Modeling the mechanisms of biological GTP hydrolysis

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A R T I C L E   I N F O

Article history:
Received 15 January 2015 
and in revised form 19 February 2015 
Available online 27 February 2015

Keywords:
GTP hydrolysis 
Ras GT-Pase 
EF-Tu 
EF-G 
Computational biology

A B S T R A C T

Enzymes that hydrolyze GTP are currently in the spotlight, due to their molecular switch mechanism that controls many cellular processes. One of the best-known classes of these enzymes are small GTPases such as members of the Ras superfamily, which catalyze the hydrolysis of the \( \gamma \)-phosphate bond in GTP. In addition, the availability of an increasing number of crystal structures of translational GTPases such as EF-Tu and EF-G have made it possible to probe the molecular details of GTP hydrolysis on the ribosome. However, despite a wealth of biochemical, structural and computational data, the way in which GTP hydrolysis is activated and regulated is still a controversial topic and well-designed simulations can play an important role in resolving and rationalizing the experimental data. In this review, we discuss the contributions of computational biology to our understanding of GTP hydrolysis on the ribosome and in small GTPases.

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Introduction

GTPases are conserved regulators of cell motility, polarity, adhesion, cytoskeletal organization, proliferation and apoptosis [1–3]. They form a large family of hydrolytic enzymes that can be classified into a number of distinct subgroups: heterotrimeric G-proteins (involved in hormonal and sensory signals), translational GTPases (involved in ribosomal protein synthesis), members of the SPR/SR family (involved in translocating peptides into the endoplasmic reticulum), tubulins and cytoskeletal motor GTPases, and monomeric GTPases such as the Ras superfamily (which are responsible for signal transduction cascades and motility) [4]. The primary biochemical function of these enzymes is to catalyze the conversion of GDP to GTP and inorganic phosphate (\( P_i \)) [5].

The most extensively studied class of small GTPases are by far the members of the Ras superfamily [6]. Small GTPases are 20–30 kDa proteins that function as molecular switches in numerous cellular functions [7]. These, are, in turn, divided into five subfamilies (Ras, Rho, Rab, Arf and Ran) that share a common fold. In GT-Pases such as Ras, GTP binding and hydrolysis typically leads to conformational transitions, such that these enzymes display a GDP bound “OFF” state, an open state, and a GTP bound “ON” state [8]. “ON” and “OFF” state regulation can be controlled by mechanisms such as switches (Ras and homologs), clocks (heterotrimeric G-proteins and subunits) and sensors (elongation factors such as EF-Tu and EF-G). In some G-proteins such as the Ras proteins and trGTPases such as EF-Tu,\textsuperscript{1} this activation is also regulated by guanine nucleotide exchange factors (GEFs) [9,10], which activate the enzyme by facilitating the exchange of GDP to GTP. Specifically, GEFs catalyze the release of the bound GDP, which is replaced by abundant cellular GTP [11] (Fig. 1). In the activated state G-proteins (also known as guanine nucleotide-binding proteins – GNBPs) interact with and activate downstream targets (effectors), which in turn trigger cellular responses [12,13]. GTP hydrolysis returns GNBPs to their inactive state, thereby terminating downstream signaling. The switch between the "OFF" and "ON" states is activated by the binding of GT-Pase-activating proteins (GAPs) [8,11]. The active and inactive forms differ in the presence or absence of the \( \gamma \)-phosphate on the nucleotide, which is reflected in considerable conformational differences in regions that contact this terminal phosphate in the GTP-bound form [14].

In parallel to the ongoing interest in Ras GTPases, the recent availability of an increasing number of crystal structures of translational GTPases such as elongation factors thermo unstable (EF-Tu) and G (EF-G) [15–24] has led to an explosion of interest in trying to understand the mechanisms of GTP hydrolysis on the ribosome [20,25–38]. Specifically, translation can be roughly divided into four phases: (i) initiation, where the ribosome binds to the messenger RNA, (ii) elongation cycles, where new amino

