programs (43), and manual refinement was performed using COOT (44).

Molecular replacement was performed using PHENIX with the protein model obtained from the x-ray crystal structure (no water molecules or ligands) and the neutron data set. The structure was then subjected to a round of rigid body refinement using the neutron scattering table in PHENIX. At this point, side chain orientations were manually altered in COOT to fit the nuclear density. The resulting PDB file was then run with phenix.readyset prior to the next round of refinement, in which occupancies were refined. Deuterium water molecules using phenix.readyset prior to the next round of refinement, in which occupancies were refined. Deuterium atoms for the nucleotide were added using phenix.ELBOW. Subsequent refinements involved adding/removing water molecules and determining which water molecules showed density...
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FIGURE 2. Nucleotide GppNHp and the magnesium ion in their respective binding sites on RAS. The neutron structure is shown in yellow with nuclear density in blue. The x-ray structure is in pink with electron density in gray. In the neutron crystal structure, hydrogen atoms are shown in light green and deuterium atoms in light green. No hydrogen atoms are shown in the x-ray crystal structure. A, GppNHp from the neutron crystal structure with $2F_o - F_c$ nuclear density contoured at the 1σ level. Note the presence of robust density at deuterium positions and breaks in the density for hydrogen atoms. B, GppNHp from the x-ray crystal structure with $2F_o - F_c$ electron density contoured at the 1σ level. The GppNHp molecule from the neutron structure (yellow) is superimposed on that of the x-ray structure (pink). For clarity, density is shown only for the nucleotide in A and B. C, Mg$^{2+}$ ion (green) with its coordination sphere on RAS and nuclear density contoured at the 1.5σ level. D, Mg$^{2+}$ ion (green) with its coordination sphere on RAS and electron density contoured at the 1.5σ level. H-bonding and electrostatic interactions within 3.2 Å are shown by black dashed lines.

for the full D$_2$O by inspecting $2F_o - F_c$ electron density map for “boomerang” density, indicative of D$_2$O. Those that were spherical were modeled as just an “O” atom. D-OMIT maps (31) were generated to check the orientation of specific water molecules and the position of deuterium atoms associated with the nucleotide and side chains of interest. The x-ray model was used originally for phasing, whereas the refinement was based solely on neutron data. Thus, the x-ray structure factors were not used at any point for joint refinement of the structure.

Results

Neutron crystallography is one of the very few methods available that allow direct visualization of hydrogen positions in proteins for sites in which hydrogen can be exchanged for deuterium (37). However, although the study of proteins using neutrons is not new, the method has produced relatively few structures due to serious challenges associated with the inherent weakness of neutron beams currently available at neutron sources worldwide. This has limited the feasibility to proteins that produce very large crystals (in the order of 1 mm$^3$) and with a relatively small unit cell (37). We solved the neutron crystal structure of RAS bound to the GTP analogue GppNHp at room temperature and pH 8.4 to a resolution of 1.9 Å. The use of a GTP analogue such as GppNHp is necessary for visualization of hydrogen positions associated with the active site of RAS in its GTP-bound state because GTP is hydrolyzed to GDP in the time frame of weeks to months that it takes for crystal growth and data collection.

Fig. 2 shows the nucleotide analogue and the magnesium ion in their respective binding sites, with nuclear density and electron density from the neutron and x-ray diffraction data, respectively. Fig. 2A shows the nuclear density for the nucleotide. Note that there are breaks in the density at hydrogen positions due to the negative scattering factor and incoherent scattering of hydrogen (30), whereas the deuterium atoms at exchangeable positions are clearly visible in the $2F_o - F_c$ nuclear density map. Fig. 2B shows the superposition of the nucleotide in the two structures, with electron density from the x-ray crystal structure. The overall main-chain root mean square deviation is 0.177 Å, with most protein and nucleotide atoms very well superimposed. There is a small rotation of the γ-phosphate and closer positioning of the nucleophilic water molecule to the nucleotide in the neutron structure. These small differences are within the error in the models and are often seen between two independently solved x-ray crystal structures of RAS. Fig. 2, C and D, shows the Mg$^{2+}$ coordination sphere obtained from the neutron and crystal structures respectively. Note that there is no nuclear density for the Mg$^{2+}$ in Fig. 2C, as the coherent neutron scattering length of a single magnesium ion is weak (45). Nevertheless, the Mg$^{2+}$ position is known from the room temperature x-ray structure determined from a crystal grown under identical conditions (Fig. 2D). The Mg$^{2+}$ ion is included in the model derived from the neutron data, because it can be inferred to be there from its clearly defined octahedral coordination sphere.

