Why Does the Ras Switch "Break" By Oncogenic Mutations?

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ABSTRACT **The elucidation of the structure of the RasGAP complex provides what is perhaps the most detailed link between protein structure and cancer causing mutations. In particular, it is known that mutations of Gln 61 destroy the GTPase activity of the complex, locks the cell in its ON state and thus, can cause cancer. It is entirely unclear however, why this specific mutation is so important. The present work uncovers the elusive role of Gln 61 by computer simulation of the GTPase reaction in Ras, RasGAP and of their mutants. Simulations of the effects of mutations of Gln 61 reproduce the corresponding observed changes in activation energies and allow us to analyze the energy contributions to these effects. It is found that Gln 61 does not operate in a direct chemical way nor by a direct electrostatic or steric interaction with the transition state (TS). Instead, oncogenic mutations of Gln 61 lead to the destruction of the exquisitely preorganized catalytic configuration of the active site of the RasGAP complex. This "allosteric" effect causes a major reduction in the electrostatic stabilization of the TS. Our findings have general relevance to other proteins that control signal transduction processes. Proteins 2004;55:1–10.** © 2004 Wiley-Liss, Inc.

Key words: RasGAP; Gln 61 oncogenic mutation; signal transduction; G–proteins; catalysis; allostery

INTRODUCTION

$p21^{ras}$ (Ras) plays a central role in the signal transduction pathway that controls cell proliferation. This protein can be considered as a switch where the signal is ON in the GTP bound form and OFF in the GDP bound form.[1–3] The switch is activated by the binding of GTPase-activating proteins (GAPs) that accelerate the relatively slow GTP hydrolysis in Ras by up to five orders of magnitude.[4,5] Mutations at either one of the 12, 13, or 61 positions slow down drastically the GTPase reaction of the Ras-RasGAP (RasGAP) complex and leave the signal in the ON state, thus, leading frequently to cancer.[2,6,7]

The elucidation of the structure of Ras[8,9] and the RasGAP[10] complex has provided what is perhaps the most detailed link between protein structure and cancer causing mutations. A spectacular clue about the control of the Ras switch was the finding that the Arg 789 residue of GAP, which is also referred to as the "arginine finger" provides a major stabilization to the transition state (TS) of the GTPase reaction.[10,11] However, despite this and other major advances it is still unclear what is the origin of the anticatalytic effect of the oncogenic residues of Ras. The most puzzling problem is the role of Gln 61. Mutations of this residue in the RasGAP complex reduce k_{cat} of the GTPase reaction by 10^{-3}–10^{-7}, depending on the specific mutation (see "Reproducing the Overall Catalytic and Mutational Effects" section). This large effect seems to be ubiquitous to different GAPs, including the Ran-RanGAP system that does not operate through an arginine finger but still contains a crucial catalytic glutamine.[12] A related glutamine is also found in other G proteins (e.g., Q204 and Q227 in $G_{i\alpha1}$ and $G_{s\alpha1}$ respectively[13]). It is clear that the proper positioning of Gln 61 is crucial for an effective catalysis. However, the nature of this positioning and its role in stabilizing the TS are entirely unclear. This means that one of the most crucial links between structure and catalysis in Ras and other signal transduction proteins is still not understood.

In principle, Gln 61 can contribute to the catalysis in several direct ways. It can act "chemically" as a general base, it can provide direct electrostatic stabilization to the TS and it can perhaps act by applying orienting entropic or steric effects (see below). However, it is also possible that this residue acts in an indirect way, whose nature is not clear.

Structural studies, mutational experiments and the use of TS analogs have not elucidate the origin of the catalytic role of Gln 61. Theoretical studies that tried to address this issue did not reach unique conclusions. Early theoretical study[14] showed that Gln 61 does not play an important catalytic role in the isolated Ras. This conclusion was subsequently confirmed by mutation experiments.[15] The effect of Gln 61 in RasGAP was addressed by Glennon et al.,[16] who suggested that this residue acts in an indirect way by stabilizing the catalytic configuration of Ras. However, the calculations were not stable enough to establish the actual role of Gln 61 or to reproduce the effect of its mutants.

Here we report the results of what is at present the most systematic simulation study of the GTPase reaction of the

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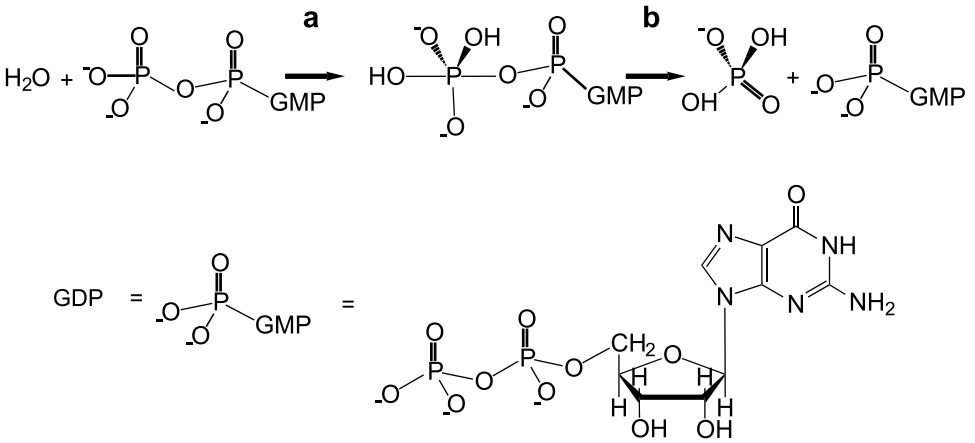

Fig. 1. A schematic description of the GTPase reaction mechanism. The reaction is considered as a two-step mechanism where the GTP serves as a general base. In the first step, **a**, the water molecule attacks the GTP and a penta-coordinated intermediate is formed. In the second step, **b**, the P_{β}-O bond is broken and the GDP and a phosphate group are formed. Note that the attack of the water molecule (step a) may be either concerted or stepwise mechanism.