\textsuperscript{1} Abbreviations used: EF-Tu, elongation factors thermo unstable; GEFs, guanine nucleotide exchange factors; GAPs, GT-Pase-activating proteins; RF, ribosome recycling factor; RF3, release factor 3; SRL, sarcin-ricin loop; WT, wild type.
acids are incorporated into the nascent polypeptide chain, (iii) termination, where the newly synthesized polypeptide is released from the ribosome, and, finally, (iv) recycling, where the ribosomal subunits dissociate and become ready to re-initiate the cycle by binding to a new mRNA. GTP hydrolysis is an essential part of all the steps mentioned above and these biologically crucial GTP hydrolysis reactions are catalyzed by a family of auxiliary proteins factors, referred to as the translational GTPases (trGTPases) [4,30,31,38]. Of these enzymes, the most mechanistically studied is EF-Tu [20,25–29,31–35,37]. Its biological role lies in the correct delivery of aminoacyl-tRNA at the A (aminoacyl) site of the ribosome. EF-Tu forms a ternary complex with aminoacyl-tRNAs and GTP that bind to the ribosome [39] (Fig. 2). Correct codon–anticodon recognition between the tRNA and the mRNA leads to a significant increase in the GTPase activity of EF-Tu by a factor of 10^5 [40]. Inorganic phosphate is released slowly after the hydrolysis [41] and release of the EF-Tu:GDP complex from the ribosome results in tRNA accommodation, which enables the correct conformation for the peptide bond formation reaction to take place [42,43].

Another rather unique translational GTPase that facilitates translocation (i.e. relocation of the tRNAs from the A and P (peptidyl) sites to the P and E (exit) sites respectively, as well as the relative movement of mRNA to the ribosome by three bases) is EF-G [44–46]. This enzyme behaves both as a molecular switch and as a motor protein [47]. Its binding to the ribosome after the new peptide bond has been formed induces an inter-subunit rotation that repositions the bound tRNAs into hybrid A/P and P/E sites [48–50]. GTP hydrolysis takes place very quickly after binding [51], followed by the completion of the translocation. The inorganic phosphoglyceric phosphate remains bound and its release is linked to the completion of the translocation [52]. Another unique characteristic of this elongation factor is the absence of a GEF, as the affinities for GTP and GDP are similar and the exchange happens spontaneously [53]. EF-G also participates in the recycling phase, facilitating the dissociation of the ribosome recycling factor (RRF) from the ribosome [54]. Release factor 3 (RF3) is a GTPase participating in the termination. GTP hydrolysis promotes the dissociation of RF1 and RF2 [55].

Note that there are many global similarities between the active sites of both translational GTPases such as EF-Tu and EF-G [56], as well as regulatory GTPases such as Ras [57–59] (Fig. 3). There has been substantial experimental and (increasingly) computational work on these systems, and yet the mechanisms of GTP hydrolysis, in particular by translational GTPases on the ribosome, remain controversial [20,29,32,37,60]. In this review, we will provide an overview of the basic challenges with studying phosphoryl transfer, as well as the specific challenges in interpreting experimental and computational data on GTP hydrolysis in biological systems.

We will present popular current mechanistic proposals and highlight the role of theory in enhancing our molecular understanding of GTP hydrolysis on the ribosome and in related biological systems.

**Challenges in elucidating the mechanisms of GTP hydrolysis in biological systems**

Phosphoryl transfer plays a critical role in signaling, protein synthesis and energy transduction, making it one of the most important classes of chemical reactions in biology [61]. As a result of this, many enzymes (phosphatases, kinases, mutases) have evolved to catalyze this class of reactions [62], and they operate via a range of different mechanisms and preferred environmental conditions such as low or high pH. Additionally, some of these enzymes utilize direct attack by water, others employ an enzyme-derived nucleophile and others still use metal ions as a catalytic tool [61,63]. Phosphorylation and dephosphorylation of a protein by kinases and phosphatases can affect the function of a protein in many ways: (i) by increasing or decreasing its biological activity, (ii) by stabilizing it or marking it for breakdown, (iii) by facilitating or inhibiting movement between subcellular compartments, or (iv) by initiating or disrupting protein–protein interactions [64]. Due to the wide range of different mechanisms that can be used in such enzymes, a comprehensive picture of the mechanism of enzyme-catalyzed phosphoryl transfer remains elusive.

One of the biggest controversies in the study of phosphoryl transfer reactions has been to distinguish between the precise molecular mechanisms involved and the nature of the corresponding transition states [61,63,65,66]. Specifically, the availability of low-lying d-orbitals on the phosphorus atom opens the door to a range of mechanistic possibilities, such that the mechanisms of phosphate hydrolysis may occur by a range of different mechanistic pathways (Fig. 4). In a fully associative mechanism (A_n + D_n, Fig. 4A), nucleophilic attack occurs prior to the departure of the leaving group, and the reaction proceeds via inversion of configuration at the phosphorus atom. In contrast, in a dissociative pathway (D_n + A_n, Fig. 4B), leaving group departure precedes nucleophilic attack and the reaction proceeds via a metaphosphate intermediate. In addition to the aforementioned stepwise pathways which proceed with intermediate formation, the reaction can also proceed via a concerted S_n2-like A_nD_n pathway (Fig. 4C), in which bond formation to the nucleophile and bond cleavage to the leaving group occur in a single transition state. Such a transition state can be dissociative or associative in nature, depending on the degree of bond formation to the incoming nucleophile and bond cleavage to the departing leaving group.

