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Analysis of GTPases Carrying Hydrophobic Amino Acid Substitutions in Lieu of the Catalytic Glutamine: Implications for GTP Hydrolysis

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ABSTRACT **Ras superfamily GTP-binding proteins regulate important signaling events in the cell. Ras, which often serves as a prototype, efficiently hydrolyzes GTP in conjunction with its regulator GAP. A conserved glutamine plays a vital role in GTP hydrolysis in most GTP-binding proteins. Mutating this glutamine in Ras has oncogenic effects, since it disrupts GTP hydrolysis. The analysis presented here is of GTP-binding proteins that are a paradox to oncogenic Ras, since they have the catalytic glutamine (Gln^{cat}) substituted by a hydrophobic amino acid, yet can hydrolyze GTP efficiently. We term these proteins HAS-GTPases. Analysis of the amino acid sequences of HAS-GTPases reveals prominent presence of insertions around the GTP-binding pocket. Homology modeling studies suggest an interesting means to achieve catalysis despite the drastic hydrophobic substitution replacing the key Gln^{cat} of Ras-like GTPases. The substituted hydrophobic residue adopts a "retracted conformation," where it is positioned away from the GTP, as its role in catalysis would be unproductive. This conformation is further stabilized by interactions with hydrophobic residues in its vicinity. These interacting residues are strongly conserved and hydrophobic in all HAS-GTPases, and correspond to residues Asp92 and Tyr96 of Ras. An experimental support for the "retracted conformation" of Switch II arises from the crystal structures of Ylqf and hGBP1. This conformation allows us to hypothesize that, unlike in classical GTPases, catalytic residues could be supplied by regions other than the Switch II (i.e., either the insertions or a neighboring domain). Proteins 2005;59:332–338.**

Key words: homology modeling; GTP-binding protein; conformational change; transition state; catalytic residues; hydrophobic stabilization

INTRODUCTION

GTP-binding proteins, or G-proteins, function as molecular switches and transducers in a variety of signaling cascades. Ras superfamily GTPases regulate a large number of events, ranging from vesicle trafficking to mitogenic activation. GTP- binding proteins are switched "ON" from their inactive GDP-bound states by the exchange of bound GDP to GTP, due to GEFs. The signaling activity is determined by the "ON" state, where it interacts with effector proteins downstream in the signal transduction cascade. It is by means of this interaction that a signal transduction cascade begins or carries on.[1,2] Hydrolysis of bound GTP returns them to the GDP-bound "OFF" state.

Ras, which often serves as a prototype for a minimal catalytic GTPase domain, was discovered as an oncogene in mammalian tumors around two decades ago, and efforts to design efficient anti-Ras drugs are still under way. Elucidating the mechanistic details of the GTPase reaction has therefore been a subject of intensive research. Gly-12[3] and Gln-61[4] are the most frequently mutated Ras residues in tumors, and the molecular basis for this oncogenic effect has been explained by the transition- state structure of the complex of Ras and RasGAP.[5] A large amount of structural information is also available to understand how Ras is regulated by GEFs[6–9] and GAPs.[10,11] Intrinsic GTP hydrolysis by GTPases is inefficient and is accelerated by orders of magnitude through the interaction with GAPs.[7] GAPs are known to introduce additional catalytic residues, an arginine in many cases (Arg^{GAP} hereafter), into the active site of the GTPase to stabilize the transition state of the phosphoryl transfer reaction[5] (Fig. 1). Variations in the

Abbreviations: EH, Eps homology domain; EngA, essential neisserial GTPase A; EngB, essential neisserial GTPase B; Era, *Escherichia coli* Ras- like protein; GAP, GTPase-activating protein; GDP, guanosine-5′- diphosphate; GDP-AlFx, GDP aluminum fluoride; GEF, guanine–nucleotide exchange factor; GMPPNP or GppNHp, 5′-guanylylimido-diphosphate; GTP, guanosine-5′-triphosphate; HAS-GTPase, hydrophobic amino acid substituted for catalytic glutamine; Hflx, high-frequency of lysogenization; hGBP1, human guanylate-binding protein1; NOG, nucleolar G protein; Φ, hydrophobic residue; PDB, Protein Data Bank; TrmE, tRNA modification.