RasGAP complex. Our study reproduces quantitatively the catalytic effect of Ras, RasGAP, and Arg 789 (relative to the uncatalyzed reaction), and then explores the role of Gln 61. This is done by simulating the effects of the Q61L, Q61A, and Q61NGln mutations (where NGln is the isosteric nitro analogue of Gln) as well as the mutation of Gln61 to its nonpolar analogue and then analyzing the corresponding energetics. It is found that Gln 61 does not act in a direct way but by occupying a pivotal position in an exquisitely preorganized catalytic configuration generated by the binding of GAP to Ras. Mutations of Gln 61 destroy this preorganization by disturbing a particularly sensitive position in the L4 loop (residues 59–65) and thus, changing the interaction between the active site residues and the TS.

METHODS

The basis of our simulation is the empirical valence bond (EVB) approach,[17] which has been used previously[16] in studies of the RasGAP system and in many quantitative studies of other enzymatic reactions (for review see Warshel[18]). The EVB potential surface allows us to take an X-ray structure of a RS a TS analogue or a product state (PS), relax the model of the substrate in a RS, and then to gradually transform the system from the RS to the PS and obtain the corresponding free energy profile. Although the present EVB surface has similar features to those used in our previous work,[16] it reflects a more systematic refinement. That is, the EVB surface of the reference solution reaction was further refined by forcing it to reproduce the known experimental and ab initio information about the hydrolysis of phosphomonoester dianions and the trend in this reaction upon changing the leaving group. The corresponding parameters are given in Table I and Figure 1 of the Supplementary material. The free energy of the EVB surface was evaluated by the umbrella sampling/free energy perturbation (UM/FEP) method.[17] The simulations were done using the ENZYMIX module of the program MOLARIS[19] with the surface constraint all atom solvent

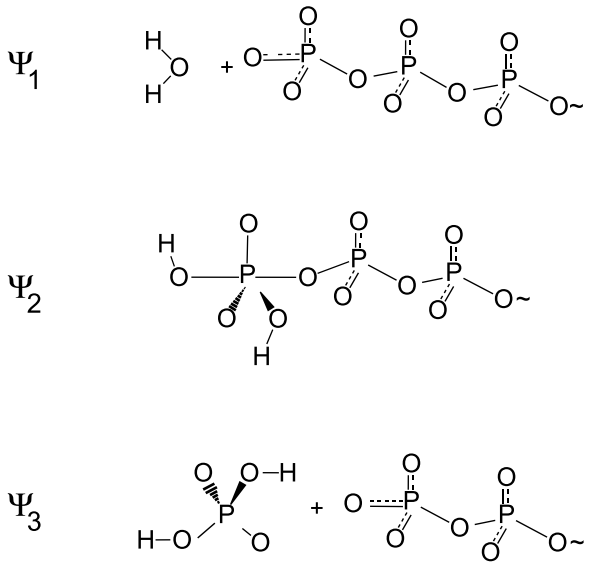

Fig. 2. The three VB structures used to describe the GTPase reaction. Ψ_1, Ψ_2 and Ψ_3 describe the reactants state, the penta-coordinated intermediate and the products state, respectively.

(SCAAS) model and its special polarization constraints[20] as well as the local reaction field (LRF) long-range treatment.[21] The hydrolysis reaction as mentioned in the text was described by considering a two-step mechanism (see Fig. 1) and three VB structures (Fig. 2) used to define the system in the EVB calculation. These structures together with their respective charges are given in Figure 1 of the Supplementary material. The coordinates used as a starting point in the simulation of the Ras and RasGAP complex correspond to codes 5P21[9] and 1WQ1[10] in the Brookhaven Protein Data Bank, respectively. The free energy profile of each step was typically evaluated after 200 ps equilibration time, followed by 31 windows of 5 ps each for moving along the reaction profile using the FEP method. Several initial conditions were used in each case

in order to ensure the stability of the results and in order to obtain a proper average over the protein configurations.

Another special feature of the present study is the use of the linear response approximation (LRA) treatment[22–24] in the analysis of the contribution to the free energy of the reactants, transition states and intermediates. This approach provides a good estimate for the free energy associated with the change between two potential surfaces (U_1 and U_2) by[22]

$$\Delta G(U_1 \rightarrow U_2) = \frac{1}{2}\left(\langle U_2 - U_1\rangle_1 + \langle U_2 - U_1\rangle_2\right) \quad (1)$$

where $\langle\ \rangle_i$ designates an average over trajectories propagated on U_i. The derivation of the LRA formulation can be accomplished by considering the microscopic equivalent of the Marcus' formula (see derivations in Sham et al.,[23] Florian et al.[24]) or by expanding the standard FEP expression.[25] Basically, this expression is an endpoints approximation for the FEP formulation,[26] whose introduction as well as first implementation in studies of biological molecules is due to Lee et al.[22] Now, the use of the LRA offers the unique ability to decompose free energies to their individual additive contributions.[24] Such a treatment cannot be accomplished by FEP approaches due to their non-additive nature. The individual LRA contribution of the $i^{\underline{th}}$ group is given by:

$$\Delta G_i(U_1 \rightarrow U_2) = \frac{1}{2}\left(\langle U_2^i - U_1^i\rangle_1 + \langle U_2^i - U_1^i\rangle_2\right) \quad (2)$$

While the LRA contribution gives the correct additive components of the total free energy, they do not relate to the corresponding mutational effects. That is, the effect of mutating a given residue should be obtained by performing LRA analysis for the native and mutant forms of this residue. A reasonable estimate of the effects of mutations can be obtained by using an effective dielectric constant (see Muegge et al.[27]) and scaling down the interactions with ionized residues by ~20 while scaling down interactions with polar residues by ~2–4.