The crystal used for data collection had a volume of 0.7 mm$^3$ with symmetry of the space group $P3_21$. The structure pushes the limits of neutron crystallography both in terms of its high resolution, allowing refinement in the absence of combined x-ray data (46, 47), and in the fact that the crystal form has a relatively large unit cell (Table 1). For this, we used the state of
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The crystal form with P3\(_2\)2\(_1\) symmetry is common among x-ray crystal structures of RAS, used originally to describe the nucleotide-binding site in detail and to formulate hypotheses for the GTP hydrolysis reaction (20). In our neutron crystal structure, the nuclear density in the active site shows the GppNHp with clear placement of deuterium atoms at exchangeable hydrogen positions. This structure provides a view of the protonation states of the α-, β-, and γ-phosphates of the nucleotide at a pH significantly higher than physiological pH and pK\(_\text{a} \text{-}\), values of the γ-phosphate for either GppNHp (6.7) or GTP (5.1) obtained by NMR spectroscopy in solution and corrected for the deuteration effect (see “Experimental Procedures”) (28). It also shows the protonation states of the amino acid residues in the P-loop and switch I that interact closely with the phosphate groups and with the bound Mg\(^{2+}\) ion, and of the well ordered water molecules that coordinate the Mg\(^{2+}\) ion. The protonation states of all residues in the conserved G-domain motifs are clear. Although switch II residues 61–66 are mostly disordered, the main chain atoms of Gly-61 are seen in the nuclear density maps, as is the nucleophilic water molecule.

The γ-phosphate of the nucleotide is located at the interface of the P-loop, switch I, and switch II where catalytic residues are found. Nuclear density associated with the γ-phosphate of GppNHp is shown in Fig. 3A. One of the three terminal oxygen atoms of the γ-phosphate (O1G in the model deposited in the Protein Data Bank) accepts an H-bond from the P-loop residue Gly-60 and Ala-61, stabilizing the initial portion of the P-loop; and the third is donated to one of the β-phosphate oxygen atoms. A second terminal oxygen atom of the γ-phosphate (O2G) simultaneously coordinates the Mg\(^{2+}\) ion and accepts an H-bond from the main chain amide of switch I residue Thr-35 (Fig. 3A). The O3G and O2G oxygen atoms of the γ-phosphate are deprotonated as expected. Remarkably, the third oxygen atom (O1G in the model) is protonated at pD 8.4, 1.7 pD units higher than the pK\(_D\) for GppNHp in the presence of Mg\(^{2+}\) in solution (Fig. 3A). The deuterium atom covalently bound to the O1G oxygen is clearly visible and directed away from the nucleophilic water molecule. Tyr-32 from a symmetry-related molecule in the crystal (Tyr-32sym) reaches into the active site with its hydroxyl group donating an H-bond to O1G. The deuterium on O1G is rotated away from Tyr-32sym, not interacting with any other group. Despite the H-bonding interaction with Tyr-32sym, the O1G atom of the γ-phosphate is not buried by the crystal contact but rather remains well exposed to solvent. On the other side of the hydroxyl group of Tyr-32sym is the GppNHp NH group that bridges the β- and γ-phosphates. Although the nitrogen atom of this group is 3.4 Å from the main chain amide nitrogen of Gly-13, the deuterium atoms associated with the amide nitrogen atoms are not directed toward each other, minimizing what is clearly an unfavorable interaction. In the case of GTP, the Gly-13 amide group is expected to donate an H-bond to the bridging oxygen atom, stabilizing the negative charge that accumulates there during the transition state of the reaction (48).

