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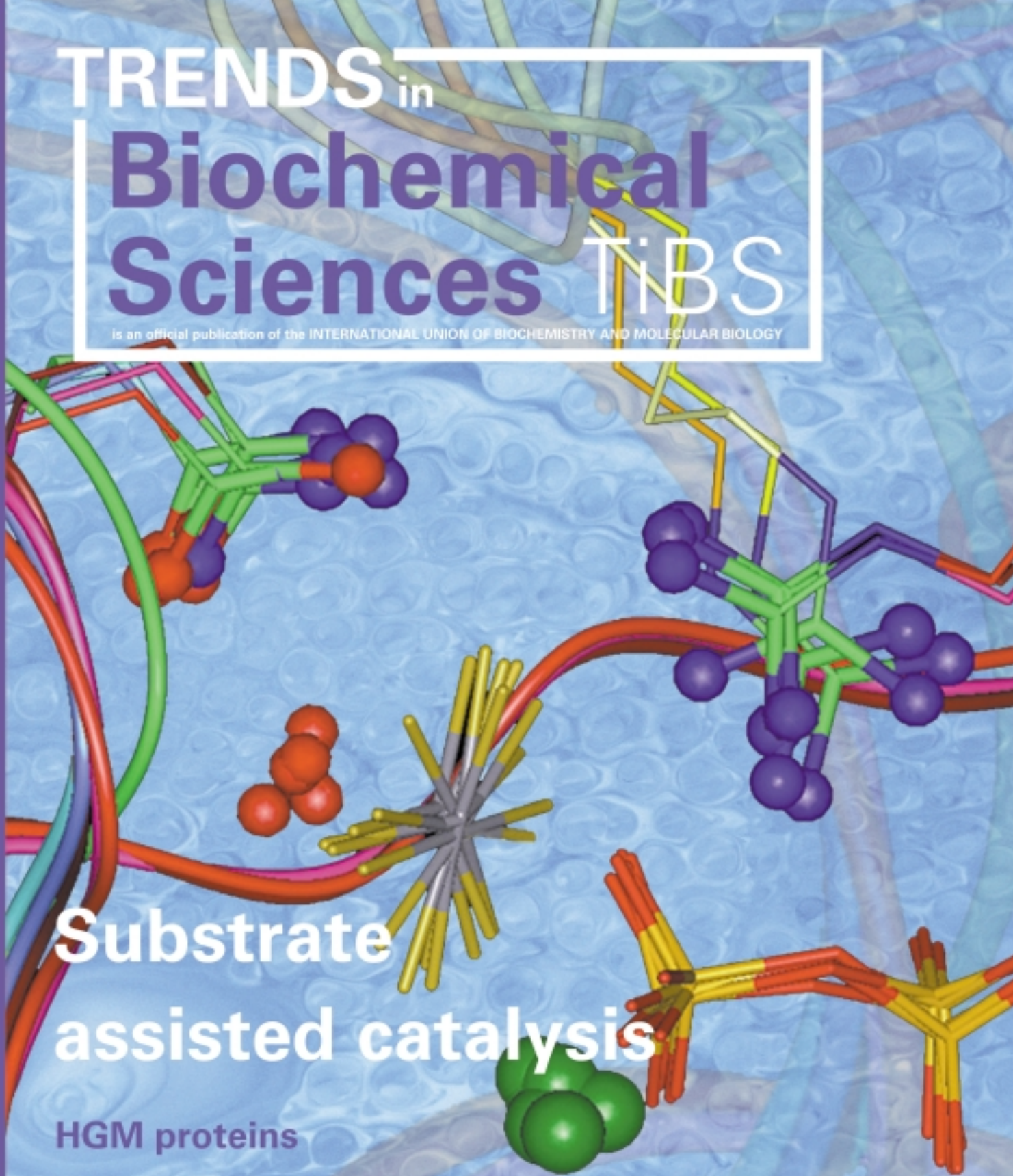

## Substrate assisted catalysis

HGM proteins

Metabolic modeling

WASP proteins

# Substrate assisted catalysis – application to G proteins

Mickey Kosloff and Zvi Selinger

**The idea that both the substrate and the enzyme contribute to catalysis (substrate assisted catalysis; SAC) is applicable to guanine nucleotide-binding proteins (G proteins). Naturally occurring SAC uses GTP as a general base in the GTPase reaction catalyzed by G proteins. Engineered SAC has identified a putative rate-limiting step for the GTPase reaction and shown that GTPase-deficient oncogenic Ras mutants are not irreversibly impaired. Thus, anti-cancer drugs could potentially be designed to restore the blocked GTPase reaction.**

Any list of the seven wonders of biology would have to include enzyme catalysis. Enzymes can convert a substrate into product $10^{10}$–$10^{15}$ times faster than the rate of the uncatalyzed reaction. This means that enzymes can accomplish in one second what would take 300–30 000 000 years in their absence[1]. Despite much effort to discover the basis for the astounding catalytic efficiency of enzymes, this issue is still hotly debated and far from being resolved[2].