REPRODUCING THE OVERALL CATALYTIC AND MUTATIONAL EFFECTS

Before exploring the role of Gln 61, it is essential to establish the reliability of our approach by calculating the catalytic effect of Ras and RasGAP. The reaction was described by considering two steps (see Fig. 1); a nucleophilic attack of the water molecule on the γ-phosphate and generation of a penta-coordinated intermediate [Fig. 1(a)], followed by completion of the hydrolysis reaction by breaking the P_β-O bond and generation of a leaving phosphate group and the GDP [Fig. 1(b)]. The calculated free energy profiles for the GTPase reaction in water, Ras and RasGAP are presented in Figure 3. The corresponding free energies are also summarized in first three entries of Table I. As seen from the figure and the table, the experimental trend is reproduced. It is interesting to see that the rate-limiting step in water is associated with the second barrier. The height of this barrier is reduced in Ras and to a greater

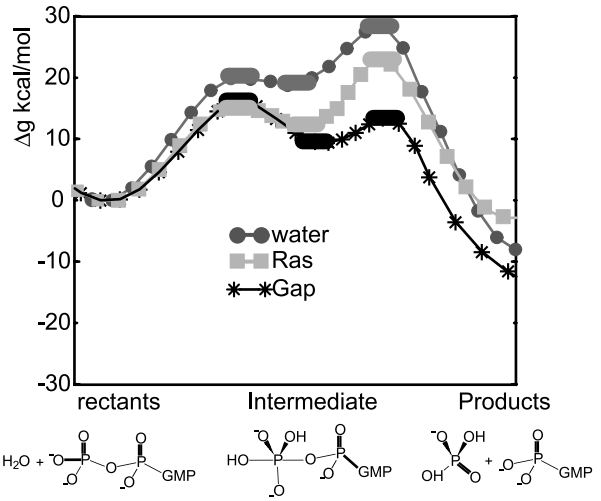

Fig. 3. The free energy profiles for the GTPase reaction in water (circles), Ras (squares) and in the RasGAP complex (stars) obtained from an average over five different profiles, calculated using the EVB FEP/Umbrella sampling procedure.

extent in the RasGAP complex upon stabilization of both the penta-coordinated intermediate and the product (GDP + P_i) states. The origin of the total effect of the Ras→RasGAP transition will be discussed in the following section.

Next we examine the effect of Arg 789 and Gln 61 mutations. Since electrostatic effects are usually the leading contribution in catalysis we considered first a simple approach of mutating the two residues to their nonpolar analogues, where all the side chain atoms have zero residual charges. This type of analysis allows us to determine what the electrostatic effect of the residues under consideration is. The results of the corresponding simulations are presented in Table I and Figure 4. It is seen from both the table and the figure that the observed effect of Arg 789 is reproduced using the nonpolar analogue mutation technique. This demonstrates that the role of the arginine finger is mainly to provide *direct* electrostatic stabilization of the TS.

The situation with Gln 61 however, appeared to be more complex. That is, the mutation of this residue to its nonpolar analogue ($Q61Q^{NP}$) did not reproduce the observed large anticatalytic effect (the activation barrier increased by only ~1 kcal/mol instead of the 5–9 kcal/mol observed effect). Note in this respect that Gln 61 interacts strongly with the reacting system (substrate plus water) in both the RS and TS. However, since the interaction is similar in the RS and TS, its overall effect is not large. It might be important to clarify this point that although the $Q61Q^{NP}$ mutation cannot be performed experimentally, it is perhaps the most effective theoretical way for examining the role of Gln 61. That is, if Gln 61 catalyses the GTPase reaction by its electrostatic effect, then removing all the residual charges of this residue will destroy the corresponding catalytic effect. However, if the catalytic effect of Gln 61 is due to indirect steric effect (keeping other residues in a correct catalytic arrangement) then it is possible that the

TABLE I. Energetics of the GTPase Reaction in Water, Ras RasGAP and Some of Their Mutants[†]

	$\Delta g_1^{\ddagger}$	ΔG_1^0	$\Delta g_2^{\ddagger}$	$\Delta g_{cat}^{\ddagger}$ (calc)	$\Delta g_{cat}^{\ddagger}$ (exp)
Water	20.0	18.4	27.9	27.9	27.5[b]
Ras	15.2	11.9	23.2	23.2	22.2[c]
RasGAP	16.1	9.0	13.5	16.1	≥15.9[c,d]
R789R^{NP} (RasGAP)[a]	21.7	17.4	21.6	21.7	20.6[e,f]
Q61Q^{NP} (RasGAP)[a]	17.1	9.0	13.4	17.1[g]	
Q61NGln (RasGAP)	19.9	11.7	18.4	19.9	≥16.0[h,d]
Q61A (RasGAP)	19.9	13.7	23.2	23.2	20.1[i]
Q61L (RasGAP)	17.3	15.8	24.2	24.2	25.2[j]

[†]$\Delta g_1^{\ddagger}$, ΔG_1^0, $\Delta g_2^{\ddagger}$ correspond to the relative free energies (in kcal/mol) of the first TS [Fig. 1(a)], penta-coordinated intermediate and the second TS [Fig. 1(b)], respectively. The activation energies were evaluated from the corresponding rate constants using transition state theory (see Warshel[17]). Parameters for the potential surfaces are given in Table 1 in the Supporting material. The calculated results are obtained from an average over at least four or five different initial conditions. The effects of different mutations on k_{cat} are not always available due to experimental difficulties and to the fact that in some cases we only have an upper limit since the chemical step is not rate limiting. Thus, we provide the estimated experimental results and their origin is given in each case.