There have been extensive experimental studies on the hydrolysis of highly charged phosphate monoester dianions, as well as ATP and GTP hydrolysis in aqueous solution [67,68]. The experimental data would largely suggest a loose, dissociative transition state, based on a steep leaving group dependence of ~1.23 in the linear free energy relationship [69], experimentally measured kinetic isotope effects [70], and a small, negative activation entropy [71]. However, quantum chemical calculations and careful theoretical analysis have suggested that the interpretation of the experimental observables is not unambiguous, as multiple different pathways can give rise to the same experimental observables [66,72]. Additionally, we recently demonstrated that in the case of phosphate monoester dianion hydrolysis, while there is a clear leaving-group dependent mechanistic preference between tighter (more associative) and looser (more dissociative) transition states, the competition between the two pathways is very close, suggesting that an enzyme could in principle use either as a solution to the
chemical problem [73]. The existence of such energetically close competing pathways has been the result of substantial controversy in the literature (for detailed discussion see e.g. Refs. [61,73] and Refs. cited therein).

Another topic that has been a subject of much debate is the nature of nucleophile activation. That is, in principle, the attacking water molecule could be activated either by general-base catalysis by bulk solvent (Fig. 5A), by direct proton transfer to the basic non-bridging oxygens of the phosphate (e.g. pKₐ of 6.5 for GTP [74], Fig. 5B), or by proton transfer to the phosphate via one or more intervening water molecules (Fig. 5C). It has also been argued that, in the case of GTP hydrolysis by for instance Ras GTPase, no general base may be needed at all [63], with no nucleophile deprotonation at the transition state.

Clearly, such controversy with regard to even the mechanism of uncatalyzed phosphate hydrolysis in aqueous solution is only amplified when moving to biological systems and, as can be seen from subsequent discussion, has also been greatly discussed in the case of biological GTP hydrolysis. We will argue in this review that careful theoretical calculations that reproduce all experimental observables in a meaningful way provide an important step to resolving these controversies.

Probing the mechanism of GTP hydrolysis by Ras GTPase

Although the main focus of this review is on modeling GTP hydrolysis on the ribosome, we will start our discussion by revising a closely related GTPase, namely Ras GTPase. As can be seen from Fig. 3 a side-by-side comparison of the active sites of the two enzymes (using Escherichia coli numbering for the EFs) shows great similarities [20,21,75,76]. Specifically, both EF-Tu and Ras harness a catalytic magnesium ion that interacts with the non-bridging oxygens of the GTP. Near the GTP, there are two important regions that change conformation upon GTP hydrolysis. These are designated Switch I and II. In Ras, Switch I (also called the G2 domain, residues 30–40) contains an important threonine residue (Thr 35) that interacts with the metal and a non-bridging oxygen of the γ-phosphate of GTP. Switch II, or the G3 domain (residues 60–76), contains a conserved glutamine (Gln61) that interacts with the nucleophilic water molecule. In EF-Tu, Switch II (residues 80–100 in E. coli variant) contains a conserved histidine residue (His84 in the case of the E. coli variant) that is in the corresponding position to Gln61 in Ras GTPase. Switch I (residues 40–62 in the E. coli variant) does not directly interact with the GTP, however, Ile60 of Switch I and Val20 of the P loop prevent the interaction of the Switch II residues with GTP prior to EF-Tu activation.

Due to its important role as a signaling protein [77] and its propensity towards oncogenic mutations [78], Ras GTpase has been studied extensively both experimentally and computationally [72,76,79–88], and the insights obtained by computational work on Ras form a basis for subsequent theoretical studies of GTP hydrolysis on the ribosome by EF-Tu [33–35,37,89,90]. As discussed before, the Ras GTPase acts as a switch, were the “ON” state corresponds to the GTP-bound and the “OFF” state to the GDP-bound state. The transition between the states is achieved by binding of the GAP, which accelerates the rate of GTP hydrolysis up to five orders of magnitude in relation to the free Ras enzyme [91]. Mutational studies have additionally shown that mutations at positions 12, 13 or 61 lead to a drastic reduction in the rate of GTP hydrolysis, leaving the enzyme in a “ON” state which can lead to cancer [92]. More insight into this enzyme came from the determination of the crystal structure of the free enzyme and of the complex with GAP [76,79,80]. Analysis of these crystals showed that a particular residue in the GAP protein (Arg789, also known as “arginine finger”) is of fundamental importance for TS stabilization during GTP hydrolysis [76]. It also showed that Gln61 interacts with a water molecule that is in a perfect position for nucleophilic attack on the γ-phosphate of the GTP molecule. However, analysis of the crystals did not provide an easy rationale for the effect of the oncogenic mutations, particularly of Gln61. This began a long [82,90,93,94] (and ongoing [63,72,86,88,95]) debate about what the catalytic mechanism for this enzyme actually is.

