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GTPase Catalysis by Ras and Other G-proteins: Insights from Substrate Directed SuperImposition

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Comparisons of different protein structures are commonly carried out by superimposing the coordinates of the protein backbones or selected parts of the proteins. When the objective is analysis of similarities and differences in the enzyme's active site, there is an inherent problem in using the same domains for the superimposition. In this work we use a comparative approach termed here "Substrate Directed SuperImposition" (SDSI). It entails the superimposition of multiple protein–substrate structures using exclusively the coordinates of the comparable substrates. SDSI has the advantage of unbiased comparison of the active-site environment from the substrate's point of view. Our analysis extends previous usage of similar approaches to comparison of enzyme catalytic machineries.

We applied SDSI to various G-protein structures for dissecting the mechanism of the GTPase reaction that controls the signaling activity of this important family. SDSI indicates that dissimilar G-proteins stabilize the transition state of the GTPase reaction similarly and supports the commonality of the critical step in this reaction, the reorientation of the critical arginine and glutamine. Additionally, we ascribe the catalytic inefficiency of the small G-protein Ras to the great flexibility of its active site and downplay the possible catalytic roles of the Lys16 residue in Ras GTPase. SDSI demonstrated that in contrast to all other Gly12 Ras mutants, which are oncogenic, the Gly12 → Pro mutant does not interfere with the catalytic orientation of the critical glutamine. This suggests why this mutant has a higher rate of GTP hydrolysis and is non-transforming. Remarkably, SDSI also revealed similarities in the divergent catalytic machineries of G-proteins and UMP/CMP kinase. Taken together, our results promote the use of SDSI to compare the catalytic machineries of both similar and different classes of enzymes.

Keywords: enzymatic catalysis; GTPase; guanine nucleotides; conformational rearrangement; structure comparison

Introduction

G-proteins function as molecular switches and transducers in a variety of signaling cascades and cellular processes. They can be classified into two families: the heterotrimeric G-proteins and the monomeric G-proteins (for which Ras often serves as a prototype).[1] Their signaling activity is determined by the identity of the nucleotide bound in their active site. As a rule, inactive G-proteins (with GDP tightly bound) are turned "on" by the exchange of GDP with GTP, catalyzed by guanine-nucleotide exchange factors (GEFs). They are turned "off" by the intrinsically catalyzed hydrolysis of the bound GTP (GTPase).[2] In order to fulfil their role as switches, G-proteins evolved an enzymatic GTPase reaction that is inefficient, sometimes to the extreme.[3] Nevertheless, the GTPase reaction can be accelerated by orders of magnitude through the interaction with GTPase activating proteins

Abbreviations used: GTP, guanosine-5′-triphosphate; GDP, guanosine-5′-diphosphate; GppNHp, 5′-guanylylimido-diphosphate; GTPγS, guanosine-5′-*O*-3-thiotriphosphate; $GppCH_2p$, guanosine-5′-(βγ-methylene)-triphosphate; GTPase, GTP hydrolysis; GAP, GTPase activating protein; GEF, guanine-nucleotide exchange factor; NTP, nucleoside-5′-triphosphate; RGS, regulator of G-protein signaling; SDSI, substrate directed superImposition; UMPk, UMP/CMP kinase.

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(GAPs).[1,3–6] Importantly, disruptions of the GTPase reaction have been implicated in many pathological disorders ranging from cholera to cancer. Specifically, mutations in the monomeric G-protein Ras impair its GTPase ability and have been implicated in more than 30% of all human tumors.[7] Elucidating the mechanistic details of the GTPase reaction has therefore been an important scientific goal and a subject of intensive research for many years.

Due to their biological importance, G-proteins have been investigated over the years using a variety of methodologies. Among these, numerous structural studies have yielded an abundance of three-dimensional structures of different G-proteins.[1,4,8,9] Significantly, structures with the transition-state analog GDP–aluminum fluoride[10] have enabled investigators to see how a G-protein stabilizes the transition state of the GTPase reaction. Previous studies have mainly scrutinized each specific G-protein structure, thereby contributing to the understanding of this particular protein (see Sprang,[1] Kjeldgaard *et al.*[11] and additional references in Materials and Methods). However, the accumulation of multiple structures suggests that it is now of interest to utilize a comparative approach in order to better understand how G-proteins function. Proteins crystallized with non-hydrolysable GTP analogs give us a snapshot of the ground state (the reactants) of the GTPase reaction while structures with GDP–aluminum fluoride show how the enzyme stabilizes the transition state. In particular, we suggest that comparison of multiple protein structures from the substrate's point of view provides a powerful tool to study how G-proteins catalyze the GTPase reaction.

Three-dimensional structural comparisons of the binding sites in guanine and adenine nucleotide-binding proteins have been explored using bioinformatic methodologies.[8,12–17] Some studies took the substrate's point of view, compared protein families with different functions and mainly centered on structural similarities of the binding sites and molecular recognition.[13,14,16–20]

Here, we explore the details of G-proteins' active sites and catalytic mechanism using a similar minded approach termed substrate directed superimposition (SDSI). SDSI entails the comparison of multiple crystal structures of enzymes by superimposing carefully selected structures using only the coordinates of the substrates, provided the latter are in a similar conformation. We advocate that using exclusively the coordinates of the enzyme's substrate for superimposition gives an unbiased comparison of the active-site environment from the substrate's point of view and enables one to draw conclusions regarding catalytic mechanisms.

SDSI shows that dissimilar G-proteins stabilize the transition state of the GTPase reaction similarly and further supports previous suggestions that the critical mechanistic step is the reorientation of two critical residues, an arginine and a glutamine.[1,3,5,6,9] Special attention is paid to the Ras protein because of its biological importance and the availability of extensive structural and biochemical data. We raise the possibility that the intrinsic catalytic inefficiency of wild-type Ras is related to the great flexibility of its active site, explore the structural basis for the deficient GTPase reaction in oncogenic Ras mutants and investigate additional residues as candidates that can contribute to catalysis. Remarkably, SDSI also revealed novel similarities in the catalytic machineries responsible for the different phosphoryl transfer reactions by G-proteins and by UMP/CMP kinase. Hence, it seems that potentially SDSI can be used to compare different classes of enzymes.

Results

The transition state of the GTPase reaction

The abundance of G-protein crystal structures enabled us to compare snapshots of discrete steps in the GTPase reaction. Moreover, SDSI of G-proteins with the transition-state analog GDP–aluminum fluoride,[10] facilitate an unbiased comparison of how different G-proteins stabilize the transition state of the reaction. We superimposed the ten available transition-state structures using the atoms of the GDP moiety and the aluminum atom (Figure 1). These crystal structures are of five heterotrimeric G-proteins and five monomeric ("Ras-like") G-proteins with their respective GAPs (see Materials and Methods).