[a]NP indicates that the corresponding residue was "mutated" to its nonpolar form.

[b]For details see Glennon et al.[16]

[c]k_{cat} is taken from Schweins et al.[5]

[d]The inequality reflects the fact that the GTPase reaction might not be the rate limiting in RasGAP.

[e]The calculated values for the mutation to the NP form are compared to the experimental value of the R789A mutation.

[f]k_{cat} is taken from Ahmadian et al.[11]

[g]The calculated value for the Q61Q^{NP} mutant does not correspond to the observed results for actual mutations (see text).

[h]Based on reported activity from Chung et al.[15] rather than on k_{cat}.

[i]A qualitative estimate based on a report of 10^{-3} reduction in the rate upon mutation Q61A.

[j]k_{cat} is taken from Schweins.[32] (A. Wittinghofer, personal communication)

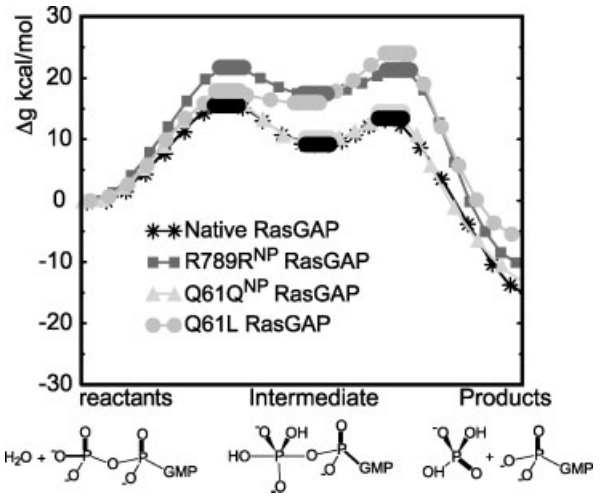

Fig. 4. Mutation effects on the GTPase reaction profile. The figure describes the free energy profiles of the GTPase reaction in the native RasGAP complex (stars) and its different mutations, R789R^{NP} (squares), Q61Q^{NP} (triangulars) and Q61L (circles). The profiles were obtained from an average over five different profiles, calculated using the EVB FEP/Umbrella sampling procedure. NP stands for mutation to the non-polar form of the indicated residue.

Q61Q^{NP} mutation will not have a large anticatalytic effect. Here indeed we find that the Q61Q^{NP} mutation has a small effect.

Obviously there is no direct experiment that can verify our finding, since a nonpolar mutant does not exist. Thus, it is essential to demonstrate that we can reproduce the anticatalytic effect of a real mutation before proceeding to analyze the significance of the finding of a small direct catalytic effect. The need to reproduce and analyze the effects of real mutations of Gln 61 is compounded by the fact that we are dealing with a problem that has not been resolved by direct experiments. At any rate, we performed extensive simulation studies of the effects of three mutations, Q61L, Q61A, and Q61NGln and the corresponding results are summarized in Table I. As seen from the table we were able to reproduce the large observed anticatalytic effects of the Q61L and Q61A mutants and the fact that the Q61NGln mutation does not lead to a large anticatalytic effect.[15] Now, with a model that reproduces the actual effect of oncogenic mutants we can go one step further and explore the origin of the calculated effects. This analysis will be given below.

GAP CHANGES THE CATALYTIC CONFIGURATION OF RAS

After validating our calculations by reproducing the overall catalytic effect and the effects of different mutations we can examine the origin of these effects. We start by exploring the overall catalytic effect of GAP focusing on its indirect effect through its interaction with Ras. Comparison of the X-ray structure of the isolated Ras to the

structure of Ras in the RasGAP complex (referred to here as Ras′) reveals some structural changes induced by the binding of GAP to Ras (e.g., Gln 61 moves closer to the γ-phosphate[10]). However, although this comparison is quite instructive, it cannot tell us what the exact functional implications of these changes are. In particular, since the enzyme is flexible, changes in the positions of various residues do not necessarily reflect changes in the activation barrier. Furthermore, although TS analogues (TSAs) can provide useful information, it is hard to deduce the exact relationship between TSAs and the corresponding TSs without extensive theoretical analysis.[28] Thus, we prefer to rely mainly on approaches which allow one to evaluate the free energy of the actual TS. The Linear Response Approximation (LRA) model (see the Methods section) is such an approach. This model allows us to assess the functional contributions of different structural changes, and to determine which of these changes has significant projections along the pathway from the reactant state (RS) to the TS. More importantly, with the LRA approach we are able to determine the individual contribution of Ras in the RasGAP complex. This is a significant advance over our previous study[16] where we only examined the difference in the properties of the reacting system in two configurations of Ras; the structure of the free Ras and a structure that was constrained to be near Ras′ but without the actual presence of GAP. With the LRA, however, we are able to focus on the more realistic and well-defined problem of comparing the catalytic effect of the isolated Ras to the contribution of Ras in the RasGAP complex. This is done by evaluating the protein-substrate electrostatic interactions in the RS and TS and decomposing them to the individual contributions from each residue. As discussed in the Method section, these additive contributions are not equal to the effect of mutating the corresponding residues. Thus, we give in the Supplementary material the actual LRA results and give here the approximated results obtained by using the optimal effective dielectric constants. These results can be related qualitatively to relevant mutation experiments. Figure 5 presents the changes in the electrostatic contributions of the Ras residues due to formation of the RasGAP complex. Figures 5a and b describe the contributions in the RS and in the penta-coordinated intermediate state (IS), respectively. Since the IS and the TS have similar properties and the calculations of the intermediate are more stable, we consider here the results for the IS as descriptors of the corresponding TS results. Inspection of Figure 5 reveals that the structural reorganization between the free Ras and Ras' is indeed functionally important. Significant changes occur in the P–loop, switch I (in particular Asp 33 and Thr 35) the β_3 region (in particular Asp 57), L4 in switch II (in particular Gln 61) and the Mg^{2+} ion (see Figure 6 for definition of the key regions). Pointing out to specific structural (rather than energy) changes is not simple, since we obtain similar trend with different starting configurations. Furthermore, we are focusing here on average energies and *not* on average structures. Nevertheless, using typical snapshots, we provide in Figure 7 a qualitative view of the trend in the structural changes. One of the interesting findings is the change in the β_3 region (in particular 57–60) of the RasGAP complex upon transition from the RS to the TS. This change (which couples Gln 61 to Asp 57) does not occur in the isolated Ras.