Early studies proposed that Gln61 could act as a base by removing a proton from the nucleophilic water molecule [80] (Fig. 6A). This hypothesis is based on Gln61 position in the crystal structures (Fig. 3C), but also on the high degree of conservation of this residue as well as mutational studies [96]. Arguments against Gln61 as a general base were presented from several corners [72,81,82,86], but the first group to quantitatively examine this hypothesis computationally were Langen and coworkers [81]. These authors showed that a hypothetical proton transfer to Gln61 would have very large barrier (larger than the corresponding reaction in water). Subsequent studies showed that substitution of this residue with a poorer base, more exactly the synthetic analog NGlu, had little effect on the GTP hydrolysis rate [61].

Fig. 2. Ternary complex of EF-Tu (red), aminoacyl-transfer RNA (aa-tRNA) in the A/T (violet), P and E sites (yellow and green respectively) as well as the non-hydrolyzable GTP analog GCP. (A) The relative position in the ribosome, where the 30s RNA (pink) and 50s RNA (blue) of the small and large ribosomal subunit respectively are noted. The two aa-tRNAs are distant before the GTP hydrolysis, allowing for the related proofreading mechanisms to take place. (B) A more detailed view of the EF-Tu site. The GTP analog, P-loop can be observed in this figure, as well as the sarcin–ricin loop (SRL) of the 50s RNA. This figure was created using the high-resolution crystal structures provided by Ref. [20] (PDB codes: 2XQD, 2XQE).
and P vibrations are coupled to other bonds). However, FTIR studies [100] were unable to find the metaphosphate intermediate expected for a fully dissociative mechanism, leading to the proposal that the reaction was concerted, but with a more pronounced dissociative character. According to this hypothesis, the nature of the base has little effect on catalysis since in a dissociative TS there is little charge development in the nucleophile, so PT from water would occur rather late (after the TS).

This discussion coincided with the growing body of evidence in favor of an alternate substrate-as-base mechanism (Fig. 6B), which is currently the most accepted mechanism for GTP hydrolysis by Ras GTPase [33,81–83,103–107] and related enzymes [33,34,37,89,94]. However, it was argued that such a mechanism would not be possible because of the underlying assumption of the dissociative nature of the TS, where protonation of an oxygen of the γ-phosphate would lead to TS destabilization and be highly unfavorable [94]. In part to address this concerns, Klahn et al. [72], used an ab initio quantum mechanics molecular mechanics free energy perturbation (QM(ai)/MM-FEP) treatment and showed that the relevant TS has an associative character in both water and enzyme (Fig. 7). The shape of the potential energy surface is similar in both the enzyme-catalyzed reaction and in the uncatalyzed background reaction; however, in the enzyme, the TS has an even more pronounced associative character. The calculated barrier to the enzyme catalyzed reaction was 14 kcal/mol, in excellent agreement with the experimental barrier of 16 kcal/mol [72].

It was furthermore argued that the origin of the oncogenicity of mutation of Gln61 lies in its interaction with the L4 loop of Switch Region II. That is, Warshel and coworkers [90,108] performed EVB calculations on the RasGAP complex and showed that mutation of Gln61 affects the conformation of this loop, destroying the catalytically favorable preorganized catalytic configuration obtained by binding of GAP to Ras. Again, although the focus of this review is on GTP hydrolysis on the ribosome, the extensive work on RasGTPase, as well as corresponding mechanistic controversies, form a basis for much of the contemporary discussion about GTP hydrolysis by EF-Tu and related enzymes.

**GTP hydrolysis on the ribosome by EF-Tu**

Following from the introductory discussion about biological GTP hydrolysis by Ras GTPase, the recent elucidation of an increasing number of crystal structures of EF-Tu and EF-G in complex with the ribosome [20–23] have led to an explosion of interest in elucidating the molecular mechanism of activation and GTP hydrolysis in these enzymes. As in other GTPases, the α- and β-phosphates of the GTP/GDP molecule bind to a conserved motif called the phosphate-loop (P-loop) [4,109]. The γ-phosphate and the Mg2+ ion interact with two loops within the G-domain designated by Switch loop I and Switch loop II, which undergo important conformational changes upon GTP hydrolysis (Fig. 8) [84]. This second loop contains a universally conserved PGH motif, which terminates with a histidine residue (His84 in the case of E. coli EF-Tu) that has been proposed to play a role as a general base for GTP hydrolysis (see discussion below) This functional residue is common to all GTPases, however it takes the form of a glutamine for other related enzymes such as Ras GTPase [76]. As shown in Figs. 3B and 9, the sidechain of this histidine interacts with both the nucleophilic water molecule, and with A2662 of the sarcin–ricin loop (SRL) of the 23S RNA [110]. Also, as with Gln61 in Ras GTPase [82], His84 has been suggested to act as a conformational switch for a transition from the inactive to active state for GTP hydrolysis. Specifically, in the inactive state of the enzyme His84 is prevented from entering the