Examination of the superposed substrates (Figure 1(a)) shows that they are all in a very similar conformation (RMSD of all the substrates' atoms <0.85 Å). This illustrates what we believe could be an important prerequisite for using SDSI, that all the parts of the substrates used must be in a similar conformation (see Materials and Methods). Previous work has identified short sequence elements that are conserved among G-proteins.[11] Visualizing this "GTP-binding motif" demonstrates that its conformation in three dimensions and its orientation towards the substrate are also highly conserved (Figure 1(b)). However, from analysis of the actual binding sites of these ten structures (defined here as all residues of the G-protein that are within 3.5 Å of the substrate) emerges a slightly different picture (Figure 1(c)). The residues comprising the actual binding site (Figure 1(b)) and the GTP-binding motif (Figure 1(c)) are not identical. Additionally, the structural elements visualized in Figure 1(c) appear to be "fuzzier"[13,21] than the conserved sequence motifs depicted in Figure 1(b). The former contains residues from the known elements of the P-loop, the guanine base binding loops and the switches I and II domains.[1] Of these, the prominent contributors to the difference between the two panels and to the fuzziness observed in Figure 1(c) are the residues belonging to the switch I region.

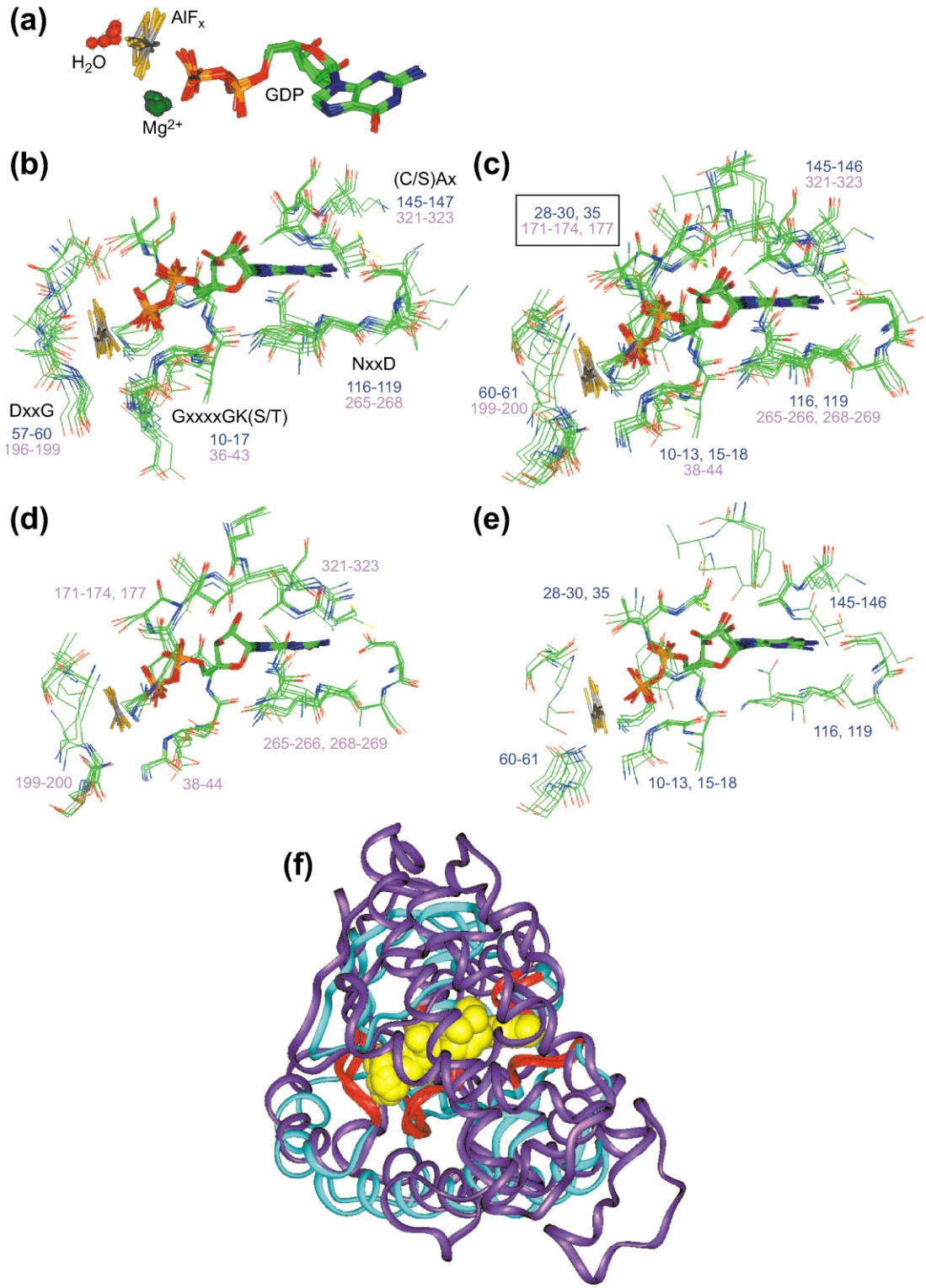

Figure 1. SDSI of transition-state structures of G-proteins. In (b)–(e), the numbers adjacent to the binding site residues are the relevant residue numbers in Ras (blue) and transducin (magenta). (a) The GDP–aluminum fluoride substrates superimposed (see Materials and Methods for details and a list of superimposed structures). (b) The GTP-binding motif conserved in G-proteins.[11] The consensus sequence residues are depicted in black. (c) The actual binding sites of the G-proteins (defined as all residues within 3.5 Å of the substrates). The additional residues of the binding site, which are not conserved in sequence throughout the structures, are depicted as ball and stick models. Most of these residues are part of the switch I region, which is not conserved between heterotrimeric G-proteins and small G-proteins (these residue numbers are marked with a black square). (d) and (e) The binding sites of heterotrimeric (d) Gα proteins and (e) small G-proteins, when visualized separately, are less "fuzzy" than in (c) the combined view. (f) Transition-state structures of Ras (cyan) and transducin (purple) superimposed by SDSI are visualized as backbone ribbon diagrams. The conserved GTP-binding motif ((b)) is colored red. The substrates are in the same orientation as in (b)–(e) and are depicted as space filling models (colored yellow).

At the sequence level, these residues are not well conserved between G-protein sub-families due to the functional divergence of switch I between small G-proteins and heterotrimeric G-proteins.[1,9] Therefore, visualizing the binding sites of each of these two sub-families separately (Figure 1(d) and (e)) shows a better structural conservation than in the combined view (Figure 1(c)). This illustrates how a structural comparison of multiple binding sites by SDSI can be used to reveal additional information not seen at the sequence level, as was also shown previously by other structural comparisons.[1,6,9] It is also interesting to note that the unbiased comparison of the active sites provided by SDSI is easily implemented, even for proteins sharing less than 15% sequence identity and having different domains, such as Ras and transducin (Figure 1(f)).