According to the conventional view of enzyme catalysis, the enzyme supplies all the functional groups that are needed to convert a substrate into a product. In a growing number of cases, however, it is evident that the substrate provides one or more functional groups that actively participate in the catalytic process. These cases, collectively termed substrate assisted catalysis (SAC), can be categorized either as natural SAC, arising through evolution (for examples, see Ref. 3); or as engineered SAC, applicable to enzymes rendered inactive by mutations (Fig. 1).

Engineered SAC entails the use of a modified substrate, bearing functional groups similar to those eliminated by mutation of the enzyme, to rescue enzymatic activity. In engineered SAC, the catalytic involvement of the substrate is self-evident, because the mutant enzyme is active only on substrates bearing the missing functional group and not on the unmodified natural substrate. As described below, G proteins are an example in which both types of SAC are relevant. Natural SAC has been studied in the small G protein Ras, whereas engineered SAC has been applied as a tool to study two G proteins: $G_s$ and Ras.

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## G proteins have evolved a useful catalytic inefficiency

G proteins are transducers of a wide range of cellular transactions, including transmembrane signaling, cell proliferation, intracellular trafficking and cytoskeletal organization. G proteins can be classified into two families: the heterotrimeric G proteins (of which the adenylyl cyclase stimulatory G protein, $G_s$, is a prototypic example) and the monomeric G proteins [for which Ras and elongation factor Tu (EF-Tu) serve as prototypes][4]. Despite their functional diversity, all G proteins behave as conformational sensors of the bound guanine nucleotide. Depending on whether they are charged with GDP or GTP, they change their conformation and consequently their interaction with other proteins in the signaling cascade[4] (Fig. 2). G proteins charged with GTP are in the 'on' state, capable of acting on their downstream effectors. Hydrolysis of the bound GTP by GTPase activity switches the G protein to the 'off' state, characterized by tightly bound GDP (Ref. 5).

The catalytic efficiency of most enzymes has been maximized through evolution. In quantitative terms, most enzymes exhibit a maximal value of $k_{cat}/K_m$ (Ref. 6). G proteins are an exception to this rule. Because they use the GTPase reaction as a switch mechanism with concomitant changes in conformation (from the GTP- to GDP-bound states), G proteins have evolved a highly flexible and mobile active site. This flexibility could be the reason for their catalytic inefficiency, although this hypothesis needs further study. The catalytic inefficiency of G proteins has physiological advantages. A G protein must remain in the active, GTP-bound state as long as is necessary for its function, sometimes for many minutes. Importantly, the lifetime of the activated state, extended by catalytic inefficiency, can be further modulated by GTPase-activating proteins (GAPs), whereas the 'off' state can be activated by guanine nucleotide exchange factors (GEFs) (Fig. 2).

The pivotal GTPase reaction, first described 25 years ago[7], has attracted considerable attention because of its role in determining the lifetime of the active G protein. Any disruption of the GTPase reaction results in a persistent activation of downstream effectors because the G protein is not switched off. Such a situation is perceived as a gain-of-function mutation at the effector level, although the primary effect is a loss-of-function at the level of the GTPase reaction. Several GTPase-deficient G protein mutants, mainly in Ras and $G_{s\alpha}$, have been implicated in tumor formation and other diseases[8,9]. In particular, the GTPase reaction of Ras is of great medical importance. Approximately 30% of all human tumors contain mutations in Ras that result in deficient GTPase activity. Furthermore, these oncogenic mutants cannot be switched off by