Of particular interest is the effect of the overall change between Ras and Ras′. It appears that the stabilizing interaction (relative to water) between the substrate and the residues of Ras is *reduced* when GAP binds to Ras and that this destabilization effect is smaller in the TS. This is an important effect since it should be noted that overall, the RasGAP system binds the substrate stronger than Ras does, and the binding is even stronger at the TS. Apparently, as can be seen from Figure 5, the interaction between GAP and Ras moves the Ras (which is part of the complex) from its stable RS arrangement towards a configuration that stabilizes the TS and the product. The corresponding catalytic effect is indirect and is thus quite different than the direct effect of the arginine finger. It is important to note in this respect previous works (e.g., Muegge et al.[27]), where it was proposed that the GAP-induced transition pushes the GTP-bound form of Ras [referred to here as Ras(GTP)] towards the structure of the GDP-bound form of Ras [referred to here as Ras(GDP)]. Now, while the effect of GAP leads to stabilization of the GDP+P_i form (Fig. 3), the structural changes upon moving from Ras to Ras′ are smaller than the changes between Ras(GTP) and Ras(GDP). Nevertheless, the structural changes are very effective in modulating the activation barrier.

Although we believe that the above analysis establishes (at least in part) the indirect allosteric effect of GAP, we move now to a more specific demonstration, focusing on the effect of Gln 61.

GLN 61 DOES NOT OPERATE IN A DIRECT WAY

As clarified in the "Reproducing the Overall Catalytic and Mutational Effects" section we were able to reproduce the observed catalytic effects of different mutants of Gln 61. Furthermore, we found that the mutation of Gln 61 to its nonpolar analogue ($Q61Q^{NP}$) does not produce a large anticatalytic effect. Although these findings point out toward an indirect effect, we must also exclude the possible direct involvement of Gln 61 in the chemistry as a general base. This option has been ruled out in the isolated Ras[14] but here we reexamined it in the RasGAP complex. The examination involved the inclusion of Gln 61 in the EVB region and consideration of a proton transfer to this residue. This study gave $\Delta G_0 \approx 23 \pm 2$ kcal/mol for proton transfer from the nucleophilic water to Gln 61. Since this ΔG_0 is higher than the actual barrier for the whole hydrolysis reaction, we can exclude the possibility of a direct chemical involvement of this residue (see related discussion in Langen et al.[14] and Glennon et al.[16]). Note also that a recent theoretical attempt to support the Gln 61 as a general base proposal involved a so-called first principle molecular dynamics (FPMD) simulations of only 0.5

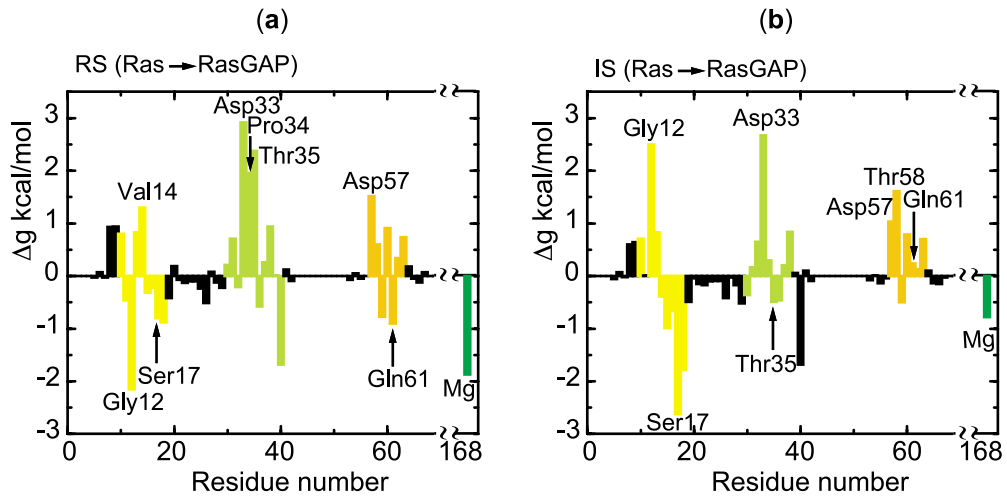

Fig. 5. Electrostatic effects due to the formation of the RasGAP complex. The figure depicts the main changes in the electrostatic contributions from the Ras residues, due to formation of the RasGAP complex, in the RS state (**a**) are compared to those in the IS (**b**). Negative and positive values stand, respectively, for stabilization and destabilization due to the structural changes upon complex formation (Ras → Ras′). The changes in the key areas are highlighted according to the notation of Figure 6; P-loop (yellow), Switch I (light green), parts of β_3 and L4 of the switch II (orange) and the Mg (dark green). The calculated changes were obtained by the LRA procedure and then scaled by a dielectric constant of 20 and 4 for the ionized and polar residues, respectively (see Methods).