The nucleophilic water molecule is positioned with its oxygen atom within H-bonding distance from O1G (2.7 Å), the main chain carbonyl oxygen atom of Thr-35 (2.8 Å), the backbone amide of Gly-60 (2.8 Å) and a nearby water molecule Wat-433 (2.5 Å) (Fig. 3A). The amide of Gln-61 is 3.9 Å away from the nucleophilic water in this room temperature structure. Despite the proximity to these four protein atoms with which it could engage in H-bonding interactions, the deuterium atoms associated with the nucleophilic water molecule are not visible in the nuclear density maps. There is no substantial nuclear density to support a strong H-bond between this water and the γ-phosphate oxygen atom or with the nearby main chain atoms of Thr-35, Gly-60, and Gln-61. Instead, the nucleophilic water molecule most likely engages in weaker interactions with all of these atoms while maintaining entropically favorable rotational freedom. Such loose interactions in the ground state for the nucleophilic water molecule are expected to facilitate its movement toward the γ-phosphorus atom as the reaction progresses toward the transition state. In fact the nucleophilic water molecule in this structure is only 3.0 Å from the γ-phosphorus atom, aligned with it more closely than previously observed, and a second water molecule is positioned within H-bonding distance of the main chain carbonyl oxygen atom of Thr-35 (Fig. 3A).

The Mg\(^{2+}\) ion is tightly coordinated by the γ- and β-phosphate atoms of the nucleotide (O2G and O2B in the model), the side chain oxygen atoms of Ser-17 and Thr-35, and two well ordered water molecules, whose deuterium atoms are clearly visible in the nuclear density maps (Fig. 4A). Wat-172 in the neutron model donates H-bonds to the main chain carbonyl of switch I residue Asp-33 and to one of the α-phosphate oxygen

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**TABLE 1**

| Data collection and refinement statistics for the neutron structure of RAS-GppNHp |
|---|
| **Space group** | P3\(_2\)2\(_1\) |
| **Unit cell dimensions** | |
| a, b, c (Å) and α, β, γ (°) | 40.1, 40.1, 160.8, 90, 90, 120 |
| **Resolution range (Å)** | 17.0–1.9 (1.98–1.91) |
| **Total reflections** | 17,490 (993) |
| **Unique reflections** | 9888 (655) |
| **Multiplicity** | 6.2 (3.0) |
| **Completeness (%)** | 82.1 (60.4) |
| **Mean I/σ(I)** | 5.6 (2.1) |
| **R-pim** | 0.093 (0.202) |
| **R-work** | 0.2453 (0.3325) |
| **R-free** | 0.2870 (0.4134) |
| **No. of non-hydrogen atoms** | 1315 |
| **Protein** | 2443 |
| **Ligands** | 47 |
| **Water molecules** | 75 |
| **Protein residues** | 162 |
| **Root mean square bonds (Å)** | 0.006 |
| **Root mean square angles (°)** | 0.9 |
| **Ramachandran favored (%)** | 98 |
| **Ramachandran allowed (%)** | 2 |
| **Ramachandran outliers (%)** | 0 |
| **Clashscore** | 2.01 |
| **Average B-factor (Å\(^2\))** | 18.7 |
| **Protein** | 18.4 |
| **Ligands** | 13.3 |
| **Solvent** | 24.9 |
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FIGURE 3. γ-Phosphate of GppNHP and its interactions with RAS. The $2F_o - F_c$ nuclear density map contoured at the 1.5σ level is shown in blue. A, protonated γ-phosphate and nucleophilic water molecule. Note the clear density supporting the deuterium atom on the γ-phosphate group, whereas density is only present for the oxygen atoms of the nucleophilic water molecule 175 and its close neighbor water molecule 433. B, Lys-16 interacts with both the γ- and β-phosphate groups of GppNHP and stabilizes the P-loop through its interactions with the carbonyl groups of Gly-10 and Ala-11. Atoms are color-coded in this and all subsequent figures containing models from the neutron diffraction data as follows: carbon, yellow; nitrogen, blue; oxygen, red; phosphorus, orange; hydrogen, light gray; deuterium, light green; and Mg$^{2+}$, green. H-bonding interactions within 3.2 Å as well as the interaction with the Mg$^{2+}$ ion are shown by black dashed lines.