However, the main objective of this work is to compare the catalytic machinery of these G-proteins. Previous work has implicated two residues as being directly involved in catalysis, an arginine and a glutamine (hence referred to as Gln_{cat} and Arg_{cat}).[1,3–5] Gln_{cat} is part of the so-called switch II region of G-proteins and has the putative role of stabilizing the nucleophilic attack of the water molecule on the γ phosphate. Arg_{cat} is thought to neutralize the negative charges that accumulate on the GTP substrate in the transition state but remarkably, Arg_{cat} is not conserved in the sequence of all G-proteins. In heterotrimeric G-proteins Arg_{cat} originates from the switch I region of the G-protein, *in cis* to Gln_{cat}.[1,3] On the other hand, small, monomeric G-proteins (for which Ras is a prototype) lack Arg_{cat} and this critical residue is supplied *in trans* by the so-called arginine finger of GAP proteins.[1,3,5,6]

In order to compare how different G-proteins stabilize the transition state, we used the same SDSI as above but visualized Gln_{cat} and Arg_{cat}, regardless of whether the latter is supplied *in cis* or *in trans* (Figure 2). Comparison of these two residues in these ten different transition-state structures shows that in all of them, the positioning of the catalytic functional groups of Gln_{cat} and Arg_{cat} is highly similar with respect to the substrate. This precise structural similarity implies similar stabilization of the transition state by all of these G-proteins. Therefore, the logical conclusion is that an analogous mechanism of GTP hydrolysis is shared by heterotrimeric G-proteins and small, Ras-like G-proteins. This similarity is especially striking considering the differences between the superimposed proteins (Figure 1(f)). In addition to their size difference (most heterotrimeric Gα proteins are more than twice as long as small G-proteins), heterotrimeric and small G-proteins share a limited sequence identity. Specifically, the small and heterotrimeric G-proteins compared here have ~15% sequence identity between them, calculated based on their structural alignment (data not shown, see also Figure 1(f)). In addition, as discussed above, the direction of insertion of Arg_{cat} into the active site is very different in these sub-families. Even more remarkable is the similar Arg_{cat} orientation employed by two bacterial GAPs, the *Pseudomonas* ExoS toxin (PDB IDs 1HE1) and the *Salmonella* SptP protein (PDB IDs 1G4U) that convergently evolved the insertion of an Arg_{cat} into the active site of small G-proteins. Even though their tertiary structure is not related to the eukaryotic GAPs,[22,23] the position of the guanido functional group of Arg_{cat} inserted by these bacterial GAPs precisely mimics the endogenous transition state stabilization. Furthermore, as some of the superimposed heterotrimeric

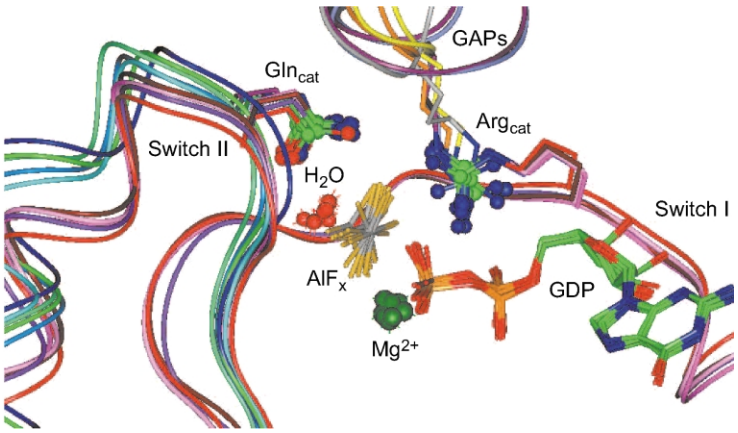

Figure 2. Gln_{cat} and Arg_{cat} orientation is similar in the transition-state structures. The orientation of the catalytic functional groups of Gln_{cat} and Arg_{cat} is highly similar in all ten superimposed structures, even though the proteins in question might differ in overall sequence and structure. The structures superimposed by SDSI are (see Materials and Methods for details): small G-proteins + GAPs: Ras (cyan) + Ras-GAP (gray), Rho (dark blue) + P50RhoGAP (yellow), CDC42 (green) + CDC42GAP (orange), Rac (blue) + ExoS (lavender), Rac (turquoise) + SptP (dark purple). Heterotrimeric G-proteins + GAPs (if present): transducin (purple), transducin (magenta) + RGS9 (not shown), transducin (pink) + RGS9 and PDE (not shown), Gi (dark red), Gi (red) + RGS4 (not shown). Backbone models for the relevant switch regions of ten G-proteins and of the finger loops of the relevant GAPs are depicted as ribbon diagrams. The functional groups of the catalytic glutamine and arginine, the magnesium atoms, and the nucleophilic water are drawn as ball and stick models. The GDP and aluminum fluoride are drawn as stick models.

G-protein structures in Figure 2 were crystallized with the catalytic domain of their respective RGS proteins (their GAPs), it is evident that, also in these G-proteins, a very similar transition state (and hence a similar catalytic mechanism) is reached, regardless of RGS participation.

Comparison of the ground states and transition states

If the different subfamilies of G-proteins employ similar transition-state stabilization, the substantial differences in catalytic rates between them[1,11,24] must correlate to differences in the rate-limiting step of the GTPase reaction. To discern the basis for these differences and for the catalytic mechanism of the GTPase reaction, one must understand the reaction pathway taking the substrate from the ground state to the transition state. We therefore compared snapshots of the ground state in the same G-proteins shown in Figure 2, provided by crystal structures with non-hydrolysable analogs of GTP (see Materials and Methods for details). Figure 3 shows the result of SDSI of the ten available ground-state structures, visualizing Gln_{cat} and Arg_{cat}. As detailed above, the sequence of small G-proteins does not contain Arg_{cat} and in most cases the affinity of GAPs to the ground state of small G-proteins is low. Therefore, there are no available ground-state structures for most small G-proteins with GAPs. For this reason, in the ground-state structures shown in Figure 3(a), Arg_{cat} is not seen in the structures of the small G-proteins, with the exception of the complex of CDC42 with P50RhoGAP (crystallized with GppNHp).[25]

In contrast to the transition-state structures, the orientation of the functional groups of Gln_{cat} and Arg_{cat} in the ground state is quite variable. More importantly, in all ten structures Gln_{cat} is not in its transition-state position (Figure 3(b)). In fact, in most of these structures the functional groups of both catalytic residues are between 2.5 Å and 4 Å away from their transition-state location, except for the small G-protein structures where Arg_{cat} is absent altogether. In the ground-state structures depicted in Figure 3(a), the average distance between the γ phosphorus atom of GTP and the C^{δ} atom of Gln_{cat} is 6.78 Å (standard deviation = 0.964). The average distance between the γ phosphorus atom and the C^{ζ} of Arg_{cat} (relevant of course only to the four structures where it is present) is 6.94 Å (standard deviation = 1.573). This contrasts with the corresponding distances and standard deviations in the transition-state structures (Figure 2), the average distance between the aluminum atom (analogous to the γ phosphorus atom of GTP) and the C^{δ} of Gln_{cat} is 4.5 Å (standard deviation = 0.134) and the average distance between the aluminum atom and the C^{ζ} of Arg_{cat} is 4.49 Å (standard deviation = 0.194). These results suggest that two general mechanistic traits of the GTPase reaction in G-proteins, as the reaction proceeds from ground state to transition state