The Supplementary Materials referred to in this article can be found at http://www.interscience.wiley.com/jpages/0887-3585/suppmat

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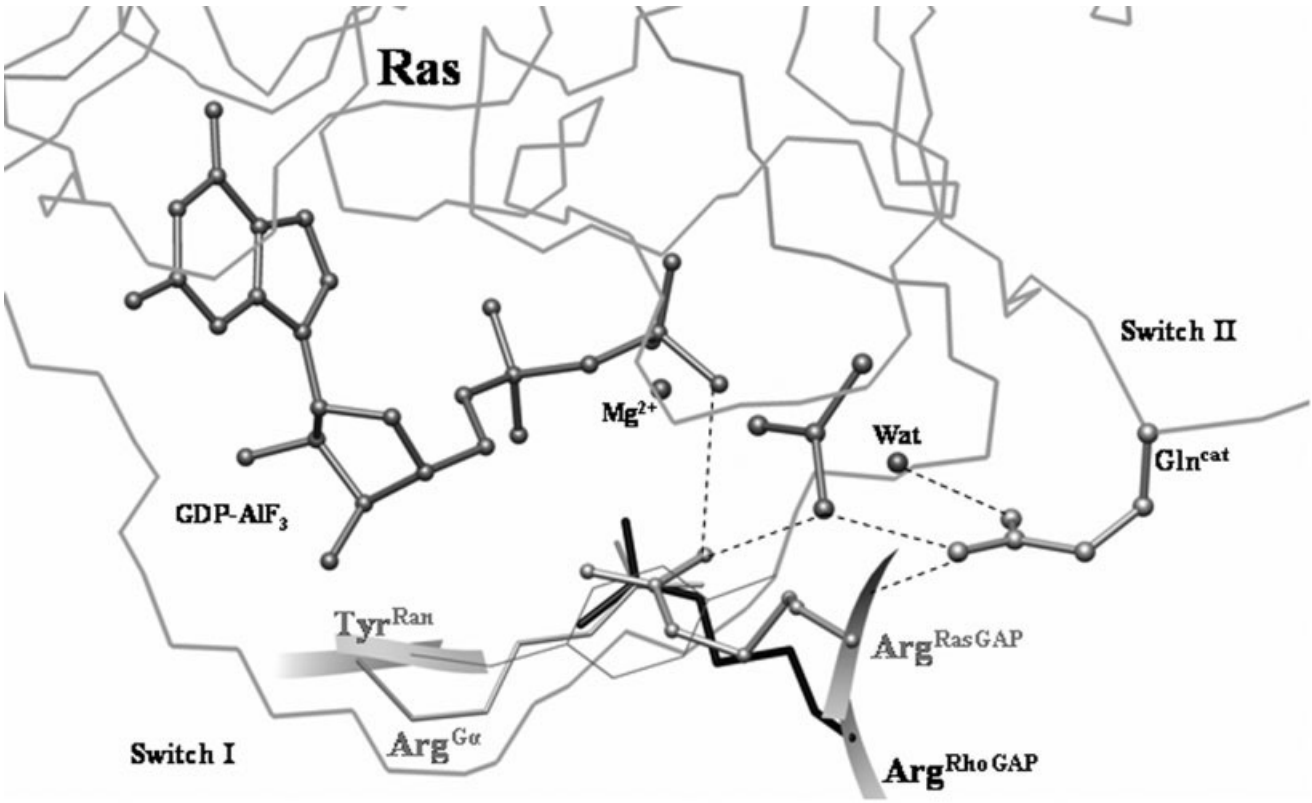