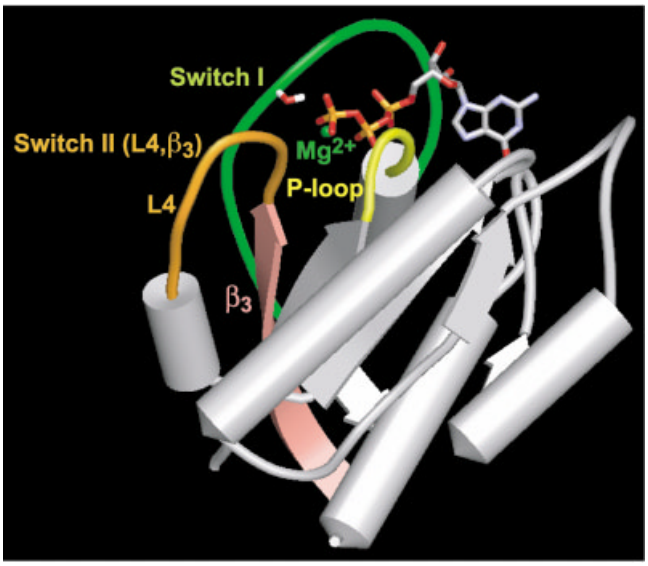

Fig. 6. A schematic representation of Ras and its key regions. The regions highlighted in different colors have substantial effect on the active site.

ps.[29] These simulations forced the water oxygen…γ-phosphate distance (the O_w-γP in the notation of Cavalli et al.[29]) to be in a bonding distance, thus creating an extremely unstable state that collapsed by ejecting the water proton toward Gln 61. Unfortunately, such an approach cannot be used to determine the free energies of different mechanistic options (see discussion of this and other inconsistent studies in Warshel[18]).

Another appealing proposal involves the idea that Gln 61 orients the attacking water molecule (e.g., Resat et al.[30]). This proposal is in fact a ground state entropic proposal, since "orientation" per se, has no meaning in terms of the corresponding change in $\Delta g^{\ddagger}$, unless it implies a ground state destabilization by entropic effects (see discussion in Villá et al.[31]). Now, while some entropic effects are possible, they are not expected to be large since Gln 61 "orients" the water fragment in the TS as well. More importantly, the entropic contributions to $\Delta\Delta g^{\ddagger}$ are included in our free energy calculations, and since mutating Gln 61 to its nonpolar form, $Q61Q^{NP}$, did not lead to a large anti-catalytic effect, we must conclude that the direct orientation effect cannot be large (no orientational effects are expected for the non-polar mutants).

WHAT IS THE ACTUAL EFFECT OF GLN 61 MUTATIONS?

Our finding that the $Q61Q^{NP}$ electrostatic mutation does not lead to a large anticatalytic effect is strengthened by the fact that the Q61NGln mutation does not lead to a large effect (see Concluding Remarks). Since the anticatalytic effects of most mutations are large, it is essential to simulate the effects of such mutations and to examine their origin. The simulations of the Q61L and Q61A mutations reproduce the large observed effect (see Table I). Now, since our calculations reproduce both the small effect of $Q61Q^{NP}$ and the large effect of Q61L mutation we must look for indirect mutational effect and in particular destruction of the catalytic configuration. Previous studies have implicated mutations of Gln 61 in changing crucial regions in the protein[16] and/or changing its interaction with the substrate and the attacking water (e.g. Resat et al.[30]). Yet, these studies were not able to actually determine in a stable way the effect of Gln 61. Moreover, no previous study was able to reproduce the effect of the actual mutation of Gln 61 and/or decompose this effect to its components. Here, we can make a major step in exploring the effect of Gln 61 mutations by applying the LRA analysis. This approach

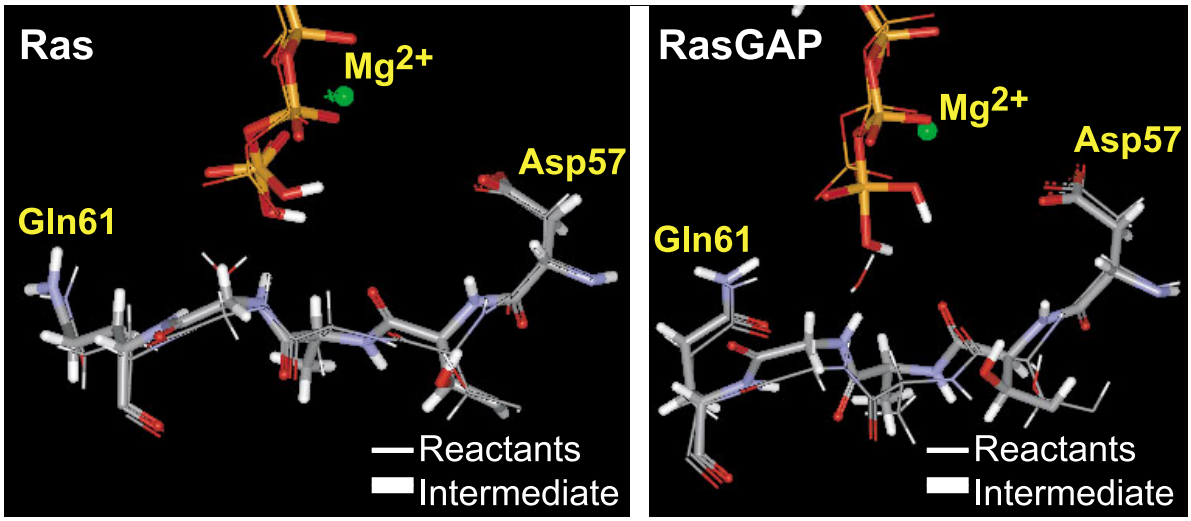

Fig. 7. Structural changes through the reaction. The figure compares RS structures of the active site in both the Ras and RasGAP complex (plain line) to their corresponding TS structures (sticks). Particular emphasis is given to the Asp57-Gln61 region. The changes in the backbone of this region in the TS of the RasGAP complex do not occur in the Ras protein alone.