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**Fig. 3.** A comparison of the active sites of (A) EF-Tu and (B) EF-G in complex with GCP (GTP analog) using E. coli numbering, as well as (C) the Ras–GAP complex, with the nucleophilic water molecule highlighted in red, and the Mg2+ ion highlighted in green (to show the interactions, we use GNP from PDB ID 1CTQ instead of GDP from PDB ID 1WQ1). SRL denotes the sarcin–ricin loop, and GAP denotes the GTPase activating protein, the relevant parts of which have been included in this figure. These structures were obtained from [20,21,75,76] (PDB codes: 2XQD, 2XQE, 4JUW, 4JUX, 1CTQ, 1WQ1). Key active site residues and interaction distances (Å) have been highlighted in this figure.

These studies led to a shift in the discussion form the nature of the base to the nature of the TS structure (although there is still debate about the nature of this deprotonation, see e.g. [34,63]). Rather, the question then became whether the corresponding TS for the GTP hydrolysis is associative or dissociative in nature. Several groups [93,97–102] argued for a dissociative TS based on FTIR and Raman spectroscopic studies, which assign vibrational modes between the P-γ2non-bridge and P-γ2non-bridge (the P-Obridge vibrations are coupled to other bonds). However, FTIR studies [100] were unable to find the metaphosphate intermediate expected for a fully dissociative mechanism, leading to the proposal that the reaction was concerted, but with a more pronounced dissociative character. According to this hypothesis, the nature of the base has little effect on catalysis since in a dissociative TS there is little charge development in the nucleophile, so PT from water would occur rather late (after the TS).
Fig. 4. A comparison of different possible mechanistic pathways for phosphate monoester hydrolysis. (A) A fully associative ($A_N + D_N$) mechanism, in which nucleophilic attack precedes departure of the leaving group and the reaction proceeds via a pentavalent intermediate with inversion of configuration at the phosphorous atom. (B) A fully dissociative ($D_N + A_N$) pathway, in which leaving group cleavage precedes nucleophilic attack, and the reaction proceeds via a metaphosphate intermediate. (C) A concerted $S_N2$-like ($A_ND_N$) pathway, in which bond formation to the nucleophile and bond cleavage to the leaving group occur in a single transition state. Such a transition state can be synchronous or asynchronous depending on the degree of bond formation to the incoming nucleophile and bond cleavage to the departing leaving group. A hypothetical scenario involving hydroxide attack on protonated GTP has been shown here for simplicity, to avoid complications with describing the deprotonation of a water nucleophile (see main text).

Fig. 5. Different plausible mechanisms for initial proton transfer during GTP hydrolysis in aqueous solution. In principle, the nucleophilic water molecule could be activated either by: (A) general-base catalysis by bulk solvent. (B) Proton transfer to the basic non-bridging oxygen atoms of the phosphate. (C) Proton transfer to the phosphate via one or more intervening water molecules.

Fig. 6. Different mechanistic hypothesis for GTP hydrolysis by Ras GTPase available in the literature. (A) A base-catalyzed mechanism in which the catalytic water molecule is deprotonated by the carbonyl group of the Gln 61 sidechain, and (B) a substrate-assisted mechanism such as that shown in Fig. 5B. Note that a similar mechanism to that shown in Fig. 5C has also been suggested in the literature (see discussion of different mechanistic options in Ref. [95]). For further details, see e.g. Ref. [61].
active site by a hydrophobic gate formed by Val20 and Ile61 [111,112]. Upon activation, when the correct codon–anticodon complex is formed, His84 moves toward the active site. Interaction with the SRL is critical for this activation step [20–23].

Again, as with Ras GTPase, due to its biological importance, there has been substantial experimental [20,29,53,110,113,114] and more recently also computational [34,35,37,89,115] work on this system. For example, the rate constants of EF-Tu-dependent binding of Phe-tRNA to the A site of poly(U)-programmed E. coli ribosomes have been measured by different research groups [41,42,48,116,117]. In 1998, Pape et al. [40], using pre-steady state kinetic experiments, determined for the first time the complete

Fig. 7. Potential energy surfaces and corresponding transition state structures for GTP hydrolysis in aqueous solution (A and B) and catalyzed by the Ras–GAP complex (C and D) (for aqueous solution, hydrolysis of the monomethyl pyrophosphate trianion was used as model). As can be seen, the background reaction is already associative in nature in the presence of a metal ion, in agreement with the prediction of Ref. [126], and this becomes even more associative in the presence of the enzyme. Reprinted with permission from Ref. [72].