atoms (O2A) of GppNHP (Fig. 4A), whereas Wat-173 donates H-bonds to the side chain of Asp-57 (the Asp residue in the conserved DXXG motif) and to the main chain carbonyl of Thr-58 (Fig. 4B). Thr-35 and Ser-17 donate H-bonds to Wat-172 and the side chain of Asp-57, respectively, thus becoming polarized to coordinate the Mg$^{2+}$ ion. Note that the side chain of Asp-57 is critical in accepting H-bonds from Wat-173 and the hydroxyl group of Ser-17, both of which coordinate the magnesium ion, explaining the highly conserved nature of this residue in the GTPase superfamily. Its protonation could very well explain the decrease in reaction rate at around pH 3 in pH titration experiments that concluded this to be the pK$_a$ of the γ-phosphate of GTP (21). A protonated Asp-57 would be expected to impair the Mg$^{2+}$ coordination sphere, resulting in the observed decrease in reaction rate. Impaired Mg$^{2+}$ binding would in turn affect the chemical shifts of the three phosphate groups as observed in the NMR experiments (21, 25).

The P-loop (residues 10–17), in addition to containing Lys-16 with a side chain that interacts with the γ-phosphate of the nucleotide as described above, provides a ring of amides that wraps around the α- and β-phosphates from residues 13 to 18 (Fig. 5). The amide hydrogen atoms of residues 15–18 that interact directly with the phosphate oxygen atoms have retained bound hydrogen atoms invisible in the positive nuclear density maps (Fig. 5A), whereas those that have been exchanged for deuterium in residues 13 and 14 are visible (Fig. 5B). Here, the fact that we soaked the crystal in deuterated buffer to allow for exchange of hydrogen for deuterium, rather than collecting data on the perdeuterated protein, provides information on the degree to which the P-loop amide ring interacts with the phosphate groups of GTP (49). The lack of exchange to deuterium, as indicated by the lack of nuclear density at the hydrogen positions, and the short distances between these amide groups and the nucleotide phosphate oxygen atoms indicate very strong H-bonding interactions.

A schematic drawing of the interactions between the rest of the nucleotide and the protein is shown in Fig. 6, with the details derived from the nuclear density associated with deuterium. Although the P-loop, switch I, and the DXXG motif bind the phosphate groups and the Mg$^{2+}$ ion, the NKXD and the EXSAX motifs come together in the structure to bind the guanosine group (Fig. 1). The guanine base and ribose moieties are protonated as expected, with deuterium atoms visible for the hydroxyl groups of the ribose and for the N1 and N2 atoms of the guanine base. These two nitrogen atoms of the guanine base donate H-bonds to Asp-119 of the NKXD motif (residues 116–119) as expected, and this interaction provides high specificity for the guanine nucleotide (20). It was previously assumed from X-ray crystal structures that the conserved Lys-117 donates an H-bond to the ribose ring oxygen atom, as its amino group is 3.4 Å from this oxygen atom (20). The neutron structure, however, shows nuclear density for the deuterium atoms associated with the Lys-117 amino group (Fig. 5B), none of which are directed to the ribose ring. Instead, Lys-117 donates H-bonds to the main chain carbonyl of Gly-13 in the P-loop (2.7 Å), whose main chain amide is likely important for catalysis (48), and to a water molecule (2.8 Å), through which it is connected to Asn-85 (not shown in Fig. 5B). We have previously studied the N85A mutant and showed that this mutation has a severe impact on hydrolysis and nucleotide binding (32). This points to the importance of Asn-85 and the associated water molecule for stabilizing Lys-117 in the nucleotide-binding pocket, as its aliphatic portion makes extensive stacking interactions with the guanine base. The side chain amide of Asn-116 of the NKXD motif donates an H-bond to the main chain carbonyl group of Val-14, and thus Asn-116 together with residue Lys-117 provide stabilization for the P-loop (Fig. 5B). Two consecutive side chains in the NKXD motif interact with two consecutive main chain carbonyl groups in the P-loop, providing additional rationale for the highly conserved nature of these residues.