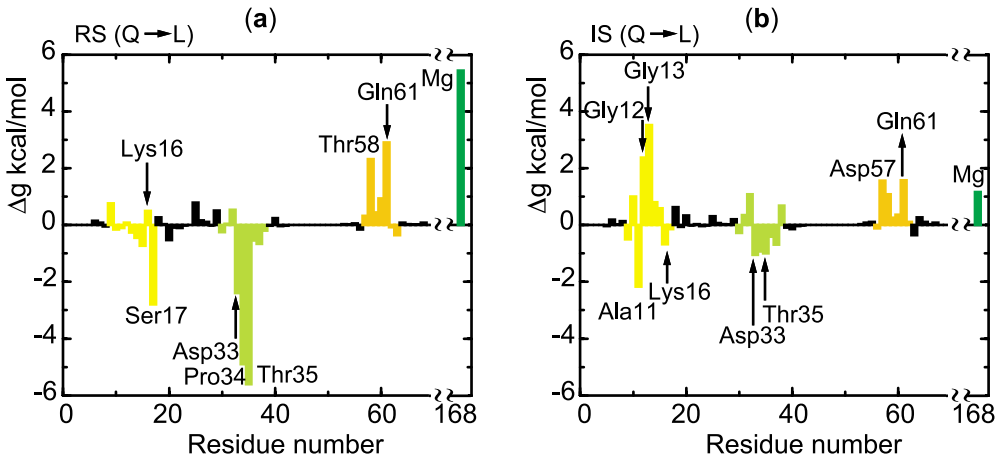

Fig. 8. Electrostatic effects due to the Q61L mutation. The figure depicts the main changes in the electrostatic contributions from the Ras residues due to the Q61L Mutation. The changes in the RS are compared to those in the IS in (**a**) and (**b**), respectively. Negative and positive values correspond, respectively, to stabilization and destabilization due to the mutation. The changes in the key areas are highlighted according to the notation of Figure 6; P-loop (yellow), Switch I (light green), parts of β_3 and L4 of the switch II (orange) and the Mg (dark green). The calculated changes were obtained by the LRA procedure and then scaled by a dielectric constant of 20 and 4 for the ionized and polar residues, respectively, (see Methods).

allows us to determine the individual contributions of the Ras residues and their mutants in the RasGAP complex. This is quite different than our previous attempt to determine the effects of different residues in two different structures of Ras in the absence of GAP.[16] Not only does the LRA analysis guarantee additivity of the different contributions, but it allows us to focus on the well-defined problem of the effect of Ras residues in the RasGAP complex. Figure 8 presents the changes in the LRA electrostatic contributions from the Ras residues due to the Q61L mutation in the RasGAP complex. Figure 8a displays the changes in the RS while Figure 8b displays the changes in the penta-coordinated IS, (note again, that the results for the IS are comparable with those of the TSs and are used here in discussing the properties of the TS). The main conclusion that emerges from Figure 8 is that the Q61L mutation leads to major changes in the interactions between several regions of the protein and the TS of the reacting substrate. The largest changes in Ras′ occur in the contributions of the P-loop, switch I, β_3, L4 and the contributions of Mg^{2+} ion. Considering the overall contributions quantitatively, it appears that the effect of the mutation is to stabilize the RS and to destabilize the IS, thereby causing loss in catalysis.

In studying protein functions we focus on the average energies over many protein configurations, rather than on averaging these configurations and reproducing the X-ray observed structures. Nevertheless, we provide in Figure 9 the structural results from two snapshots generated during the simulations. The most relevant structural changes include a change in the position of the Mg^{2+} ion relative to

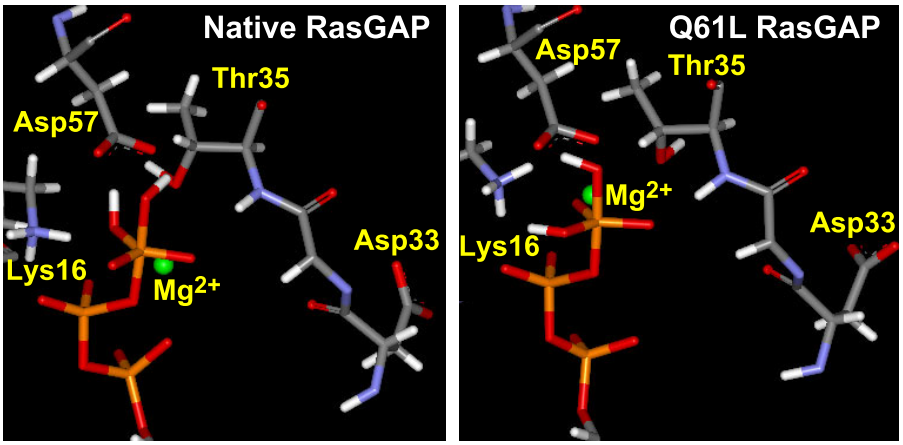

Fig. 9. Structural changes due to the Q61L Mutation. The figure compares the structure of the active site of the native RasGAP complex to that of the corresponding Q61L mutant. The relative positions of Thr35, Asp57 and the Mg^{2+} ion, that undergo the largest structural changes are emphasized.

the γ-phosphate coupled to a displacement of several residues and in particular Asp 33 and Thr 35. These changes are not surprising since the through space distance between Gln 61 and Thr 35 is not large and these residues are "connected" by a water molecule. Furthermore, the changes in 59–61 region are easily propagated through the β_3 loop.