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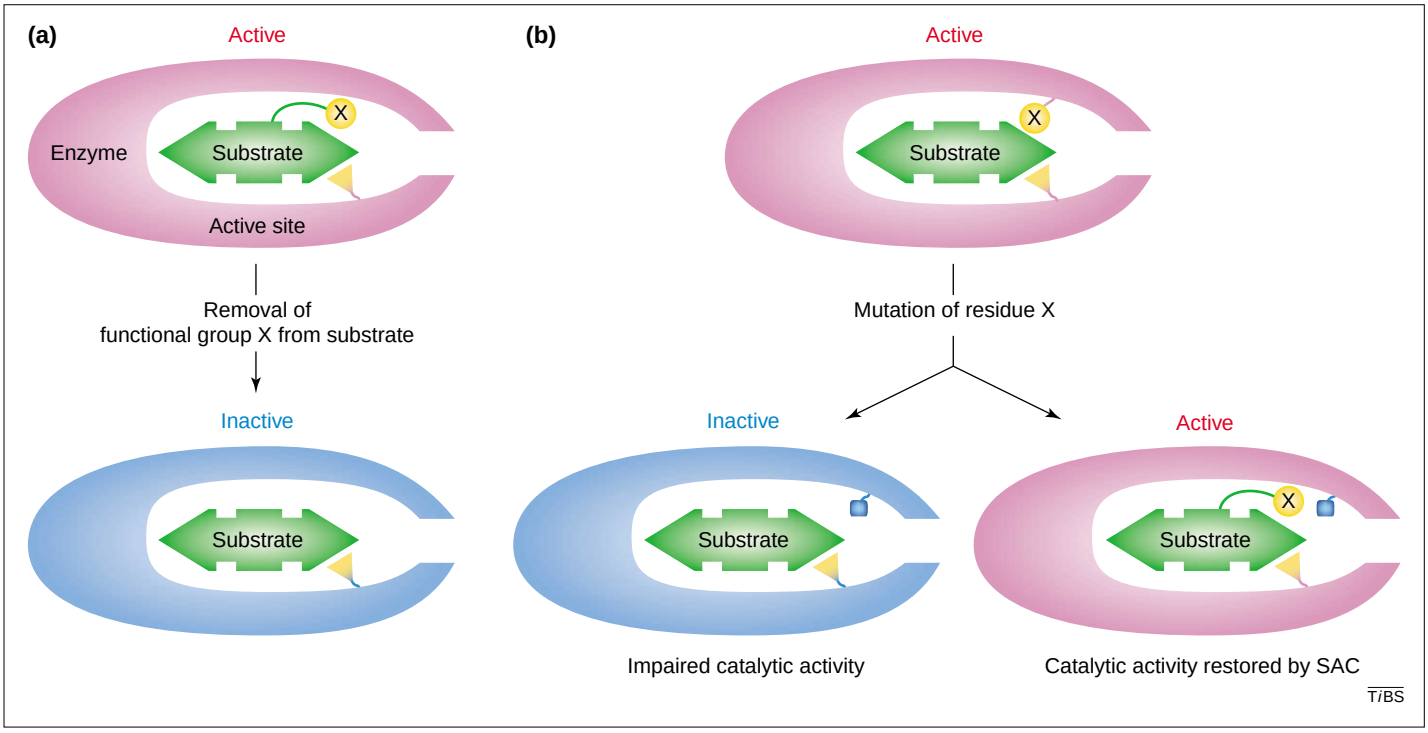


**Fig. 1.** (a) Natural substrate assisted catalysis, SAC (experimental verification). In some enzymatic reactions the substrate bears a functional group (marked X) that actively participates in the catalytic process. Any change in the substrate that removes or changes this functional group results in impairment of the catalytic activity, thus providing an experimental verification for SAC. (b) Engineered SAC. Enzymatic catalysis, the conversion of a substrate into product, is dependent on several functional groups in the active site of the enzyme. When a residue bearing a catalytic functional group (marked X) is eliminated by mutation, catalytic activity is impaired. Placing the missing functional group correctly in the mutated active site restores catalytic activity. In engineered SAC, this functional group is brought into the active site by the substrate bearing the group eliminated by mutation.

GAPs. Consequently, GTPase-deficient G proteins persistently drive cell division and contribute substantially to the neoplastic phenotype of tumor cells. This has given rise to the idea that restoring the GTPase activity of oncogenic Ras mutants might be a useful approach to cancer therapy[10].

### Anatomy of the active site of G proteins

To achieve catalysis, an enzyme has to reduce the free energy of activation for the enzymatic reaction, relative to the spontaneous uncatalyzed process. Therefore, the residues crucial for catalysis must differ in their interaction with the substrate in the ground state (reactants) and the transition state[2,11]. The two residues in G proteins that are directly involved in catalysis are a glutamine and an arginine ($Gln_{cat}$ and $Arg_{cat}$, respectively), which have been conserved throughout the G protein family and relevant GAPs (Ref. 4). In heterotrimeric G proteins, both residues originate in *cis* from the G protein itself. By contrast, the Ras-like monomeric G proteins lack $Arg_{cat}$ and this crucial residue is supplied in *trans* by the so-called 'arginine finger' of GAP proteins[12] (Figs 2b and 3). The orientation of these two residues is similar in different crystal structures of G proteins with the transition state analog GDP–aluminum fluoride[13] (Fig. 3). The positioning of the catalytic functional groups of the conserved $Gln_{cat}$ and $Arg_{cat}$ in relation to the substrate is highly similar in all cases. This precise structural similarity implies that the same transition state, and therefore a similar mechanism of GTP hydrolysis, is shared by all of these G proteins. The suggestion that such a common mechanism evolved from a shared ancestor is corroborated by the high sequence similarity of the active site residues of different G proteins[4].