Fig. 1. The position occupied by residues stabilizing the transition-state structures is invariant. RasGAP and RhoGAP provide Arg^{789} (dark gray ball and stick conformation) and Arg^{85} (black sticks), respectively, to stabilize the transition-state structures. In heterotrimeric Gα-proteins, Arg^{178} (dark gray sticks) resides in the same protein in an α-helical domain adjacent to the G-domain. Intriguing variation arises in the Ran-GTPase system, where a Tyr^{39} (dim gray sticks) from the Switch I region of Ran plays a role similar to Arg^{GAP}. In these structures, aluminium fluoride (AlF_3) mimics γ-phosphate, where Al and F atoms represent the phosphate and oxygen atoms, respectively. Its orientation is such that the fluoride ligands contact the side-chain amino groups of Gln^{cat} and Arg^{GAP}. The carbonyl group of Gln^{cat} interacts with the nucleophilic water molecule (Wat) and amino group of Arg^{GAP}, with the oxygen of β- phosphate. The backbone oxygen of Arg^{GAP} interacts with NH_2 group of Gln^{cat}. GDP- AlF_3 is shown as a ball and stick conformation. The nucleophilic water molecule and magnesium ion are shown as balls. The Gln^{cat} (dark gray ball and stick conformation) side-chain is also indicated.

way GAPs introduce such stabilizing residues have been offered by the heterotrimeric G proteins, where this residue is supplied by an α-helical domain[12] built within the protein (Fig. 1). Evidence is now accumulating that in Ras family G proteins, the stabilizing GAP residue is not necessarily an Arg. A Tyr39 (Tyr^{Ran} hereafter) within the GTPase domain of Ran[13] (Fig. 1) and an Asn290 in RapIGAP[14] (GAP of Rap1) have been shown to perform a function similar to Arg^{GAP}.

Apart from the residue supplied by GAP, the rest of the machinery required for hydrolyzing GTP or, in other words, the machinery responsible for intrinsic hydrolysis, residues within the GTPase domains. Rooted in these domains are 5 highly conserved signature motifs—G1, G2, G3, G4, and G5—that participate in stabilizing the bound guanine nucleotide and its hydrolysis.[15] The G5, a weakly conserved SAK/L motif (conserved only in the Ras family) and the G4 (N/TKxD) motifs provide contacts to the guanine base of the nucleotide. The G1 region, with a consensus sequence Gx(4)GKS/T, is also known as the P-loop. This is because it provides residues K and S/T to stabilize the phosphates of GTP/GDP. The G2, with only a single conserved Thr residue, and G3, with "DxxG" motif, are known as Switch I and Switch II, respectively, due to the large conformational changes observed between the GDP and GTP bound forms.[15] In the classical GTPases, the switches participate in interaction with their regulators, and Switch I specifically mediates effector binding. Gln-61, (Gln^{cat} in this work) adjacent to the DxxG motif in Switch II/G3, is found to be mutated in oncogenic Ras and is a key player in hydrolyzing GTP to GDP.[15] Hydrolysis of GTP is due to a nucleophilic attack by a water molecule, and the role of Gln^{cat} is to stabilize the transition state by orienting the relative positions of the nucleophilic water and the γ-phosphate[5] (Fig. 1). This idea is supported by the structures of GTPases and their GAPs in the transition state of hydrolysis. The hydrolysis reaction also requires crucial stabilizing residues like Arg^{GAP} provided by the GAP. Arg^{GAP} and Gln^{cat} work together to achieve GTP hydrolysis.[5,16–18]

Given the importance of this cardinal Gln^{cat} for GTP hydrolysis, it is not surprising to find oncogenic mutations at this position, where the protein gets locked in its GTP bound form and remains constitutively active. A hydrophobic amino acid substitution (of Gln^{cat}) disables GTP hydrolysis, as it can no longer form the hydrogen bonds to stabilize the transition state. However, the presence of a subset of GTPases with definite biological roles, carrying

TABLE I. Conserved Hydrophobic Interactions in HAS-GTPase Subfamilies.

Name of the subfamily	No. of sequences analyzed	Residue prior to P-loop	Residue next to DXXG	Residues in helix (α3)			
Ras	—	V^{9}	Q^{61}	D^{92}	I^{93}	Q^{95}	Y^{96}
Hflx	35	V^{203}	F^{255}	A^{297}	X	X	V^{301}
EH	9	V^{122}	I^{215}	C^{261}	X	A^{264}	L^{265}
Era	60	I^{159}	L^{211}	V^{257}	X	L^{260}	I^{261}
EngA	124	V^{9}	I^{61}	V^{103}	X	L^{106}	L^{107}
EngB	12	A^{9}	F^{56}	M^{116}	F^{117}	X	L^{120}
TrmE	69	V^{227}	I^{279}	I^{321}	X	X	V^{325}
NogI	8	C^{174}	I^{225}	L^{271}	X	X	I^{275}
FeoB	9	I^{9}	V^{16}	L^{100}	X	X	L^{104}
Rsr1	2	L^{9}	I^{61}	X	L^{293}	X	L^{296}
Rb25	3	I^{18}	L^{71}	X	X	X	L^{107}