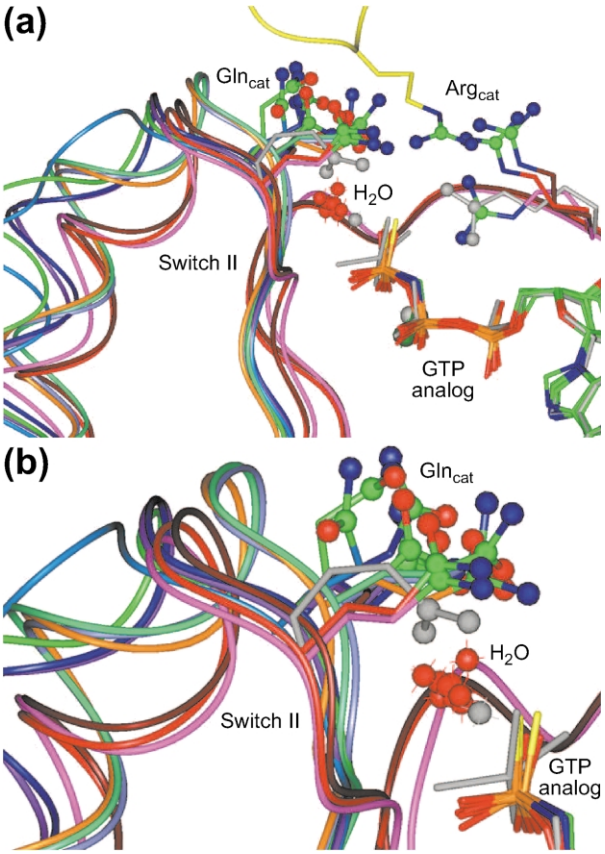

Figure 3. Gln_{cat} and Arg_{cat} orientation is variable in the ground-state structures. (a) The orientation of the catalytic functional groups of Gln_{cat} and Arg_{cat} is highly variable in the ten structures superimposed by SDSI, in contrast to their orientation in the transition-state structure (shown in gray for comparison). The structures are (see Materials and Methods for details): small G-proteins, Ras-GppNHp (green), Ras-GppCH$_2$P (blue), Ras-GTP at 100 K (dark blue), Ras-GppNHp at 100 K (purple), RhoA (lavender) + PKN (not shown), CDC42 (orange) + p50RhoGAP (yellow), Rac (turquoise). Heterotrimeric G-proteins, transducin (magenta), Gi-GTPγS (red), Gi-GppNHp (dark red). Backbone models for the relevant switch regions are depicted as ribbon diagrams. The functional groups of the catalytic glutamine and arginine, the magnesium atoms, and the nucleophilic water are drawn as ball and stick models. The GTP analogs are drawn as stick models. (b) Close-up of Gln_{cat}, showing its variable orientations, which are not similar to its transition-state orientation (shown in gray).

are: (1) the reorientation of Gln_{cat} and Arg_{cat} (2) variable position in the ground state as opposed to a specific orientation in the transition state. In fact, proceeding from the ground state of small G-proteins that do not include Arg_{cat} to the transition state (as depicted in Figure 2) can be considered an extreme case of Arg_{cat} reorientation between ground state and transition state. Interestingly, in many of these ground-state structures Gln_{cat} (and Arg_{cat} when present), and some of their surrounding residues and backbone are highly mobile as indicated by their high *B*-factors (see

below).[1,26] Therefore, it is possible that in some of these G-proteins the contribution of the reorientation of Gln_{cat} and Arg_{cat} to the free-energy barrier between the ground state and the transition state has some entropic component. The latter will be a result of the loss of entropy inherent in proceeding from a putative multiple conformation ground state to a single conformation transition state.

Flexibility in the ground state of Ras

To further explore these hypotheses we examined in more detail the GTPase reaction in the Ras protein. This proto-oncogene is an ideal target for SDSI because of the many Ras crystal structures available, both in the ground state and in the transition state. A close-up of Gln_{cat} in four different ground-state structures of Ras (Figure 4(a)) shows that the same protein can adopt different orientations in the ground state. In

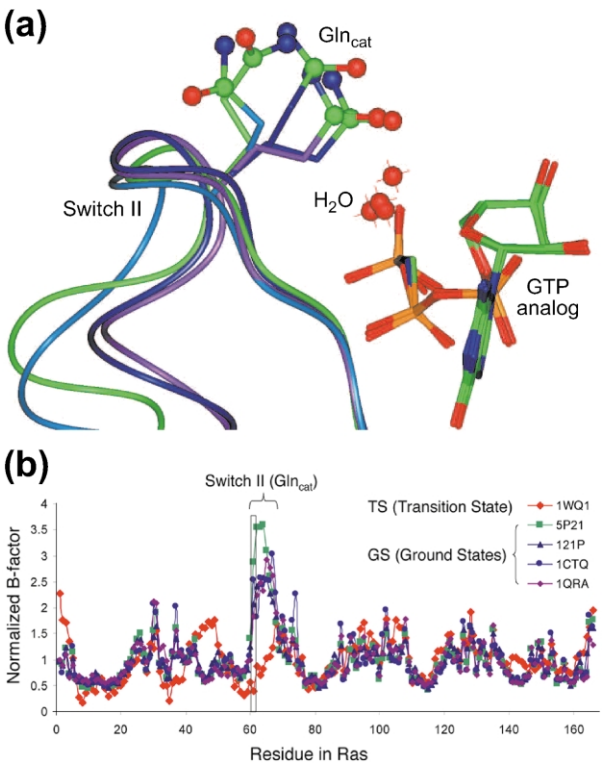

Figure 4. Multiple Gln_{cat} conformations in the ground states of Ras. (a) Gln_{cat} in four ground-state structures of Ras superimposed by SDSI, showing its variable orientation. The structures are: Ras-GppNHp (green), Ras-$GppCH_2P$ (blue), Ras-GTP at 100 K (dark blue), Ras-GppNHp at 100 K (purple). Backbone models for the relevant switch regions are depicted as ribbon diagrams. The structures are rotated 45° about the *Y*-axis compared to Figure 3(a). The functional groups of the catalytic glutamine and arginine, the magnesium atoms, and the nucleophilic water are drawn as ball and stick models. The GTP analogs are drawn as stick models. (b) Thermal factors of these four ground state Ras structures *versus* the transition-state structure. Normalized *B*-factors were calculated by dividing the *B*-factor for each residue by the average *B*-factor for the whole structure.

all of these orientations, the C^{δ} of Gln_{cat} is between 2.7 Å and 3.7 Å away from its transition state orientation. Furthermore, Gln_{cat} is part of the switch II domain and the latter was found to adopt multiple conformations in crystal structures.[27,28] By comparing the *B*-factors in the four Ras structures shown in Figure 4(a), one can see that in these ground states, not only Gln_{cat} but the whole switch II domain seem to be the most mobile in the whole protein (Figure 4(b)). The hyper-flexibility of this part of the Ras active site is corroborated by previous experimental studies using other methods: heteronuclear NMR found interconversion between conformers of Ras termed "regional polysterism" in large portions of the active site, including switch II.[29] Time-resolved crystallographic studies of Ras showed multiple conformations in switch II[30] and molecular dynamics simulations emphasized the highly flexible nature of the active site.[31–34] Also, the use of fluorescent GTP analogs led to a conclusion (that was not generally adopted) that there is a conformational rearrangement of Ras-GTP preceding and controlling the GTPase rate.[35,36] It is important to note that in the different studies, this ground state flexibility is not restricted to the side-chains but also involves the backbone itself, resulting in many possible conformations of this domain. In contrast, in the transition-state structure of Ras the switch II domain and Gln_{cat} are quite rigid, stabilized by their interaction with GAP (Figure 4(b)).[37] This suggests that the rearrangement of Gln_{cat} is not simply a switch from one orientation to another but rather the attainment of a specific transition-state orientation out of many possible ones. This can contribute an entropic component to the free energy barrier of the GTPase reaction in Ras and combined with the absence of Arg_{cat}, might explain why the intrinsic GTPase rate of Ras (without GAP) is so slow.