A comparison of G-protein structures with GDP–aluminum fluoride, which mimic the transition state, with structures of G proteins in the ground state, highlights the catalytic importance of $Gln_{cat}$ and $Arg_{cat}$. It reveals that the interaction of these two residues with the substrate is different in the two states. $Gln_{cat}$ is located in a region of G proteins termed switch II (Fig. 3)[4]. It is crucial for efficient catalysis, because mutation of the conserved glutamine to almost any other amino acid decreases the intrinsic GTPase rate by up to two orders of magnitude, and abolishes GTPase acceleration by GAPs (Refs 4,10,14). The general view is that the function of $Gln_{cat}$ is to polarize the nucleophile and orient it for a more efficient in-line attack on the γphosphate[4,15–17]. In other words, $Gln_{cat}$ is a major contributor to an electrostatic 'envelope' around the nucleophile and substrate that stabilizes preferentially the transition state, and thus lowers the activation energy for its formation. A different role was suggested in a recent computer simulation study, proposing that $Gln_{cat}$ has an indirect structural effect[18].

$Arg_{cat}$ is the second substantial contributor to the electrostatic 'envelope', stabilizing the transition state. Absence of $Arg_{cat}$, its mutation or its modification directly affects the GTPase rate. The

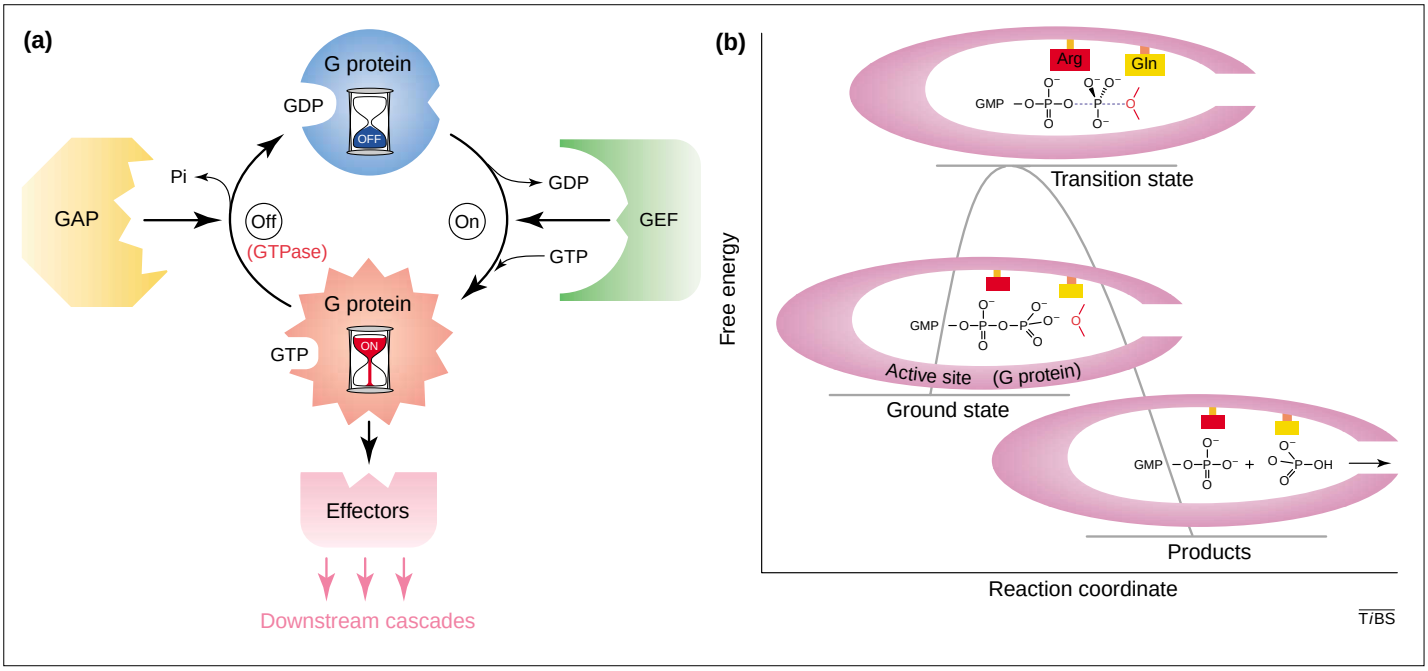


**Fig. 2.** (a) The regulatory GTPase cycle. A G protein functions as a molecular switch[23]. It is held in the 'off' position by GDP, which is tightly bound in the active site. Exchange of the bound GDP for GTP turns the G protein to the 'on' position. This checkpoint reaction is enhanced by guanine nucleotide exchange factors (GEFs) specific to each G protein. When it is turned 'on', the G protein interacts with the various effectors of the downstream signaling cascades. The duration of the G-protein action on the cascade is controlled by an internal timer – the intrinsic GTPase reaction that gives rise to free inorganic phosphate and tightly bound GDP. Turning 'off' the internal timer can be accelerated by orders of magnitude by GTPase-activating proteins (GAPs). Conversely, impairment of the GTPase reaction will slow down the timer. The result will be excess G protein in the 'on' state and hyperactivation of the downstream cascades. This can lead to abnormal and sometimes pathological phenotypes. (b) The GTPase mechanism. When in the 'on' state, GTP is bound in the active site of the G protein (ground state). The β and γ phosphates of GTP are shown in detail. The hydrolysis of GTP is a concerted reaction involving an in-line nucleophilic attack by a water molecule on the γ phosphate, and a configuration inversion of the latter[4]. The phosphoanhydride bond is hydrolyzed via a pentavalent transition state. The transition