The conserved residues involved in hydrophobic interactions are tabulated with reference to Ras. The numbers (in superscript) indicate the position of the residues in the corresponding protein. The residue next to DxxG in Rsr1 (I^{61}) does not interact with its hyrophobic core, as it attains an unproductive conformation due to a deletion (see text). X indicates that the residue does not participate in hydrophobic interactions.

hydrophobic substitutions in place of Gln^{cat} and possessing the ability to hydrolyze GTP, presents a clear contradiction. HAS- GTPases hydrolyze GTP, perhaps by using variations on the well- known catalytic mechanisms or by altering it significantly. The work reported here is an analysis of such HAS-GTPases, with an attempt to understand how these proteins function in the absence of a key Gln^{cat}.

RESULTS AND DISCUSSION

The role of conserved residues of the G1–G5 motifs of classical GTPases (those that contain Gln^{cat}) in stabilizing and providing contacts to the nucleotide is now well understood.[19] In this analysis, in order to understand the effect due to the absence of Gln^{cat}, we have restricted ourselves to those GTPases that retain the conserved residues of the G1–G5 motifs, except the Gln^{cat}. GTPases that carry a hydrophobic amino acid after the DxxG motif constitute the initial set of HAS-GTPases for our analysis (see Supplementary Material). Significant enhancement in understanding the role of catalytic residues Gln^{cat} (from Switch II) and Arg^{GAP} (from GAP) is due to the structures with the transition-state analog GDP-AlFx, which have elucidated how these 2 residues need to be reoriented in order to stabilize the transition state of hydrolysis.[5,18]

The abundance of currently available sequence data enabled us to look for a large number of GTPases lacking the crucial catalytic residue Gln^{cat}. In general, catalytic residues are strongly conserved even in distantly related proteins. However, it is intriguing that despite lacking this crucial residue, HAS-GTPases hydrolyze GTP efficiently.[20–23] The simplest way to address this contradiction is to reason that the catalytic residue would be relocated to a different region in the protein or be supplied by an interacting protein.

To begin addressing these aspects, HAS-GTPases were grouped into 10 subfamilies based on the sequence similarity (Table I). We constructed a multiple sequence alignment for each subfamily, taking special care to align the secondary structure elements (Fig. 2). The striking feature of these alignments is the presence of several conspicuous insertions in the G-domain, mainly in loops away from the nucleotide-binding pocket and in loops around the nucleotide-binding pocket (see Supplementary Material).

To gain further insight into the role of insertions in GTP hydrolysis by HAS- GTPases, we generated homology models of a representative of each of the subfamilies, using the X-ray structure of Ras as a template.[5] In most cases, the models present fewer differences with respect to Ras, except for large deviations in the loop regions (see Supplementary Material). It was not obvious to find uniquely conserved residues in either any of the insertions or in regions around the nucleotide-binding pocket, which can substitute the role of Gln^{cat}. It is likely that the insertions around the γ-phosphate (i.e., Switch I and Switch II) could be strong contenders to effectively modify or provide significantly new interactions at the phosphate side of GTP. Hence, a thorough analysis of the insertions in these regions is necessary to understand their role in GTP hydrolysis by HAS-GTPases.

Effect of Hydrophobic Substitutions in Lieu of Gln^{cat}

The analysis of crystal structures of GTPase-GAP complexes[5,13,16–18] determined so far clearly indicates that the points of entry for GAP residue and Gln^{cat} can be delineated. In these structures, the functional groups of Tyr^{Ran} in the Switch I of Ran- Rangap,[13] Arg^{GAP} of Ras-RasGAP,[5] Rho-RhoGAP,[16–18] Rac-Exos,[24] and the heterotrimeric G-proteins[12] superpose remarkably well on each other (Fig. 1). It seems that irrespective of the genesis and nature of the stabilizing GAP-like residue, the final three-dimensional (3D) position they occupy relative to the γ-phosphate is invariant. This comparison of the of GTPase-GAP complexes suggests the Switch I side of the GTP binding pocket to be the specific specific site of entry for the GAP residue. In HAS-GTPase models, the insertions in the Switch I regions do not provide additional contacts to the γ-phosphate and the nucleophilic water molecule, and hence do not clearly identify any residues that fulfill the role of a GAP.