Point mutations in Ras impair the GTPase reaction

The critical catalytic role of Gln_{cat} is also supported by point-mutation studies, showing that mutations of Gln_{cat} reduce the intrinsic GTPase activity of Ras by about 100-fold and prevent GTPase acceleration by GAPs,[1,38–40] leading to a GTPase rate that is six to seven orders of magnitude slower that that of wild-type Ras *in vivo*. Moreover, the small G-protein Rap1A contains a threonine residue instead of the conserved Gln_{cat}, resulting in reduced GTPase activity. Mutation of this threonine residue to glutamine increases the GTPase rate to its value in Ras.[41] However, it is less obvious why the most common oncogenic mutations in Ras, mutations of glycine 12, also impair the GTPase reaction. A perplexing exception is the Gly12 → Pro mutation that, while also quite resistant to GAPs, has a higher intrinsic GTPase rate than wild-type and is uniquely not oncogenic.[42] Previous structural studies of the

oncogenic Gly12 mutants proposed that such mutations push Gln_{cat} away or block the approach of functional Gln_{cat}.[39,42] Model building of the mutant structures[43] or modeling of the Gly12 mutations on the transition-state structure suggested steric clashes with Gln_{cat} or Arg_{cat}.[37] Similar suggestions were put forth for the corresponding mutations in heterotrimeric G-proteins.[40] In contrast, it was recently put forward that mutations of Gly12 might interfere with a putative catalytic role of Gly13[33] and that Gly12 mutants do not introduce steric clashes in the active site.[44]

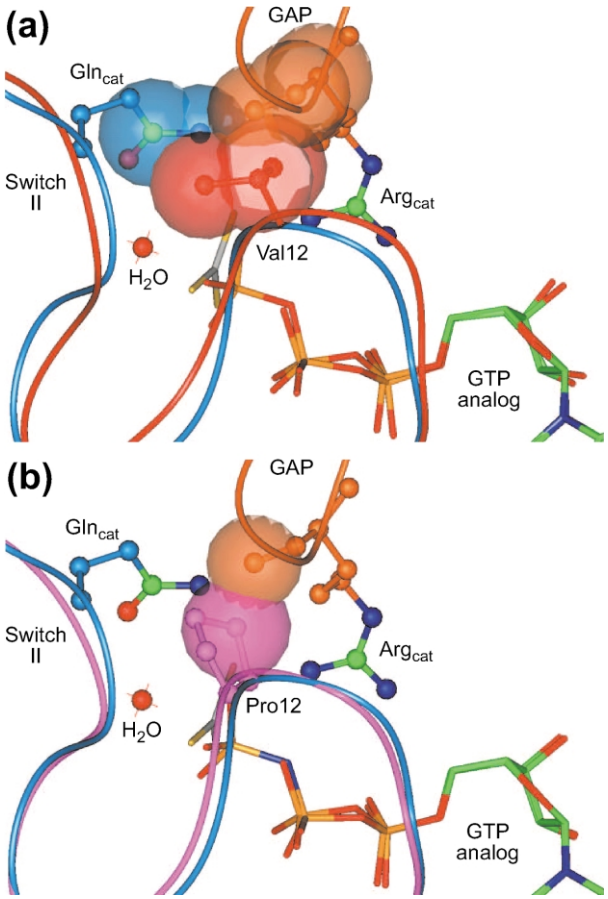

Figure 5. Steric interference with Gln_{cat} and Arg_{cat} reorientation in mutant Ras proteins. Structures of mutant Ras superimposed by SDSI onto the transition-state structure of Ras (see Materials and Methods for details). Gln_{cat} and Arg_{cat} in the transition-state structure and the mutated residue in position 12 are depicted as ball and stick models. Transparent spheres mark atoms of the mutant Ras structures and atoms of Gln_{cat} and Arg_{cat} in the transition-state structure that have a steric clash between them. (a) The structure of the Gly12 → Val oncogenic Ras mutant with GTP (red) superimposed onto the transition-state structure of Ras-RasGAP (blue). The valine side-chain is projected to interfere with the reorientations of Gln_{cat} and Arg_{cat} to their transition-state orientation. (b) The structure of the Gly12 → Pro non-transforming Ras mutant with GppNHp (magenta) superimposed onto the transition-state structure of Ras-RasGAP (blue). The proline side-chain is not projected to clash with atoms of Gln_{cat} in its transition-state orientation but is expected to present some interference with Arg_{cat} in its transition-state orientation, particularly with its backbone carbonyl hydrogen bond with Gln_{cat}.

To address these questions, we visualized the effect of these mutations on the GTPase mechanism in Ras by SDSI of mutant Ras structures onto the transition-state structure of Ras with Ras-GAP (Figure 5). In the most prevalent oncogenic mutant, Gly12 → Val, the added volume of the valine side-chain results in a marked steric clash with the transition-state position of both Gln_{cat} and Arg_{cat} (Figure 5(a)). Therefore, SDSI gives a direct visual rationalization for the impairment of both the intrinsic GTPase and the response to GAPs. Similar results are seen with other structures of oncogenic Ras mutants (data not shown).[39,42]

In contrast, the pyrrolidine ring of proline in the Gly12 → Pro mutant occupies a different position than the side-chain of valine (Figure 5(b)). Because of this difference, while still presenting some steric hindrance (albeit smaller) to the insertion of Arg_{cat} as in the transition state, Pro12 does not clash with the catalytic orientation of Gln_{cat}. On the other hand, the phi and psi angles of the proline or valine in position 12 of the mutants are similar to the corresponding angles of the glycine residue in the wild-type Ras. Therefore, SDSI indicates that steric interference to the proposed critical step in the GTPase reaction (the reorientation of Gln_{cat} and Arg_{cat}) does correlate with the biochemical data on oncogenic Ras mutants, as suggested previously.[37,39,42,43] Importantly, SDSI explicitly shows why the Gly12 → Pro Ras mutant does not have a reduced intrinsic GTPase rate. Additional support to this view is the observation that the oncogenic mutations in glycine 12 decrease the rate of GTPase towards the lower limit set by the slower GTPase rate in Gln_{cat} mutants.[45] Another backing for this structural interference hypothesis can be deduced from additional mutations in residues close to Gln_{cat} (that are expected to perturb its reorientation to its transition-state position), that also show a decrease in the intrinsic GTPase rate.[43]

Additional catalytic residues in Ras?