On the opposite side of the guanine base from Lys-117 is Phe-28 positioned perpendicular to the aromatic ring of the nucleotide and stacking in van der Waals interaction with Lys-147, the last residue in the conserved EXSAX motif. The side chain of Ala-146 is in a tight pocket with no room for any larger side chain and is probably conserved due to its unique fit in this pocket and the fact that a glycine at this position would confer undesirable flexibility to the nucleotide-binding pocket. Its main chain amide is 2.9 Å from the O6 of the guanine base, and the fact that its hydrogen was not exchanged for deuterium in our structure is indicative of a strong H-bond interaction. The hydroxyl group of Ser-145 is deuterated and donates an H-bond to the side chain of Asp-119 (the specificity residue in the
NKXD motif), helping position it for interaction with the guanine base. The side chain of the adjacent Thr-144 donates an H-bond to Asn-116, also in the NKXD motif (Fig. 5B). Recall that Asn-116 interacts directly with the P-loop, so here we have a direct network between three of the highly conserved motifs in the nucleotide-binding pocket (Thr-144 in the EXS/AK motif, Asn-116 in the NKXD motif, and Val-14 in the P-loop) (Fig. 5B). Residue Glu-143 is relatively far from the nucleotide-binding site, and although it is highly conserved across the superfamily, its specific function has not been discussed in the literature.

**Discussion**

In addition to providing insight into an important and prominent reaction in biology, the neutron crystal structure of RAS represents a technical achievement with respect to neutron diffraction as a method for visualization of hydrogen in enzyme-active sites. Unlike x-rays that interact with electrons, leaving electron-poor hydrogen atoms invisible in the electron density maps, neutrons interact with the nuclei of atoms, yielding strong coherent scattering for deuterium, with scattering length comparable with that for carbon, oxygen, and nitrogen atoms (30). Although the last 10 years have brought remarkable technical advances, including perdeuteration (51), the possibility of low temperature data collection (52), and more powerful beams and detectors as exemplified by the IMAGINE beam line at the Oak Ridge National Laboratory (37), the unit cell length continues to be a severe limitation in studying proteins that crystallize with large unit cells. As a result, the majority of the 83 neutron crystal structures currently present in the Protein Data Bank (PDB) (corresponding to 32 unique proteins) have the longest unit cell length less than 100 Å and feature proteins that are relatively rigid in structure, able to form crystals with low mosaicity or irregularities. This is due to the following: 1) the fact that spot overlap becomes a serious problem in diffraction from crystals with long unit cell dimensions in the Laue or quasi-Laue geometry on instruments with fixed crystal-to-detector distances, and 2) the requirement for larger crystals in cases of large unit cell volume systems (53). Only six neutron protein crystal structures are currently available from crystals with one of the unit cell lengths greater than 100 Å (Table 2). All six were
solved in the last 5 years as advances in technology have allowed increasingly challenging structures to be solved with neutron diffraction data (50). RAS represents a protein of intense interest due to its central role in signal transduction associated with disease (2). It also represents a quantum leap from what has so far been achieved in neutron crystallography by the fact that its crystals have the longest unit cell length, $c = 160.8$ Å. Despite the long unit cell $c$ axis length, the RAS neutron data set has an overall completion of 82.1% and redundancy of 6.2, with the highest resolution shell being 60.4% complete with a multiplicity of 3.0, allowing remarkable clarity in the visualization of deuterium atoms in the structure at 1.9 Å resolution. This achievement was made possible by the low density of reflections along the other two axes, $a = b = 40.1$ Å, and the tunability of the IMAGINE beam line (37). With this neutron crystal structure of RAS, we push forward into the realm of signaling molecules, revealing hydrogen positions in the active site and water-mediated H-bonding networks across the structure at an unprecedented detail. The quality of the structure is remarkable, given that RAS is a dynamic protein with multiple sites of protein-protein or protein-membrane interactions (54). Its presence in the PDB extends possibilities for a group of proteins that had until recently been unsuitable for this methodology.