state is preferentially stabilized by $Gln_{cat}$ and $Arg_{cat}$. $Arg_{cat}$ can reach the active site in two ways: (1) from the G protein itself, in *cis* to $Gln_{cat}$, as occurs in heterotrimeric G proteins; and (2) originating from GAP, in *trans* to $Gln_{cat}$, as occurs in Ras-like small G proteins (Fig. 3; Ref. 12). The products of GTPase are GDP and inorganic phosphate. The phosphate is rapidly released from the active site, leaving behind the turned 'off' G protein that stably binds GDP. $Gln_{cat}$ and $Arg_{cat}$ are not marked in the ground state and in the product state to indicate that they are not in catalytic orientation.

first case occurs in small Ras-like G proteins, in which $Arg_{cat}$ is absent and is introduced into the active site by GAPs (Ref. 12; Fig. 3). Point mutations or modification of $Arg_{cat}$ by cholera toxin reduce the GTPase rate by up to two orders of magnitude[19–22]. By its positive charge and donation of H-bonds, $Arg_{cat}$ neutralizes the negative charge that builds on the phosphate oxygen atoms of GTP during the reaction[18]. The absence of $Arg_{cat}$ is a significant contributor to the slower intrinsic GTPase rate (in the absence of GAP) of small G proteins.

### SAC restores activity to mutant G proteins

The rate of GTP hydrolysis directly determines the lifetime of the 'on' state of the G protein[23]. Elucidating the rate-limiting step of the GTPase reaction in physiological conditions is necessary for understanding the intrinsic reaction, as well as for understanding how different GAPs act and for targeting the blocked GTPase by drug design[10]. Engineered SAC offers a unique functional tool to investigate the identity of the rate-limiting step in the GTPase reaction and the roles of the catalytic residues. However, limitations are inherent in the use of substrate analogs to study the mechanisms of enzymatic reactions[24].

Using modified GTP analogs, SAC was applied to two representatives of G-protein families: the heterotrimeric $G_s$ (Ref. 25) and the prototypic small G protein Ras (Ref. 10). The first study showed that the GTP analog 3,4-diaminobenzophenone-phosphoroamidate-GTP (DABP-GTP) restored activity to the GTPase-deficient Gln227Leu $G_s$ mutant missing $Gln_{cat}$ (Fig. 4). The hydrolysis rate of this mutant was restored to the wild-type level. Covalent modification of $Arg_{cat}$ (Arg201 in $G_s$) by cholera toxin reduced the GTPase rate by 25-fold, as measured either with GTP or with DABP-GTP. These findings show that DABP-GTP substituted for the function performed by $Gln_{cat}$, but did not bypass the catalytic requirement for $Arg_{cat}$. Further studies showed that an exocyclic aromatic amine or, alternatively, a hydroxyl group, covalently attached to the GTP substrate (Fig. 4), is necessary and sufficient for functional replacement of $Gln_{cat}$ (Ref. 24).