Fig. 8. (A) Overall conformational change of the G-domain of EF-Tu when the ternary complex (tRNA:EF-Tu:GTP) binds to the ribosome. This structural change can be seen by superimposing the tRNAs of the free ternary complex (PDB code 2C78 [19]) with that of the ribosome-bound complex (PDB codes: 2XQD, 2XQE [20]). (B) Active site of EF-Tu during GTP hydrolysis as seen in the high-resolution crystal structure (green), and as computed by MD simulations during an initial pre-reaction state (magenta), an intermediate rotated state (yellow) and the final activated state (cyan). The sarcin–ricin loop (SRL, orange), His84, and Val20 and Ile60 (which form the hydrophobic gate) are highlighted. Reprinted with permission from Ref. [37].
kinetic mechanism. According to these authors, initial binding to the ribosome is rapid and readily reversible. This is followed by codon recognition, which then induces the EF-Tu conformational change and GTP hydrolysis. In this work, the conformational change and GTP hydrolysis were grouped together (GTP hydrolysis was proposed to occur instantly after the protein rearrangement). The measured rate constants at 20 °C were $500 \pm 100 \text{ s}^{-1}$ at 10 mM Mg$^{2+}$ concentration and $55 \pm 15 \text{ s}^{-1}$ at 5 mM Mg$^{2+}$ concentration, which would correspond to activation free energies of 13.5 and 14.8 kcal/mol respectively. The authors measured also putative rates for the conformational change from the GTP to GDP bound form ($60 \pm 20 \text{ s}^{-1}$), aa-tRNA accommodation and peptidyl bond formation ($7 \pm 1$ and $8 \pm 1 \text{ s}^{-1}$, depending on the Mg$^{2+}$ concentration) and dissociation of EF-Tu ($3 \pm 1$ and $4 \pm 1 \text{ s}^{-1}$). A schematic for the different states along the peptide elongation path is shown in Fig. 10. Rodnina and co-workers having identified up to seven distinct steps on the pathway from initial binding of the ternary complex to the peptidyl transfer [118,119] (see Fig. 10B). This revised scheme includes initial binding and codon recognition to bring the system to its first selection point, where the off-rates between forming the cognate and near-cognate ternary complexes differ by a factor of up to 1000 [119,120]. This leads to GTPase activation and rate-limiting GTP hydrolysis, which is rate-limiting for the non-cognate complexes but not for the cognate complexes [119,120], and a proofreading step in which the aa-tRNA can either continue to form the peptide bond or be rejected with unequal probabilities for correct and incorrect substrates.

Additional major breakthroughs in this area came in 2009 and 2010 [20,110], with the elucidation of medium-resolution (3.2 Å) crystal structures of EF-Tu in complex with the ribosome. In particular, the resulting structure showed His84 as being positioned in an apparently ideal position to accept a proton from the nucleophilic water molecule (Fig. 3B), causing the authors to propose...
that the imidazole sidechain plays an important role in the activation of GTP hydrolysis on the ribosome as a general base catalyst (see Fig. 11 for a summary of currently popular mechanistic proposals). It should be noted here that the catalytic center of trGTPases, and, in particular, the histidine residue of the invariant PGH motif in their active sites is strongly conserved [36,49,56,121] and, therefore, the translational GTPases are expected to operate through a universal GTP hydrolysis mechanism. Following from this, a mechanism such as that proposed by Voorhees et al. would intuitively seem to be fully reasonable, in particular as Rodnina and coworkers [29] have shown that mutation of His84 to an alanine residue reduces the rate of GTP hydrolysis more than 10^6 fold. The preceding steps are only slightly affected, suggesting that the effect of His84 is in the catalytic step itself.