The analysis of GTPase-GAP complexes also suggests that Gln^{cat} is introduced from the Switch II side of the GTP-binding pocket, with its functional groups close to the γ-phosphate (Fig. 1). In this context, we examined the insertions in Switch II to determine whether they can provide residues to substitute for the loss of Gln^{cat} in HAS-GTPases. In the ground-state (GTP-bound) structures of GTPases, the orientation of the functional groups of Gln^{cat} is quite variable. However, in the transition-state structures (i.e., GTPase-GAP complexes), Gln^{cat} is reoriented to stabilize the γ- phosphate and the nucleophilic water molecule[5] (Fig. 1). As catalytic residues such as these can be identified from the transition-state structures, we have generated homology models for the representative members of all HAS-GTPase subfamilies using the Ras-GDP-

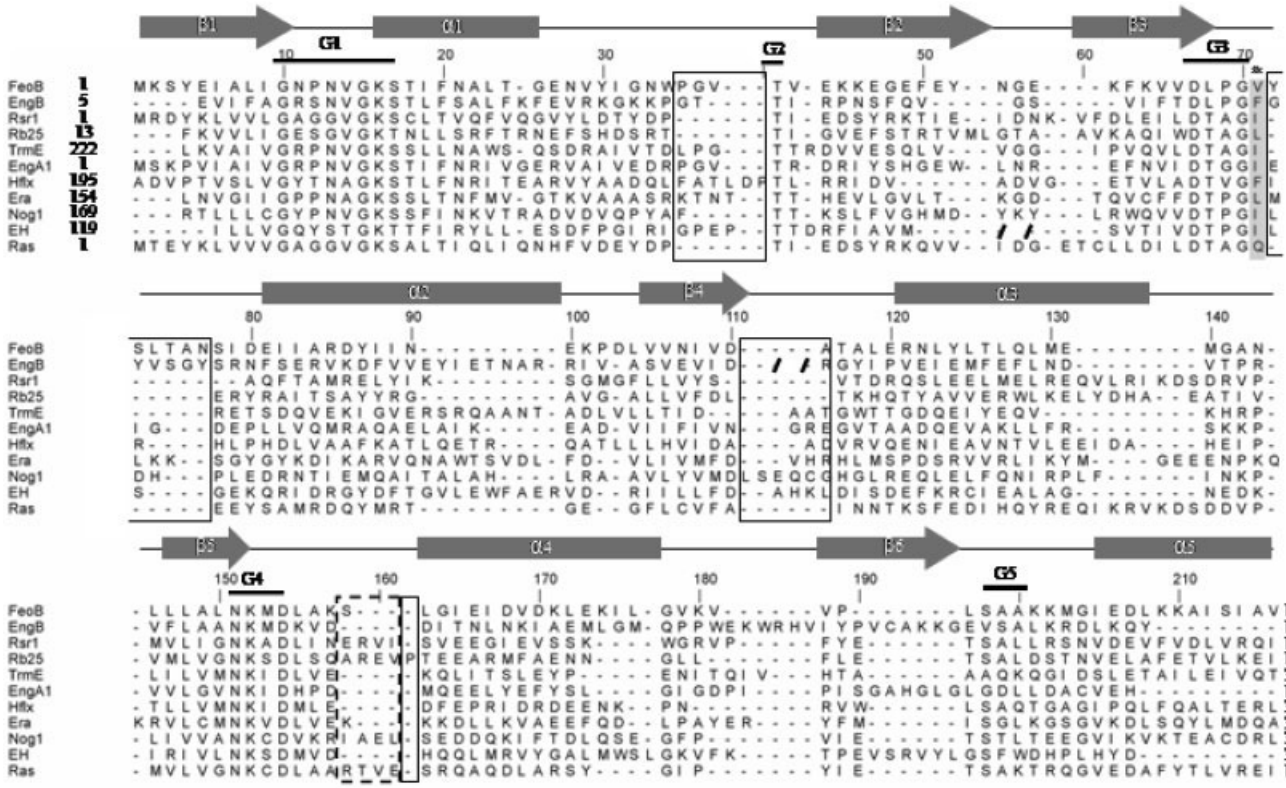

Fig. 2. Multiple sequence alignment of HAS-GTPases. Secondary structure based sequence alignment of representative members of all HAS-GTPase subfamilies, along with Ras, is shown. Secondary structure elements (α-helices as cylinders, β-sheets as arrows) are numbered in accordance with their sequential occurrence in the 3D structure of Ras (PDB code: 5p21). Boxed regions indicate insertions, while boxes with broken borders indicate deletions. The region where Gln^{cat} and hydrophobic substitution occur is highlighted in gray. The 5 conserved motifs, G1–G5 (solid black lines) are also marked. Numbers at the N- and C-terminus indicate the position of the G-domain in the corresponding sequences. Certain long-length insertions that prohibit reliable homology modeling have been deliberately deleted. These regions (46 residues in EH and 13 in EngB) are indicated by **//** along the sequence of these proteins.

AlF_3 part of the structure of Ras-GDP-AlF_3-GAP334[5] as a model. Among HAS-GTPases, the length of insertions in Switch II ranges from 6 residues (FeoB[25] sub-family) to 1 residue (EngA[20] subfamily) (Fig. 2 and Supplementary Material). In all the HAS-GTPase homology models, it was not possible to identify residues that provide interactions with the γ-phosphate and are reasonably well conserved within a subfamily. It would require experimental studies and transition-state structures to precisely identify the catalytic residues.