Recently, additional or alternative residues to Gln_{cat} and Arg_{cat} have been suggested as candidates for major catalytic contributors to GTPase catalysis by Ras. These suggestions were based either on infrared spectroscopy,[46–50] electron spin-echo envelope modulation (ESEEM)[33] or on *ab initio* modeling of the active site.[51] Specifically, Lys16 has been suggested as having a direct catalytic role in stabilizing the transition state.[49–51]

Visualization of Lys16 in Ras structures superposed by SDSI shows that in contrast to Gln_{cat}, its orientation compared to the substrate is invariant in the ground-state structures and in the transition-state structure (Figure 6(a)). Specifically, its distance from the γ phosphate that it binds does not vary. In fact, the position of this conserved lysine is practically invariant between ground

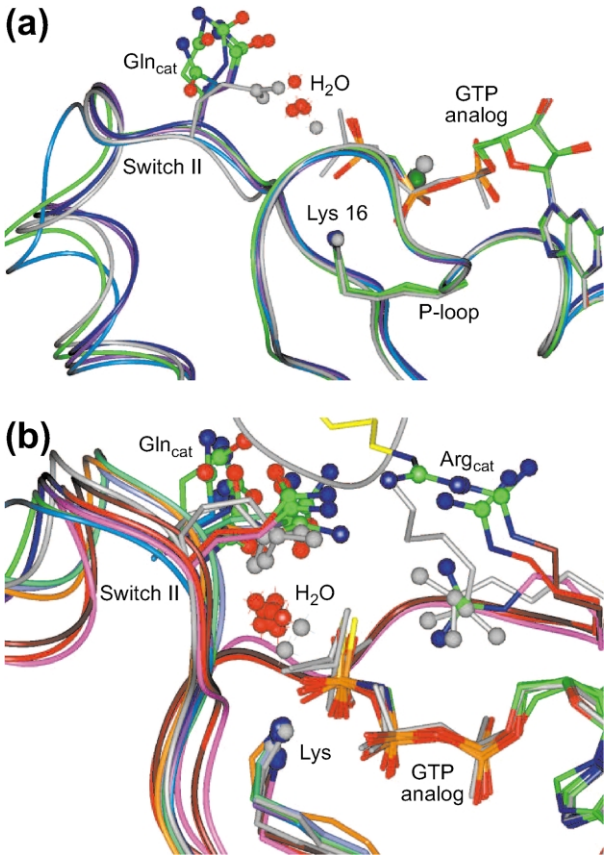

Figure 6. The orientation of the conserved P-loop lysine is invariant. (a) SDSI of four ground-state structures of Ras onto the transition-state structure of Ras-RasGAP (in gray). In contrast to Gln_{cat} and switch II, the backbone of the P-loop and the Lys16 residue contained in it are invariant between the different structures. The structures are (see Materials and Methods for details): Ras-GppNHp (green), Ras-GppCH_2P (blue), Ras-GTP at 100 K (dark blue), Ras-GppNHp at 100 K (purple). (b) The P-loop lysine position relative to the substrate is invariant in small G-protein and in heterotrimeric Gα proteins, whether in the transition-state structures or in the ground-state structures. The structures additional to (a) are Gi-GDP + aluminum fluoride (in gray), RhoA (lavender) + PKN (not shown), CDC42 (orange) + p50RhoGAP (orange), Rac (turquoise). Heterotrimeric G-proteins, transducin (magenta), Gi-GTPγS (red), Gi-GppNHp (dark red).

state and transition state in all the G-proteins we investigated (Figure 6(b)). The average distance in the ground-state structures between the γ phosphorus atom and the C^{ζ} of this lysine is 3.79 Å (standard deviation = 0.064) with lower than average *B*-factors. These results corroborate the critical role Lys16 is known to have in the binding of GTP.[1] On the other hand, a catalytic residue must preferentially stabilize the transition state over the ground state. During GTPase, the more significant changes in the charges of GTP will be either on the β-γ oxygen or on the γ phosphate, depending on the reaction's mechanism.[24] Our results do not show a change in the interaction between Lys16 and the substrate during GTPase. Additionally, we see that the interaction of this lysine with the substrate is invariant in different G-proteins in the ground state and in the GAP-stabilized transition state (Figure 6(b)). This invariance does not correlate with the slow intrinsic GTPase rate of Ras *versus* the considerable rate acceleration by GAPs. Therefore, if Lys16 has any catalytic role, its action does not seem to be influenced by GAP. However, it is possible that Lys16 or its equivalent in other G-proteins contributes to GTPase catalysis by a mechanism of ground state destabilization (see Discussion).

Insights from SDSI of Ras and UMP/CMP kinase

SDSI can also be used to compare distantly related enzymes that share little sequence identity. The nucleoside monophosphate kinase UMP/CMP kinase (UMPk) has less than 12% sequence identity with Ras, calculated based on their structural alignment (data not shown). UMPk catalyzes a different phophoryl transfer reaction than G-proteins, the reversible trans-phosphorylation between a nucleoside triphosphate and a nucleoside monophosphate (bound simultaneously at the active site).

We superimposed by SDSI the aluminum fluoride transition-state structures of Ras (with Ras-GAP) and UMPk. The SDSI resulted in alignment of most of the β-sheet core of the proteins (not shown) and of the P-loops of Ras and UMPk (Figure 7(a)). Such a correlation is not surprising considering that both enzymes share a common three-dimensional fold (the "P-loop containing nucleotide triphosphate hydrolase" fold). This is not the case for the LID and NMP-binding domains of UMPk[52,53] or the switch II domain of Ras (see above), containing the respective catalytic residues. These domains are specific and unique to each enzyme (Figure 7(a)). On the other hand, it is evident that the orientation of the conserved P-loop lysine in UMPk (Lys19) is practically the same as the orientation of this residue (Lys16) in Ras (and therefore in the other G-proteins, see Figure 6). This similarity is consistent with the ubiquitous role of the P-loop lysine in binding the terminal phosphate groups of nucleoside tri-phosphates[15,54] and does not support a unique catalytic role for this lysine in GTP hydrolysis by different G-proteins.

As depicted in Figure 7(a), the active site of UMPk contains five arginine residues with putative catalytic roles.[52] Unexpectedly, in the transition-state structures superposed by SDSI, the functional groups of two of these arginine residues occupy a similar position as the functional groups of Gln_{cat} and Arg_{cat} in Ras. The transition-state position of UMPk-Arg148 corresponds to Gln_{cat} while that of UMPk-Arg131 corresponds to Arg_{cat} (Figure 7(b)). In UMPk the distance between the aluminum atom and the C^{ζ} of Arg148 is 4.08 Å while in Ras the distance between the aluminum atom and the C^{δ} of Gln_{cat} is 4.48 Å. Furthermore,