These studies were extended by using SAC to investigate the intrinsic GTPase in the Ras protein[10]. This protein has long been of interest because of the high percentage of GTPase-deficient Ras mutants in human tumors. DABP-GTP rescued oncogenic, GTPase-deficient Ras mutated in $Gln_{cat}$ (Gln61→Leu/Ala/Asn). The hydrolysis rate was restored to wild-type levels, alleviating the need for the missing $Gln_{cat}$. In Ras mutated in Gly12, the GTPase rate is severely impaired[10]. In fact, Gly12 mutants of Ras are the most common oncogenic Ras mutations found in human tumors. Strikingly, in some of these mutants the rate of DABP-GTP

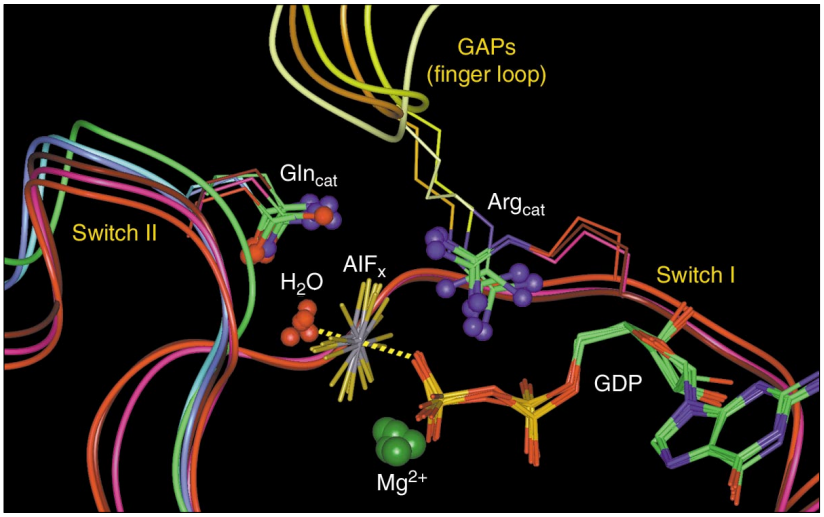


**Fig. 3.** Superimposition of six different G-protein structures complexed with the transition state analog GDP–aluminum fluoride and with relevant GTPase-activating proteins (GAPs). All G proteins are complexed with GDP and aluminum fluoride, placing the proteins in a conformation mimicking the transition state for GTP hydrolysis. Broken lines show the position of the covalent bonds broken and formed during the reaction. The positioning of the catalytic functional groups of the conserved $Gln_{cat}$ and $Arg_{cat}$ in these six structures is highly similar, even though the proteins in question might differ in overall sequence and structure. This leads to the conclusion that the catalytic mechanism in these G proteins proceeds via a similar transition state and hence by a similar catalytic mechanism. Backbone models for the relevant switch regions of six G proteins and of the finger loops of the relevant GAPs are shown as backbone ribbon diagrams. The crystal structures are of the following proteins complexed with aluminum fluoride [Protein Data Bank (PDB) accession numbers are in square brackets]. Small G proteins with GAPs: Ras (blue) complexed with Ras-GAP (light yellow) [1WQ1]; Rho (green) complexed with P50RhoGAP (yellow) [1TX4]; and CDC42 (cyan) complexed with CDC42GAP (orange) [1GRN]. Heterotrimeric G proteins: transducin (magenta) [1TAD]; $G_i$ (dark red) [1GFI]; and $G_i$ (red) complexed with RGS4 (not shown) [1AGR]. 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. This figure was prepared using the Insight II software package.

hydrolysis was increased by three orders of magnitude compared with the hydrolysis of GTP. Indeed, SAC not only restores activity to mutant Ras, but also achieves more efficient catalysis than does the slow intrinsic rate of normal Ras.

### Mechanistic implications

In contrast to the use of engineered SAC, the role of natural SAC was investigated in the mechanism of GTP hydrolysis by the wild-type Ras protein[14]. The γ phosphate of GTP was implicated as the general base that abstracts a proton from the nucleophilic water[26]. A similar mechanism has been described for other enzymes that catalyze phosphoryl and nucleotidyl transfer. These include t-RNA synthetase, aspartate carbamoyltransferase and the group II restriction endonucleases EcoRI and EcoRV (reviewed in Ref. 3).

Furthermore, a series of GTPase-deficient Ras mutants were shown to fall on a straight line when the logarithm of their activity at pH 7.4 was plotted against the $pK_a$ determined for the γ phosphate of the bound GTP (Refs 27,28). This type of plot, referred to as a linear free energy relationship (LFER; see Ref. 6 for an eloquent explanation), means that the rate of the GTPase reaction at neutral pH is directly related to the capacity of the γ phosphate for proton abstraction. Surprisingly, when proton transfer was investigated by replacing the water solvent with the $D_2O$ isotope, no effect on the rate of the reaction was observed[27]. Although negative results are difficult to explain, this question was raised again when the isotope effect was tested in the hydrolysis of DABP-GTP. With this analog, a strong isotope effect was found – a twofold reduction of the rate[10]. This could be caused by different rate-limiting steps in the hydrolysis of GTP and DABP-GTP or by dissimilar reaction mechanisms.