However, an interesting observation is that in the models of all HAS-GTPases, the hydrophobic residue that substitutes Gln^{cat} points away from the nucleotide-binding pocket (Fig. 3). To facilitate further discussion, we wish to define 2 possible scenarios for the conformations of Switch II and/or the residue after DxxG (i.e., substituting Gln^{cat}), which we term the "active conformation" when the residue participates in hydrolysis, and a "retracted conformation" when it is oriented away from the binding pocket. While an "active conformation" is observed in classical GTPases with Gln^{cat} taking part in hydrolysis [Figs. 1 and 3(a)], what we observe in all the models of HAS-GTPases is a "retracted conformation." Here the substituted hydrophobic residue (in place of Gln^{cat}) is positioned away from the γ-phosphate to avoid unwanted interactions due to its hydrophobic nature, and in doing so, it does not occupy the position that Gln^{cat} does in Ras-like GTPases [Fig. 3(a)]. In this "retracted conformation," the Switch II loop is also oriented upward in comparison to Ras and other classical GTPases.

In addition, the "retracted conformation" is further stabilized due to hydrophobic interactions with other residues in its vicinity. For instance, in Hflx,[26] the hydrophobic residue F^{255} of Switch II is stabilized by interactions with hydrophobic residues V^{203} before P-loop (V^{203}GYTNAGKS^{211}), A^{297}, and V^{301} in α3. It is fascinating to note similar stabilizing interactions in other HAS-GTPase models [Fig. 3(a)] where the position of these hydrophobic residues is identical (Table I). Overall, the hydrophobic residue (replacing Gln^{cat}) is stabilized by interactions with a hydrophobic pocket formed by residues arising from 2 regions: a hydrophobic residue prior to the P- loop (ΦGxxxxGKS/T) and 2 from helix α3 [Fig. 3(a)]. The latter 2 residues correspond to D92 and Y96 in the helix α3 of Ras. A summary, presented in Table I, for all the subfamilies of HAS-GTPases, suggests that these positions are uniquely hydrophobic in nature. In some subfamilies, such as EngA[20], Era[21], EngB[27] and EH[28], the hydrophobic pocket is further strengthened by interactions from additionally conserved hydrophobic residues of the same