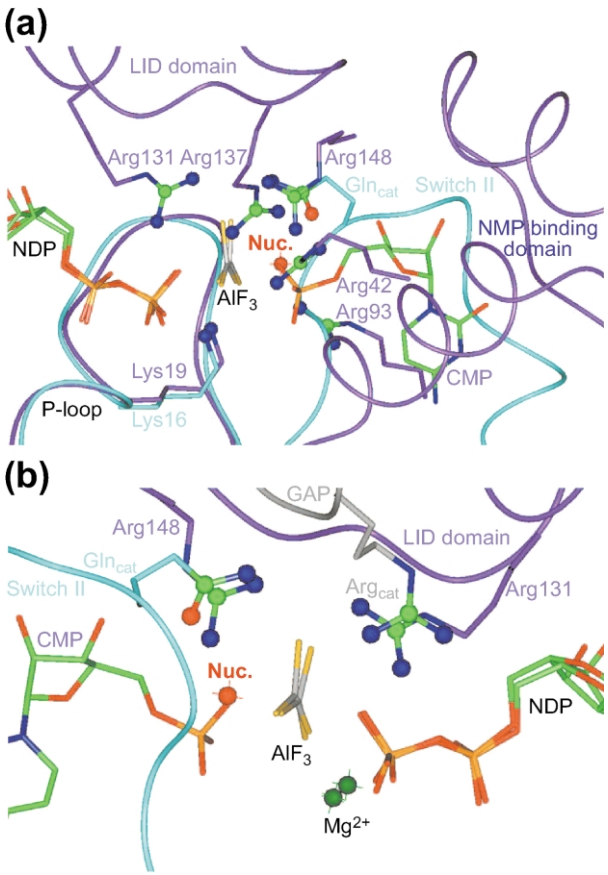

Figure 7. SDSI of the transition-state structures of Ras (cyan) and UMP/CMP kinase (UMPk) (purple). Backbone models for the P-loops, switch II of Ras and the LID and NMP-binding domains of UMPk are depicted as ribbon diagrams. The functional groups of the catalytic glutamine and arginine residues, the magnesium atoms and the nucleophilic water are drawn as ball and stick models. The nucleotides and aluminum fluoride are drawn as stick models. The nucleophiles (water in Ras and the oxygen atom of the α phosphate of CMP in UMPk) are labeled by Nuc. (a) A similar conformation of the P-loops and P-loop lysine residues (residues 16 in Ras and 19 in UMPk) in seen in both structures. The switch II domain in Ras and the LID and NMB-binding domains in UMPk have no correlated domains in the superimposed structures. Also visualized are Gln_{cat} of Ras and the five putative catalytic arginine residues of UMPk. (b) The orientation of the functional groups of Gln_{cat} (Ras) and Arg_{cat} (Ras-GAP) relative to the substrate is highly similar to that of Arg148 and Arg131 (UMPk), respectively. The position of the nucleophilic oxygen atoms is also nearly the same. The finger loop of Ras-GAP (gray) is depicted as a ribbon diagram. The structures are rotated 180° about the *Y*-axis compared to (a).

in both UMPk and in Ras the distance between the aluminum atom and the C^{ζ} of the respective arginine residue is exactly the same, 4.64 Å. Consistent with a critical catalytic role, mutation of either of these two arginine residues in UMPk results in 10^4–10^5 decrease in catalytic efficiency.[52] These findings highlight the fact that these superimposed residues, even when they are chemically different, apparently play similar mechanistic roles in these phosphoryl transfer reactions. Arg131/Arg_{cat} have the role of stabilizing the negative charges that accumulate on the NTP substrate in the transition state while Arg148/Gln_{cat} reduce the energy barrier for the nucleophilic attack on the γ phosphate of the NTP. With the benefit of hindsight, it is logical that in UMPk the role played by a neutral Gln_{cat} in G-proteins is taken instead by a positively charged arginine, since more negative charges are present on the nucleophile and in its immediate environment in UMPk.

Interestingly, there is another resemblance between G-proteins and UMPk. The conserved arginine residues in UMPk also undergo rearrangement as part of their catalytic cycle and are in domains of UMPk shown to be flexible.[52,53] This flexibility was suggested by quantum chemical investigations to be crucial for catalysis.[55] In contrast to G-proteins, however, it is difficult to correlate this rearrangement solely with a direct catalytic role, as these conformational changes are also known to be related to substrate binding.[56]

Discussion

In contrast to most enzymes, the enzymatic GTPase reaction in G-proteins is used as a regulatory mechanism for their signaling functions rather than as a mean to accelerate the making of product. Accordingly, compared to other enzymes, G-proteins rank between mediocre catalysts and very inefficient catalysts (Ras being a prototypical example of the latter).[3,24] In view of these facts, any model for the mechanism of GTPase should answer the following questions: (1) which residues provide the major contribution to catalysis? (2) Why is the intrinsic GTPase so slow in small G-proteins? (3) What limits the rate of GTP hydrolysis and thereby results in different rates in different G-proteins? (4) How do GAPs (and RGSs) accelerate the GTPase rate by orders of magnitude? (5) Why do specific point-mutations in G-proteins impair the GTPase mechanism? (6) What additional catalytic contributions could be responsible for the remaining activity in mutant G-proteins?

This work illustrates how SDSI can complement existing knowledge to answer these questions by comparing multiple G-protein structures and understanding the commonalities and differences in their active sites and catalytic mechanisms. SDSI reveals that different G-proteins (both small and heterotrimeric) stabilize the transition state of the GTPase reactions similarly, as has already been observed by comparing the structures of Ras and Gi[37] and CDC42 and Gi.[57] This stabilization is achieved by employing two critical catalytic residues that directly interact with the substrate in the transition state, Gln_{cat} and Arg_{cat}. A different role was suggested in a recent computer simulation

study proposing that in Ras, Gln_{cat} has an indirect structural effect.[58] We show that variability in the positioning of Gln_{cat} and Arg_{cat} in the ground state and reorientation of these residues as the reaction proceeds from ground state to transition state is a mechanistic trait common to all these G-proteins. Combined with the essential role of Gln_{cat} and Arg_{cat} in GTPase catalysis,[1,3,5] our results augment these previous suggestions by assigning the role of the critical step in the intrinsic GTPase reaction to the slow conformational change in the G-protein, preceding and necessary for the exact positioning of these functional groups in their catalytic positions.

Our study of the ground state of Ras raises the possibility that its catalytic inefficiency is more pronounced because of the flexibility of its active site in the ground state. Previously, connections between enzyme motion and catalysis have been suggested as components of catalytic mechanisms.[59,60] However, here we suggest that the dynamic flexibility of the switch domains in G-proteins, which is known to be important for the switching and signaling activity of G-proteins,[1,9] may well have an additional role. This flexibility might also enable a slower turn-off of the Ras protein, permitting it to reach and activate its downstream effectors. Therefore, the model our results support maintains that the function of GAPs is not only to insert Arg_{cat} into the active site of small G-proteins but also to reduce the variability in the orientation of the catalytic residues and anchor Gln_{cat} at its catalytic position.[1,3,5,6,9] This model is also supported by evidence showing that mutation of Arg_{cat} in GAPs reduces their GTPase acceleration by three orders of magnitude, leaving ~40% of the rate enhancement unaccounted for.[61,62] It is therefore logical that the remaining activity is mainly an affect on Gln_{cat} reorientation, as suggested previously (see above). Distinctly, the GAP activity on small G-proteins not attributed to Arg_{cat} ($\sim 10^2$ rate acceleration) is comparable to the GAP activity of RGS proteins on heterotrimeric G-proteins.[4,63] As RGS proteins were also suggested to accelerate GTPase by reducing the flexibility of the switch regions and by stabilizing the catalytic orientation of the catalytic residues,[4,9,63] it seems that indeed both families of GAPs share this mechanistic trait.

Our results also show the structural basis for the impairment of GTPase in oncogenic Ras mutants and why GAPs can no longer accelerate their "turn off". As seen in the Ras-RasGAP structure, Gln_{cat} also reciprocates by binding and stabilizing Arg_{cat}.[37] Mutant Ras either had Gln_{cat} mutated to another residue (that cannot stabilize the transition state) or contains mutations that are shown by SDSI to sterically prevent both the insertion of Arg_{cat} and the reorientation of Gln_{cat} (Figure 5), as suggested previously.[37,43,64] Conversely, SDSI shows that a much-reduced steric interference can explain why the Gly12 → Pro Ras mutant has a higher intrinsic GTPase rate than the other Gly12 Ras mutants and why it retains some responsiveness to GAPs.[42]

Another question that needs addressing is whether there are additional contributions to GTPase catalysis other than those made by Gln_{cat} and Arg_{cat}. G-proteins can accelerate the measured GTPase k_{cat} by about 10^{10} at the most, as compared to GTP hydrolysis in solution.[24] Seeing that in mutant G-proteins without a functional Gln_{cat} and Arg_{cat}, GTPase rate is 10^6 slower, it seems that additional contributions account for $\sim 10^4$ rate acceleration. One such contribution was suggested to be the involvement of the Ras-bound GTP substrate, acting as a general base for its own hydrolysis.[65,66] While this was shown not to be the rate-limiting step,[67] linear free energy relationships showed a correlation between the pK_a values of the bound GTP and the GTPase rate in Ras mutants.[68] These results supported the proposed contribution of the enzyme-bound GTP to catalysis.