One possible explanation for this difference is that the rate-limiting step of the intrinsic GTPase in Ras is the optimal positioning of the catalytic functional group of $Gln_{cat}$ in the active site. Only when this crucial residue is properly oriented can it efficiently stabilize the transition state containing the nucleophile attacking the γ phosphate. SAC by DABP-GTP bypasses this slow rearrangement step by introducing the aromatic amine already in a catalytic orientation[10]. Consistent with this idea, wild-type Ras hydrolyzes DABP-GTP about ten times faster than it hydrolyzes GTP, further supporting the idea that engineered SAC bypasses the original 'bottleneck' in the GTPase reaction and explaining why proton transfer becomes the new rate-limiting step in DABP-GTP hydrolysis[10].

A possible connection between a conformational rearrangement of the catalytic residues and the rate of the GTPase reaction has previously been mentioned[4,17,18,29,30] but has not been generally accepted, possibly owing to the lack of evidence from functional studies. The fact that these flexible parts include the crucial catalytic residues means that multiple orientations are possible for these residues in the ground state. The specific conformation of these residues in the transition state (Fig. 3) suggests that a conformational rearrangement takes place along the reaction pathway. A few examples of experiments that support this hypothesis are described below.

Using fluorescent GTP analogs led to the conclusion that there is an isomerization (conformational rearrangement) of Ras-GTP preceding and controlling GTPase rate[30,31]. At the time, this finding was not fully appreciated because it had not yet been demonstrated that Ras-GAP functions partly by inserting an $Arg_{cat}$ into the active site of Ras.

Time-resolved crystallographic studies of the GTPase reaction in Ras showed multiple conformations for the L4 loop containing $Gln_{cat}$ (Ref. 32). Simulation studies based on crystal structures illustrated a hypervariability of the 60–65 loop in Ras and emphasized the flexible nature of the active site[33]. The anisotropic temperature factors (B factors) in different crystal structures of G proteins show that $Gln_{cat}$ and its surrounding residues (L4/switch II) are highly mobile compared with other parts of the protein, even under the confines of crystal-packing interactions.

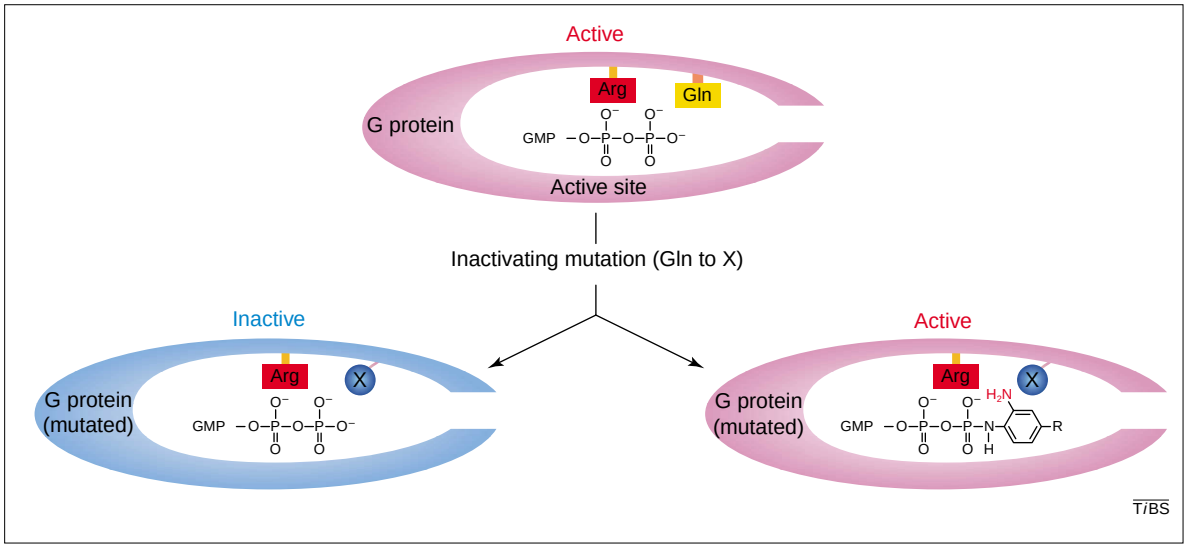


**Fig. 4.** Substrate assisted catalysis (SAC) employed to investigate GTPase mechanism. GTP hydrolysis in G proteins is dependent on $Gln_{cat}$ and $Arg_{cat}$ for optimal activity. When $Gln_{cat}$ is mutated to almost any other residue (marked by X), catalytic activity is impaired (left branch). Using the modified GTP analogs places an aromatic amine (shown in red) correctly into the mutated active site and restores catalytic activity (right branch). The aromatic amine (or alternatively a hydroxyl group – not shown) functionally replaces $Gln_{cat}$. The figure shows engineered SAC employed in heterotrimeric G proteins[24,25], in which $Gln_{cat}$ and $Arg_{cat}$ come in *cis* from the same protein. When using engineered SAC to investigate the intrinsic GTPase in Ras (Ref. 10), Ras-GAP is inactive and hence $Arg_{cat}$ is not present. Abbreviation: GAP, GTPase-activating protein.