Protonated GppNHp at pH 8.4 Is Consistent with a Protonated GTP at Physiological pH—To capture the GTP-bound form of RAS it is necessary to use a GTP analogue, due to the fact that RAS catalyzes the hydrolysis of GTP to GDP. RAS hydrolyzes GTP with a rate constant of 0.03/min (32, 55, 56). Although this is a relatively slow reaction, GTP would certainly be fully hydrolyzed to GDP during the lifetime of a neutron crystallography experiment, which is in the order of months, including crystal growth and data collection. Using active site oncogenic mutants bound to GTP is also not viable, as the mutants still catalyze the hydrolysis reaction, albeit more slowly than wild type (7), and the mutations likely change the environment of the $\gamma$-phosphate of GTP. There are challenges associated with using analogues, however, due to the fact that they differ, even if slightly, from GTP. Detailed structural comparisons have been made between RAS-GTP (obtained by Laue crystallography with a caged substrate) and RAS-GppNHp (57). With the exception of local adjustments due to the presence of an NH group instead of an oxygen bridge to the $\gamma$-phosphate, the two structures are the same. Still, any changes in the $pK_a$ value for ionizable groups in the analogue relative to those groups in GTP must be kept in mind. Thus, in addition to a structural similarity to GTP when bound to RAS in terms of the interactions with conserved elements of the nucleotide-binding pocket, GppNHp has the advantage of having known $pK_a$ values for the $\alpha$, $\beta$, and $\gamma$-phosphate groups (28). The $pK_a$ value in solution for protonation of the $\gamma$-phosphate of GppNHp in the presence of Mg$^{2+}$ is 6.7, 1.6 pH units higher than the $pK_a$ value of 5.1 for GTP-Mg$^{2+}$, both determined by NMR in deuterated medium (28) (corrected for the isotope effect, see under “Experimental Procedures”). Note that the $pK_a$ shift required in

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**FIGURE 6.** Schematic diagram of the GppNHp interactions in the active site of RAS. The nucleotide is in black, and the protein residues are colored as in Fig. 1 according to the conserved motifs to which they belong. Water molecules are in blue. Deuterium atoms visible in the neutron density maps are shown in bold. Note that only residues in the P-loop (mauve), switch I residues (light gray), and residues in the NKCD motif (teal) interact directly with the nucleotide. Residues in the DTAG motif (including Asp-57) and the ETSAK motif are critical in positioning the directly interacting residues.

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**TABLE 2**
Current neutron structures in the PDB with longest unit cell edge greater than 100 Å

| Protein/complex                  | PDB code | Resolution | Longest unit cell edge length |
|----------------------------------|----------|------------|-------------------------------|
| D-Xylose isomerase               | 4DVO     | 2.0        | 102.8                         |
| Urate oxidase                    | 4N9M     | 2.0        | 105.5                         |
| Cytochrome c peroxidase          | 4CVI     | 2.4        | 107.6                         |
| $\alpha$-Thrombin complex with   | 3VXF     | 2.75       | 107.8                         |
| bivalirudin                      |          |            |                               |
| Inorganic phosphatase            | 3Q3L     | 2.5        | 113.7                         |
| Trypsin complex with bovine      | 3OTJ     | 2.15       | 122.6                         |
| pancreatic trypsin inhibitor     |          |            |                               |

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| bivalirudin                      |          |            |                               |
| Inorganic phosphatase            | 3Q3L     | 2.5        | 113.7                         |
| Trypsin complex with bovine      | 3OTJ     | 2.15       | 122.6                         |
| pancreatic trypsin inhibitor     |          |            |                               |