There are, however, a number of complications with this proposal. The first is the close proximity of His84 to several negatively charged species (Fig. 3B), including the phosphate group from the SRL loop, the highly charged polyphosphate, and the sidechain of Asp21, which would lead one to believe that the imidazole sidechain is most likely protonated. As would be expected, calculations uniformly suggest an upshift of at least three units in the pK_a of this residue [33,35,37]. We also recently performed free energy calculations to examine the energetic cost of ground state proton transfer from the catalytic water to His84, and demonstrated that this is strongly disfavored by 17.5 kcal/mol (compared to the process in aqueous solution) [87], as a result of electrostatic repulsion between the resulting hydroxide ion and the negative charge on the γ-phosphate of the GTP [37]. Additionally, and as also pointed out by Liljas et al. [32], careful examination of the EF-Tu active site shows that the coordination geometry of the water molecule would rather suggest that one of its water molecules is involved in a H-bonding interaction with the backbone carbonyl group of Thr61 (Fig. 3A), whereas the other is very close to one of the non-bridging oxygens of the γ-phosphate. Tying in with this, we demonstrated that a ground state proton transfer from the water molecule to the γ-phosphate is favorable by –8.3 kcal/mol (compared to the same process in aqueous solution) [89], and that once the GTP is protonated, the active site seems optimally arranged to stabilize the negative charge on the resulting hydroxide ion (Figs. 8 and 9).

Finally, the fact that there is no experimentally observed pH-dependence in the pH region where the imidazole sidechain of His84 would normally be expected to ionize (6–8.5) [29] provides further evidence against a mechanism such as that shown in Fig. 11A. Remarkably, our recent MD simulations [37] demonstrated that interaction with the SRL appears to spontaneously drive His84 into an activated conformation for GTP hydrolysis (Fig. 9), which is only attained if the simulations are performed with His84 in its protonated form. Therefore, structural, biochemical and computational evidence strongly suggest that His84 is positively charged by the time it has reached the active site.

An alternative to nucleophile activation by histidine is a substrate-as-base mechanism such as that suggested for Ras GTPase [81–83]. This can, in principle, involve either a concerted (A_start A_end) or a stepwise (A_start A_end + A_end) pathway, as shown in Fig. 4C and A. Such a mechanism is supported by both computational work of Adamczyk and Warshel [33] and by our recent ground state free energy calculations [37]. In both cases, His84 plays an important role in activation of GTP hydrolysis, whether through direct electrostatic interactions as described above [37], or through indirect (“allosteric”) interactions as proposed by Adamczyk and Warshel [33]. In theory, the reaction could also proceed through a mechanism such as that suggested by Aleksandrov and Field [11C] [35], which involves a scenario in which the protonated histidine transfers a proton to the substrate via the nucleophilic water molecule and then this water molecule attacks a monoanionic phosphate, with His84 acting as a general base to activate the nucleophile. Such a mechanism would in principle not be incompatible with the observed pH independence, even though it involves very complex movement of protons. Finally, one can always ask whether the substrate-assisted pathways occur through direct deprotonation of the nucleophilic water molecule Fig. 5B or via an intermediary water molecule that provides a bridge between the nucleophile and non-bridging oxygen of the phosphate Fig. 5C [34], or whether a general-base is necessary at all, as has been argued for Ras GTPase [63]. For comparison, our recent calculations on the uncatalyzed hydrolysis of phosphate monoester dianions suggest that, for good leaving groups, such a dissociative solvent-assisted pathway in which there is no deprotonation of the nucleophile at the transition state is preferred over a substrate-assisted associative pathway [73]. In the case of EF-Tu, both these mechanisms would require the presence of at least one additional water molecule in the EF-Tu active site, which there is no space to accommodate (see also Fig. 9).

As can be seen from the mechanistic options outlined above, as with Ras GTPase, the mechanisms of GTP hydrolysis on the ribosome are highly complex and subject to extensive discussion. A further complication to this mechanistic picture is the availability of recent crystal structures of the GTP-bound forms of free eukaryotic and archaeal EF-Tu homologs, which clearly show the presence of a monovalent cation between the β- and the γ-phosphates of the
bound GTP [24]. This cation was not present in e.g. previous EF-Tu structures [20,110], however, the authors argued that the GTP analogs GDPcD or GDPNP analogs used in previous work disrupt the ion coordination, which was why it was not observed [24]. Clearly, if this ion really exists in that position in the activated state, it could potentially have major impact on the energetics of the GTP hydrolysis and affect the postulated mechanism.

To explore this issue, we recently performed an exhaustive analysis of different possible mechanisms for GTP hydrolysis in EF-Tu on the ribosome [89]. These calculations support the previously suggested substrate-assisted pathway [20], and emphasize that, for optimal catalysis, His84 has to be doubly protonated to stabilize the negative charge developing on the water nucleophile during the reaction. Examining the contributions of different residues to the calculated activation barrier (Fig. 12) also highlighted the importance of key active site residues in assisting the charge migration during the chemical reaction, with His84 and the PGH backbone acting as a “pull” for the negative charge on the γ-phosphate, whereas Asp24 acts as a “push” to repel the negative charge in the same direction. Finally, Lys24 eventually stabilizes the final double negative charge on the resulting GDP (see Figs. 3B and 10 for positions of key residues). Tying in with this stabilization of charge migration is an important structural change predicted in our previous work [37], where we found a backbone flip of the PGH motif to accommodate the catalytic confirmation stabilizing the oxyanion hole shown in Fig. 9. This structural change has now been experimentally confirmed [21,22], and our more recent study demonstrates that it is crucial for obtaining reliable energetics [89].