Nuclear magnetic resonance (NMR) studies can give a more dynamic view of protein structures. Various studies of Ras using NMR corroborate the previous conclusions by showing that parts of Ras (including switch II containing $Gln_{cat}$) are mobile in solution. This contrasts with the specific conformation that these domains adopt in most crystal structures of G proteins (not detailed here). Therefore, some crystallographic studies might lead to the erroneous assumption that $Gln_{cat}$ has a specific orientation in the ground state. A recent study using heteronuclear NMR found interconversion between conformers of Ras termed 'regional polysterism'[34]. The mobile regions were loops L1, L2 and L4 of the protein that comprise a large portion of the active site and include (not surprisingly) $Gln_{cat}$. It should be emphasized that here, as well as in other structural studies, the mobility of the switch II region is not restricted to the side chains but involves movement of the backbone itself.

## Catalytic inefficiency of G proteins and control by GAPs

As mentioned above, a possible reason that G proteins are inefficient GTPases is that they have a flexible active site. An additional reason that holds true for monomeric Ras-like G proteins is the absence of $Arg_{cat}$ in the active site. The intrinsic GTPase reaction of Ras without GAP is indeed very inefficient ($k_{cat} = 0.02\ min^{-1}$)[10] and is even less efficient in other small G proteins. GAPs can both insert a catalytic residue ($Arg_{cat}$ in most small G proteins) and stabilize an optimal positioning of $Gln_{cat}$. This is evident in the transition state structure of Ras with Ras–GAP, which revealed that the main chain of $Arg_{cat}$ is H-bonded to $Gln_{cat}$, thus stabilizing its position[12,16]. Additional protein–protein contacts were seen to brace and therefore stabilize the catalytically active conformation of Ras. These two functional roles of GAP can be separated experimentally. Mutation of $Arg_{cat}$ in different GAPs retained partial GAP activity on the small G proteins Ras, CDC42 and Rho (Refs. 22,35–37). This duality was also shown by computer simulation studies[18].

The regulators of G-protein signaling (RGS) family of GAPs probably functions solely by affecting the positioning of the catalytic residues in the G protein and not by the insertion of catalytic residues[38]. Indeed, the crystal structure of $G_{i\alpha1}$ with RGS4 shows a reduction in the flexibility of all three switch regions[39].

## Conclusions

Nature developed SAC in the evolution of enzymatic machinery long before biochemical research arrived at this concept. Engineered SAC extends the application of this approach to answer questions that are difficult to address by other methods. It combines both structural and functional analysis to investigate the inner workings of enzymes. The use of SAC as an analytical tool has led to a hypothesis for the rate-limiting step of the GTPase reaction.

It is likely that most members of the G-protein family catalyze GTP hydrolysis through a similar transition state. The difference in catalytic rates between members of the family lies in the identity of the residues participating in the catalytic process and in the specific path that the different G proteins use to progress from the ground state to the transition state. This hypothesis suggests a common mechanism of action for the different GAPs (including RGSs). GAPs complement the active site (when necessary) and affect the rate-limiting step by

stabilizing the 'active' conformation of the protein, placing the crucial functional groups in their proper orientations. This model needs to be further tested using methods that enable time-resolved analysis (such as NMR or other spectroscopic analyses) that can directly investigate the connection between the process of positioning of the catalytic residues and the reaction rate.

G proteins are an excellent example of how the mechanism of enzymatic GTPase has evolved to maintain a low catalytic efficiency, which enables this family of proteins to act as malleable molecular switches. The hypothesis that conformational rearrangement is rate limiting and linked to catalytic inefficiency has unique evolutionary advantages: (1) small mutations in the protein can cause large differences in the intrinsic rate of GTP hydrolysis; (2) it enables adaptation of the 'inner clock' of each G protein to its specific function; (3) interaction with GAPs accelerates the 'off' rate to effectively terminate G-protein activity; and (4) effectors with GAP function enable bi-directional interplay, achieving both transmission and termination of the signal. This diverse family can thus achieve highly elaborate temporal and spatial resolution. Further work will tell whether other proteins that are 'enzymatically self-regulated proteins' are similar not only in concept but also in the finer mechanistic details.

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