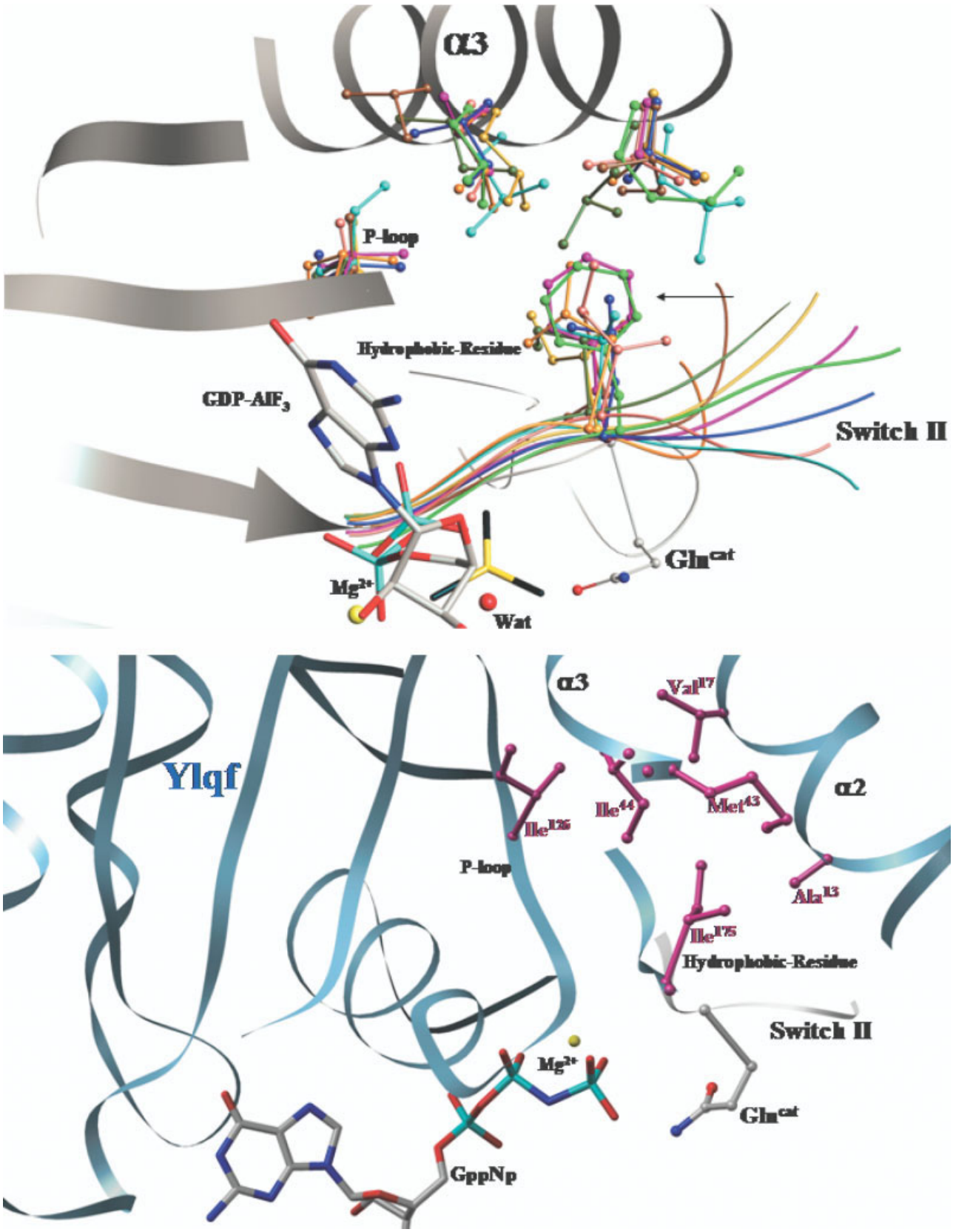

Fig. 3. Retracted conformation of the hydrophobic residue in Switch II. (**a**) The hydrophobic residue and Switch II of all HAS-GTPases are shown. It adopts a retracted conformation (indicated by an arrow). The interactions stabilizing this conformation are conserved within and across the subfamilies of all HAS- GTPases, as depicted here (also see Table I). Hflx is shown in dark magenta, EH in golden rod, Era in blue, EngA in dark salmon, EngB in lawn green, TrmE in sienna, Nog1 in dark olive green, FeoB in cyan, Rsr1 in pink, and Rb25 in dark orange. (**b**) In the crystal structure of Ylqf, a circularly permuted GTPase, the hydrophobic residue Ile^{175}, adopts a retracted conformation similar to that observed in all HAS- GTPases. The hydrophobic residue Ile^{175} is stabilized by interactions with residues A^{13}, V^{17}, M^{43}, I^{44}, and I^{126} from helices α3, α2, and the P-loop, as indicated. The active conformation of the Gln^{cat} of classical GTPases is shown in gray for comparison.

helix (Table I). Interestingly, in the classical GTPases, residues corresponding to D_{ras}^{92} and Y_{ras}^{96} are largely charged or, at most, only one of them is hydrophobic. The fact that a very small number of classical GTPases contain hydrophobic residues at both positions precludes us from concluding that this is a feature unique to HAS-GTPases. On the whole, we conclude that the presence of hydrophobic residues in helix α3 is a feature contributing to stabilization of the hydrophobic residue (substituting Gln^{cat}), resulting in its "retracted conformation."

The only exception to this scenario comes from Rsr1,[22] where, although the hydrophobic pocket exists, the hydrophobic substitution (for Gln^{cat}), Ile^{61}, does not interact with it. This is because Rsr1 contains a 2-residue deletion in the Switch II region. Homology models of Rsr1, the only HAS-GTPase with a deletion in Switch II, suggests that the consequence of this deletion is in drawing the loop away from the nucleotide-binding pocket, which results in Ile^{61} not occupying the same position as Gln^{cat}. Due to this withdrawal of the loop, Ile^{61}, although not stabilized by interactions from a similar hydrophobic pocket, seems to achieve a "retracted conformation" (see Supplementary Material).