Alternatively, Raman and IR difference spectroscopy studies have shown that the nucleophilic water seems to be already positioned for attack in the ground state of Ras, with the beginning of a bond formed between it and the γ phosphate.[46,69] Slightly different results from Fourier transform IR spectroscopy have correlated stronger binding of the γ phosphate of GTP in Ras with a putative early state in GTP hydrolysis.[47–50] Lys16 was advocated as a principal contributor to catalysis by participating in this interaction. However, these methods can only observe either the ground state or the products of the GTPase reaction. Therefore, application of these methods to predict the interaction of the protein with the extremely short-lived transition state and for drawing extensive conclusions on the reaction mechanism is not conclusive. As our results show, Lys16 does not change orientation between the ground state and the transition state. Additionally, the more significant changes in the GTP's charges will be either on the β-γ oxygen atom or on the γ phosphate, depending on the reaction's mechanism.[24] Therefore, our results do not offer support for the proposed major catalytic role of Lys16 because in order for it to be catalytic and reduce the free energy barrier, it must have a significantly stronger interaction with the transition state than with the ground state. Moreover, the invariance of the spatial interaction between Lys16 and GTP does not correlate with the former being a major contributor to catalysis because it does not explain the slow intrinsic GTPase rate of Ras *versus* the considerable rate acceleration by GAPs.

On the other hand, either of the above proposals is consistent with a mechanism of ground state destabilization as a minor contributor to catalysis, in addition to the major contribution of Gln_{cat} and Arg_{cat}. By stabilizing a higher-energy ground-state structure, which is closer in free energy to the transition state, an enzyme can contribute to a reduction in the free energy barrier. It has been

argued that such a mechanism is anti-catalytic because it reduces the free energy of activation at the expense of substrate binding.[70] However, G-proteins are an exception to this rule when compared with other enzymes. Their binding of substrate requires interaction with GEFs in order to take place and is therefore completely separated in time from catalysis. Therefore, ground state destabilization is a valid and possible contributor to catalysis in G-proteins and can account for some of the enzymatic activity not due to Gln_{cat} and Arg_{cat}. Ground state destabilization is also supported by the fact that the nucleotide dissociation rate constant for Ras was found to be threefold higher for GTP than for GDP (at 37 °C),[71] implying that the affinity of Ras for GTP is lower.

Intriguingly, SDSI enabled us to compare the catalytic machinery of G-proteins with that of UMPk. The highly similar orientation of the conserved P-loop lysine in both UMPk and Ras emphasize the ubiquitous role of the P-loop lysine in substrate binding and at the same time does not corroborate the unique catalytic role suggested for this residue in GTPase catalysis by Ras. Unexpectedly, the similarities we found in the positions of catalytic functional groups in UMPk and Ras highlight a generic role in phosphoryl transfer reactions for these residues and indicate what are the probable catalytic roles played by Arg131 and Arg148 in UMPk. Moreover, our comparison between UMPk and Ras underscores how SDSI enables an unbiased comparison between distantly related enzymes that share little sequence identity, while binding comparable substrates.

Thus, our study advocates the use of SDSI as part of the arsenal of available structure analysis methodologies that can be applied to other families of proteins in order to comparatively analyze their active sites and learn more about their catalytic mechanisms.

Materials and Methods

Selection of G-protein structures

SDSI refers to the superimposition of protein structures that bind comparable substrates, using only the coordinates of the substrates. The protein structures for superimposition were carefully selected according to the substrate they bind. For this study, we exclusively used G-proteins for which both transition-state structures (with the transition-state analog GDP–aluminum fluoride) and ground-state structures (with non-hydrolysable GTP analogs) were available, because these are the two states relevant for deciphering the GTPase mechanism. Structures with the reaction's product (GDP) are not informative for this specific question. On the other hand, when a G-protein had been crystallized with different GTP analogs (e.g. GppNHp and GTPγS), all representative structures of that G-protein were utilized under the assumption they can give different snapshots of what is in reality a dynamic structure. Only protein structures with resolution better than 3.0 Å were used.

The transition-state G-protein structures (with GDP–aluminum fluoride) selected are: 1WQ1, Ras + RasGAP120;[37] 1TX4, Rho + RhoGAP;[72] 1GRN, CDC42hs + CDC42GAP;[57] 1HE1, Rac + ExoS;[23] 1G4U, Rac + SptP;[22] 1TAD, Gt;[73] 1FQK, Gt + RGS9;[74] 1FQJ, Gt + RGS9 + PDE;[74] 1GFI, Gi;[26] 1AGR, Gi + RGS4.[63] The ground state G-protein structures selected are (with the GTP analog in parentheses): 5P21, Ras (GppNHp);[27] 121P, Ras ($GppCH_2p$);[75] 1QRA, Ras (GTP, at 100 K);[28] 1CTQ, Ras (GppNHp, at 100 K);[28] 1AM4, CDC42Hs + p50RhoGAP (GppNHp);[25] 1CXZ, RhoA + PKN (GTPγS);[76] 1MH1, Rac (GppNHp);[77] 1TND, Gt (GTPγS);[78] 1GIA, Gi (GTPγS);[26] 1CIP, Gi (GppNHp, at 100 K).[79] Mutant Ras structures are: 521P, Gly12 → Val mutant;[39] 821P, Gly12 → Pro mutant.[42] The transition-state structure (with ADP, CMP and aluminum fluoride) of UMP/CMP kinase selected is 3UKD.[52]

Substrate-directed superImposition (SDSI)

Prior to superimposing the different structures, each was examined and all the atoms in the substrates that differ significantly in their chemical structure were not used for the superimposition. For example, in the structures of G-proteins with the transition-state analog GDP and aluminum fluoride, the latter is either AlF_3 or AlF_4^-, depending on the pH of crystallization.[80] Therefore, only the atoms of the GDP moiety and the aluminum atom were used for the superimposition. Superimposition and visualization were performed using InsightII (Accelrys).

After superimposition, the conformations of the superposed substrates were inspected to ensure they are indeed comparable (see Figure 1(a) for an example). Using GTP analogs as the superimposed substrates (~30 superposed atoms), an RMSD of less than 1.0 Å was considered adequate if the superposed substrates are identical. For non-identical substrates (e.g. GppNHp and GTPγS) an RMSD of less than 1.5 Å was considered adequate. If the substrates are not in a similar conformation, superimposition was performed using only the regions of the substrates that are comparable (in structure and conformation) and only the protein environments of these zones were considered. Accordingly, in the superimposition of Ras and UMPk (Figure 7) only the coordinates of the phosphate groups and the aluminum were used for superimposition. This resulted in a slightly improved superimposition as judged visually (RMSD = 0.223 Å) because the riboses and bases of the bound nucleotides are in a somewhat different conformation (the RMSD for the superimposition of the whole di-nucleotides and aluminum was 1.217 Å).

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