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Implications of a Protonated GTP γ-Phosphate—When thinking about the role of RAS in the hydrolysis of GTP, it is helpful to distinguish between the RAS contributions that are common to both intrinsic and GAP-catalyzed reactions from those features specific to each one. Upon binding RAS, GTP loses the flexibility that it has in solution, with accompanying vibrational uncoupling of the α-, β-, and γ-phosphates as observed by FTIR spectroscopy, and structural and charge shifts consistent with a dissociative reaction mechanism (27). It has been demonstrated by a combination of FTIR experiments and QM/MM calculations that GTP on RAS has a slightly longer bond between the oxygen bridging the β- and γ-phosphates and the γ-phosphorous atom, bringing the structure closer to that expected in a hydrolysis reaction with a dissociative transition state, aided by an interaction between the bridging oxygen and the amide of Gly-13 (14). The Mg\(^{2+}\) ion is critical in achieving this effect, and aided by Lys-16, it also induces a shift of negative charges from both the α- and γ-phosphate groups toward the β-phosphate, thus bringing the substrate closer to the transition state in terms of charge distribution as well (14, 58). The QM/MM simulations have been performed with a deprotonated γ-phosphate bearing a local charge of −2 and consistently show a Na\(^{+}\) ion neutralizing one of the charges. The authors point out that a less negative γ-phosphate helps the shift in charge toward that found in the transition state and may facilitate the approach of the nucleophilic water molecule. The neutron crystal structure presented here supports this effect being achieved through a protonated γ-phosphate and explains the discrepancy between the RAS-induced band shifts in the FTIR spectra and QM/MM simulations for one of the γ-phosphate modes (14). In further support of a protonated γ-phosphate, a more recent QM/MM study of the GAP-catalyzed hydrolysis reaction on RAS based on a two-water mechanism in the presence of the entire protein complex finds that a protonated GTP having a charge of −3 is more stable in the context of the active site than the −4 charge usually assumed for the pre-catalytic state (19). Interestingly, the shift in bond lengths and charges observed for GTP on RAS are increased in the complex with GAP, suggesting that GAP brings the ground state even closer to the transition state of the GTP hydrolysis reaction than does RAS alone (58). It appears that GAP accentuates effects already observed in the RAS-bound GTP ground state, propelling the reaction forward toward the transition state and onto product formation. The intrinsic hydrolysis of GTP on RAS has the same starting point but is activated by other factors such as ordering of switch II through the allosteric switch mechanism (6). Thus, although the details of the hydrolysis mechanism may not yet be resolved, it should evolve from a ground state with a protonated γ-phosphate if indeed RAS induces a pKₐ shift in GTP as we observe for its analogue GppNHp. This ground state is promoted by features that are conserved across the entire superfamily of GTPases and thus are expected to be common not only to the intrinsic and GAP-catalyzed reactions on RAS but to GTP hydrolysis catalyzed by GTPases in general.

The neutron crystal structure presented here does not resolve the mechanistic details of the GTP hydrolysis reaction catalyzed by RAS, but it does provide some guiding features. Once the idea that Gln-61 served as a base to activate the nucleophilic water molecule was put to rest (21, 48), GAP itself was proposed as the base, with various substrate-assisted mechanisms put forth in the literature (6, 21, 57). Given that all of the residues near the active site that could possibly serve as a catalytic base in the reaction had been shown not to be involved, a deprotonated γ-phosphate was the only candidate left (21). Chemical shift changes were observed by NMR for the three phosphate groups of GTP at around pH 3 due to a change in the environment that was attributed to protonation of the γ-phosphate of GTP (21). Furthermore, the reaction rate in the same study was shown to be constant over a wide pH range, from pH 3 to pH 7.5. These experiments could indeed be interpreted as supporting a γ-phosphate pKₐ of 3, but they are equally consistent with a scenario in which the γ-phosphate remains protonated over the entire pH range tested and where the change in chemical shifts of the three phosphate groups of GTP below pH 3, with accompanying decrease in reaction rate, is due to protonation of Asp-57 resulting in the loss of Mg\(^{2+}\) binding. In the first scenario RAS would decrease the pKₐ value of the nucleotide relative to that in solution (from 5.1 to 3.0), whereas in the latter interpretation the pKₐ is significantly increased. Given that the γ-phosphate of GppNHp is protonated at pH 8.4, the neutron crystal structure presented here strongly supports the notion that factors other than the protonation of the γ-phosphate explain the changes observed in the NMR experiments. It is highly unlikely that the RAS active site significantly decreases the pKₐ for GTP while promoting a large increase in its close analogue GppNHp. Note that the observed constant reaction rate in the wide pH range of 3.0–7.5 with an unchanging chemical shift for the α-, β-, and γ-phosphates (21) is consistent with a constant protonation state of GTP on RAS in this range. This supports our conclusion that the γ-phosphate remains protonated in the physiological pH range of 7.0–7.2 if one accepts that changes below pH 3 are due to other factors, such as protonation of Asp-57.