Taken together, a "retracted conformation" of the hydrophobic residue and/or Switch II seems to be a conserved molecular event for HAS-GTPases. It is indeed heartening to note that there exists an experimental support for such an event as observed in the GTP-bound structure of Ylqf[29] (PDB code: 1puj). Ylqf is a conserved hypothetical protein of *Bacillus subtilis,* with a circular permutation of G1–G5 motifs, and in principle, it can be defined as a HAS-GTPase due to the hydrophobic residue, Ile^{175} that replaces Gln^{cat}. We have not considered proteins such as Ylqf in our analysis due to the rearrangement of the G1–G5 motifs. In this structure, most interactions with GTP, except those from Switch I (G2), are similar to the observed interactions in classical GTPases. In the structure Ile^{175} that replaces Gln^{cat} of classical GTPases is drawn away into a "retracted conformation" and interacts with hydrophobic residues A^{13}, V^{17}, M^{43}, I^{44}, and I^{126} in the vicinity. Here, too, M^{43} and I^{44} are presented by the helix α3, and I^{126} lies just prior to the P-loop [Fig. 3(b)]. A similar scenario is also observed in the structure of hGBP1[30] (see Supplementary Material), another HAS-GTPase not considered in our analysis, as it lacks the NKxD motif. The interactions observed in the structures of Ylqf and hGBP1 are hence in tune with the proposed hydrophobic stabilization of "retracted conformation" based on our models. Experimental studies on HAS-GTPases would verify the presence of a retracted conformation and the proposed stabilization.

The retraction of Switch II away from the nucleotide results in creating a space that is usually occupied by the Gln^{cat} in classical GTPases, and this space may be used to similarly introduce a catalytic residue. This residue (which substitutes for the role of Gln^{cat}) could be offered by the insertions or the neighboring domains or by another interacting protein. For Arg^{GAP}, which is provided either *in cis*[12] by the same molecule or *in trans*[5,16–18] by a different molecule, such scenarios are well known. Surprisingly, all HAS-GTPases contain extra domains in addition to the G-domain. If the role of these domains would be an *in cis* supply of catalytic Gln^{cat}-like residue, it will be a fascinating variation on the known catalytic mechanisms used by GTP- binding proteins. Therefore, it is possible to conclude that novel variations in catalytic mechanisms are invoked by HAS-GTPases.

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