Considering the above analysis we can provide a new view on the origin of the oncogenic effects of mutations of Gln61. Obviously, a part of the catalytic effect of the GAP is associated with a direct electrostatic contribution of Arg 789 (although we note cases where the arginine finger is not involved[12]). However, in addition to the direct effect of GAP, it clearly has a major contribution by forcing Ras to a preorganized catalytic configuration. Now Gln 61 seems to play an important role in this preorganization since mutation of this residue destroys catalysis and leads to major changes in the interaction of Ras (in the RasGAP complex) with the reacting system.

CONCLUDING REMARKS

The present work examines the elusive origin of the oncogenic mutation of Gln 61. It is found that this residue does not operate in a direct chemical way and that even its direct electrostatic interaction with the TS does not account for a major part of its overall catalytic effect. Thus, the action of Gln 61 must involve a significant indirect effect. That is, this residue is a crucial part of the polar framework that forms the active site of the RasGAP complex. This configuration is preorganized as a catalytic configuration only when GAP activates Ras. Now, as is demonstrated in this work, Gln 61 is coupled to other residues in the P-loop switch I, β_3, and switch II and mutations of Gln 61 lead to a major disturbance in the preorganized environment. It is perhaps best to consider Gln 61 as a crucial pivot in a delicately designed environment, so that any serious perturbation (e.g., mutation) destroys the perfect balance. This picture is quite different than a model where the actual interaction between the given residue and the TS is crucial to catalysis.

In view of the significance of our conclusions it is important to examine the validity of our simulations. In this respect it should be realized that we do not contradict any experimental fact, but rather complement these facts by providing new information that could not be obtained experimentally. Each of our individual calculations by itself is not sufficient to establish the current conclusion. For example, it is possible to argue that something might be missing in the $Q61Q^{NP}$ calculations (e.g., some quantum mechanical effects) and that in "reality" the direct interaction is much larger. However, the experimental fact that the Q61NGln mutation does not lead to a significant anticatalytic effect lends a major support to the conclusion from the $Q61Q^{NP}$ simulations. Here we have both experimental and computational evidences that a change of the charge distribution of Gln61 with a small change in the shape of this residue does not lead to a large change in activity. It is significant, however, that we do not find a support for the idea that Q61NGln and the native protein act by distorting the GTP toward its TS structure[15] (the tetrahydral geometry of the γ-phosphate in the RS was found to be similar in the native and in the Q61NGln mutant).

At any rate, after establishing that Gln 61 does not act in a direct way and reproducing the large effect of the Q61L and Q61A mutations, we believe that the computational analysis of the effect of these mutations is a valid sequence-structure-function correlation.

In view of our findings, it might be useful to perform double mutations of Gln 61 and different key residues that produce "hot spots" in Figure 8. Experimental and theoretical analysis of such mutations might quantify the indirect coupling between Gln 61 and the TS by showing how the changes at the Gln 61 site are propagated in the active site.

An additional indirect support for our finding comes from the observation (A. Wittinghoffer, personal communication) that GAP forms complexes in the active site of Gln 61 mutants of Ras and GTP (which correspond to the GS of our reaction), but it does not form complexes with such mutants and transition states analogues (TSAs). This

finding indicates that TSAs are not stable in the active sites of Gln 61 mutants of the RasGAP complex. This is consistent with the idea that the TS is unstable in the mutant enzyme (relative to its stability in the native system). However, we have here more than just a support for the idea that the enzyme works by transition state stabilization. That is, TSAs (e.g., GDP · AlF3) are quite different than the actual TS. Thus, the possible specific interaction between Gln 61 and the TS (which presumably leads to the direct effect of Gln 61) cannot stay so specific and large when the TS is replaced by the TSA. This means that the assumption that Gln 61 has a very specific chemical interaction with the TS is inconsistent with the destabilization of the TSA (the TSA would not be destabilized significantly by the mutation since the native enzyme is unlikely to have the same strongly stabilizing Gln61-TSA interaction). The destabilization of the TSA is consistent, however, with a more delocalized change in the interaction between the active site residues and the TS upon mutation of Gln 61 (such changes are also likely to reduce the electrostatic interaction with the TSA).

Another source of support to our finding that Gln 61 acts in an indirect way comes from studies of the effects of mutations of $G_{s\alpha}$. Q227L mutation (that is thought to have similar role to that of the Q61L mutation in RasGAP) only leads to a reduction of about 10^2 in k_{cat}[32,33] compared to the 10^6 effect of the Q61L mutation. If the catalytic Gln was involved in a special direct interaction with the TS one would have expected similar contributions in the two related systems.

The mechanism of the GTPase reaction of the Ras system has been the subject of a significant discussion (e.g., see Glennon et al.[16]). The mechanistic considerations ranged from examination of linear free energy correlations (LFERs)[35] to considerations of the effect of the arginine finger on the associative and dissociative mechanisms.[16,35] The present study simulated an S_N2 type associative mechanism and succeeded to reproduce the catalytic effects of the different key residues. However, as shown in our previous work[16] in a somewhat more qualitative way, similar results are obtained with a more dissociative type of the TS. Thus, the conclusions of the present work are not largely dependent of the exact mechanism of the GTPase reaction.

In view of the present findings we consider the indirect effect of GAP as the major factor in the activation of the signal transduction switch. Apparently, relatively small structural rearrangements in switch I, II β_3 and the P-loop are sufficient to change in a drastic way the stabilization of the TS by the RasGAP complex. The system can be considered as metastable with regards to its native sequence, where mutations of some residues change the energy landscape and lead to destruction of the catalytic effect. Mutations of Gln 61 are particularly effective in destroying the catalytic configuration, leaving the system in its ON state and thus causing cancer.

There are probably some conformational changes that can restore the catalytic activity of Gln 61 mutants. Finding small molecules capable of inducing these conformational changes, might be an important therapeutic strategy.[36]

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