If we eliminate the idea of substrate-assisted activation of the nucleophilic water, we are left to conclude that the attacking water molecule in the reaction is sufficiently nucleophilic, in the context of a substrate that is partially activated toward the transition state, for the reaction to proceed without the involvement of a base. This may facilitate the catalyzed reaction because a neutral nucleophile would not be repelled by the overall negative charge of the γ-phosphate. Note that some of the previously proposed mechanisms are still possible, given
that the reaction begins with a protonated γ-phosphate rather than it becoming protonated in the process of substrate-assisted catalysis. In our previously proposed mechanism for intrinsic hydrolysis, for example, the proton on the γ-phosphate is shuttled during the reaction to a bridging water molecule that donates H-bonds to Tyr-32 in switch I and Gln-61 in switch II, transiently placing a partial positive charge where negative charge accumulates in the transition state of the reaction (6). This is still feasible within the new context presented here (Fig. 7A).

In the RAS-GAP complex where AlF3 is used as a transition state mimic for the GAP-catalyzed hydrolysis reaction, the positively charged arginine finger is found near the γ-phosphate oxygen atom equivalent to the one we find protonated in GppNHp and to the GDP β-phosphate oxygen that in GTP would be the β,γ-bridging oxygen atom (59). The authors cited the proximity of the positively charged arginine to the γ-phosphate mimic in support of an associative transition state, where negative charge accumulates mostly at the terminal phosphate group in the pentacovalent transition state (48). A protonated γ-phosphate would be expected to shift the interaction of the arginine finger toward the β,γ-bridging oxygen atom (Fig. 7B), where most of the negative charge accumulates in a dissociative-like transition state (48). A dissociative-like mechanism is supported by experiments using 18O isotope effects for both the RAS intrinsic and GAP-catalyzed hydrolysis of GTP (22, 23). A protonated γ-phosphate would be a key player in promoting this mechanism.

The neutron crystal structure of RAS, with the GppNHp analogue used to study its GTP-bound form, brings light to the possibility that the pre-catalytic state of the GTP hydrolysis reaction catalyzed by RAS is different than previously thought. Although the protonation state of GTP on RAS needs to ultimately be determined with GTP itself, our results question the status quo, providing evidence that we should look into this question further. A protonated γ-phosphate of GTP on RAS would provide a paradigm shift in our understanding of the RAS active site. Given the highly conserved nature of the nucleotide-binding site across the entire superfamily of GTPases (3), a protonated GTP provides a new perspective from which to study the details of the catalytic mechanism of GTP hydrolysis on RAS and other GTPases, with impact on a large range of biological functions (12).

Author Contributions—R. K. purified and crystallized protein, did the exchange to deuterated conditions, collected neutron data, refined and analyzed the neutron structure, and wrote the paper. G. H. optimized the crystal growth conditions to obtain large crystals suitable for neutron data collection. K. W. helped with large scale protein production. F. M. designed and performed the neutron data collection experiments, did the data processing, and guided R. K. through the structure refinement. C. M. conceived the project, analyzed the neutron structure, and wrote the paper. All authors approved the final version of the manuscript.

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