In conclusion, from our recent work [89], we surmise that the proton transfer is not the rate-limiting step, in agreement with the kinetic isotope effect measurements of Rodnina and coworkers [38]. Additionally, there is no indication of a significant catalytic effect due to the presence of a monovalent metal ion near the α and γ-phosphate oxygens from either experimental (independence of the rate on the K+ concentration [38]) or from our computational results. Following from this, we also examined two key mutations: H84Q and D21A (see Fig. 3A for the position of these residues). We find that mutating H84 to Q most probably changes the identity of the most feasible mechanism from the hydroxide mechanism towards the neutral concerted water attack (with an activation energy of 20 kcal/mol). In contrast, the D21A mutation can affect the mechanism in two different ways, both of them in quantitative agreement with the determined experimental rates. Therefore, our hypothesis is that the absence of this negatively charged residue disrupts the favorable movement of charge observed in the wild type (WT) enzyme, making the proton transfer from the water to the γ-phosphate oxygen less favorable and, consequently, raising the free energy barrier. Finally, removal of the Asp21 can potentially lead to a drop in His84 pKα, rendering this histidine neutral, making the concerted water attack the most feasible mechanistic hypothesis.

**Conclusions and future perspectives**

GTPases are essential for many vital processes within the cell. Their functions include controlling protein biosynthesis, growth control, differentiation, cytoskeleton reorganization, and regulating transport processes. They act, for example, by regulating cell signaling cascades, such as Ras, or by regulating transcription (in the case of EF-Tu). In Ras and Ras related proteins the GTP bound conformation is acting as a clock, while the switch is “ON” the downstream cascade and subsequence cellular function is activated. In EF-Tu the GTP bound conformation it is working as a sensor. GTP hydrolysis only takes place activation by the SRL and movement of His84 towards the active site [20–23]. It is interesting how the cell uses this common and universal switching mechanism to activate all these different GTPases, with similar conformational changes between conserved loops within these enzymes regulating GTP hydrolysis and consequently the exchange between active and inactive conformations. The extremely important role of the Ras proteins in cellular homeostasis is highlighted by the plethora of oncogenic mutations related to them [78]. EF-Tu has also been recently identified as a potential cancer
biomarker and there are studies correlating EF-Tu expression levels with various carcinomas [122–125]. All of these factors make GTPases very attractive systems to study, both experimentally and computationally. In fact, the history of the study of these enzymes is very vast, although not free of controversy. A major point of debate lies in the details of the GTP hydrolysis catalytic mechanism and more specifically in the nature of the transition state structure and associated base.

Most studies trying to tackle the mechanism of GTP hydrolysis used either Ras [75,86,88,94,97–100] or Ras-related enzymes [94,102] and EF-Tu [29,35,40,41] as model systems. Early studies on these enzymes posited analogous hypotheses that nearby active site residues, specifically the conserved Gln61/His84 residues in Switch loop II would fulfill the role of a catalytic base. However, these hypotheses were mostly discarded based on deeper analysis [93,97–102]. transition state structures have been suggested based on how the available experimental and computational data are predicted. However, irrespective of the precise nature of the TS, we share the view of Refs. [33,34,37,90,95,108] that the catalytic effect of these enzymes is likely to be primarily electrostatic in nature, and that mutations to active site residues would disrupt the perfect balance in charge distribution these enzymes have evolved to provide.

There is still a long road ahead in the study of GTPases. Although there now exist detailed descriptions of the catalytic mechanism of the WT enzymes [33,35,37,63,72,81,103], as well as characterization of the effect of some mutations in atomic detail [29,90,96,108], there still exist several oncogenic mutations [78] that remain an open question in terms of the rationale behind their deleterious effects. Therefore, there is plenty of scope for further work in modeling these reactions, both focusing on the actual chemical step of catalysis and also on large-scale conformational dynamics and the effect of interactions with GEFs, GAP and effector proteins, which becomes increasingly feasible to study with constant advances in computational power.

Acknowledgments

The authors would like to thank the Swedish Research Council (VR) and the Knut and Alice Wallenberg Foundation (through a Wallenberg Academy Fellowship and the RibosomeCore consortium) for funding and support. S.C.L.K. is an ERC Starting Grantee (306474).

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