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On the Mechanism of Guanosine Triphosphate Hydrolysis in *ras* p21 Proteins†

Ralf Langen, Thomas Schweins, and Arieh Warshel*

Department of Chemistry, University of Southern California, Los Angeles, California 90089-1062

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ABSTRACT: The residue Gln61 is assumed to play a major role in the mechanism of *ras* p21, and mutations of this residue are often found in human tumors. Such mutations lead to a major reduction in the rate of GTP hydrolysis by the complex of *ras* p21 and the GTPase activating protein (GAP) and lock the protein in a growth-promoting state. This work examines the role of Gln61 in *ras* p21 by using computer simulation approaches to correlate the structure and energetics of this system. Free energy perturbation calculations and simpler electrostatic considerations demonstrate that Gln61 is unlikely to serve as the general base in the intrinsic GAP-independent reaction of p21. Glutamine is already a very weak base in water, and surprisingly the $GlnH^+$ OH^- reaction intermediate is even less stable in the protein active site than in the corresponding reaction in water. The electrostatic field of Glu63, which could in principle stabilize the protonated Gln61, is found to be largely shielded by the surrounding solvent. However, it is still possible that Gln61 is a general base in the GAP/*ras* p21 complex since this system could enhance the electrostatic effect of Glu63. It is also possible that the γ-phosphate acts as general base and that Gln61 accelerates the reaction by stabilizing the OH^- nucleophile. If such a mechanism is operative, then GAP may enhance the effect of Gln61 by preorienting its hydrogen bonds in the transition-state configuration.

ras p21 proteins constitute a group of highly conserved proteins that play a major role in signaling cell growth and differentiation in eukaryotes. Like other G-proteins these proteins have the ability to bind GTP and hydrolyze it to GDP. Their intrinsic GTPase activity can be enhanced over 1000-fold (Bollag & McCormick, 1991) by physical interaction with the GTPase activating protein (GAP). The GTP-to-GDP conversion leads to a significant conformational change in several regions of the *ras* protein. The GTP-bound conformation seems to represent the active, growth-promoting state of the proteins whereas the GDP-bound form appears to be inactive.

Mutations in positions 12, 13, and 61 of *ras* p21 have been found in a large number of human tumors. X-ray crystallography studies (De Vos et al., 1988; Pai et al., 1989, Krengel et al., 1990; Milburn et al., 1990) have located these residues in close proximity to the GTP γ-phosphate (Figure 1). Biochemical analysis has shown that these mutations impair the protein's ability to hydrolyze GTP (Fasano et al., 1984) and render it insensitive to activation by GAP (Adari et al., 1988). Therefore, the protein remains in the active, growth-promoting GTP-bound state, which can lead to cancer. Thus an understanding of the GTPase mechanism of p21 and the effect of GAP on this mechanism is of importance for an understanding of the molecular basis of cancer. The role of Gln61 in the mechanism of p21 is of particular interest since mutations of this residue in the GAP/*ras* p21 complex reduce the rate of GTP hydrolysis by more than 4 orders of magnitude (Sigal et al., 1988) and are found to be responsible for cell transformation (Fasano et al., 1984). The same mutations in the isolated p21 change the hydrolysis rate by only 1 order of magnitude (Fasano et al., 1984).

A recent study (Pai et al., 1990; Krengel et al., 1990) proposed that Gln61 helps to facilitate the nucleophilic attack on the γ-phosphate by activating a water molecule. Although the proposed mechanism is not explicitly stated, it seems to imply that Gln61 is the general base in the hydrolysis. Such a mechanism [which is referred to here as the general base (GB61) mechanism] is consistent with the observation of a water molecule between Gln61 and the GTP perfectly positioned for a direct in-line attack on the γ-phosphate. However, despite the appealing structural evidence this mechanism is not fully established. For example, structural studies of p21 mutants have been recently used as an argument against the role of Gln61 as a general base (Prive et al., 1992). In order to validate the proposed mechanism, it is essential to correlate the structure of the system with its energetics.

This work examines the GB61 mechanism by using the structure of *ras* p21 to evaluate the energetics of the assumed proton-transfer process. This is done by using the empirical valence bond (EVB) approach that has been used previously in semiquantitative studies of the closely related hydrolytic reaction of staphylococcal nuclease. The EVB results are further verified by well-defined electrostatic considerations. The present study indicates that Gln61 is not likely to be the general base in the reaction of isolated p21. Alternative mechanisms and the possible effect of GAP are considered as well.

SIMULATION STUDIES

Simulations of the Proton-Transfer Step. The GB61 mechanism can be formally described as

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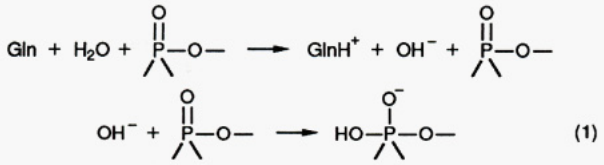

(1)

The energetics of this or any other assumed mechanism can be examined by the EVB method [see, e.g., Warshel et al. (1988) and Warshel (1991)]. This method describes the reacting system as a mixture of resonance structures representing different feasible bonding and charge configurations. The potential surfaces of these resonance structures are calibrated by experimental information of solution reactions and then transferred without any change in parameters to the protein environment. The free energy surface for the enzyme reaction is then evaluated by a combined free energy perturbation (FEP) and umbrella sampling formulation. The EVB/FEP approach is described in detail elsewhere (Warshel et al., 1988), and its reliability has been demonstrated in several test cases [see, e.g., Warshel et al. (1991)], including studies of the catalytic reaction of staphylococcal nuclease (Åqvist & Warshel, 1989), which is related to the GB61 mechanism. The parameters and conditions used in the present simulation are given in Table I.

The EVB/FEP free energy surface of the GB61 mechanism for the GTP hydrolysis in *ras* p21 is depicted in Figure 2. Details of the simulations are outlined in the caption of Table II. The overall rate constant of the reaction is determined by the activation barrier for the proton transfer and the nucleophilic attack stages, which are designated by $\Delta g_1^{\ddagger}$ and $\Delta g_2^{\ddagger}$, respectively. Of particular interest is the fact that the overall activation barrier cannot be lower than $\Delta g_1^{\ddagger}$. Keeping this in mind, we find it very significant that the calculated value of $\Delta g_1^{\ddagger}$ is $\sim 30 \pm 4$ kcal/mol, while the observed activation free energy, $\Delta g_{obs}^{\ddagger}$, for the overall reaction of p21 is 23 kcal/mol. This value is deduced from the observed rate constant of 3.3×10^{-4} s^{-1} (Temeles et al., 1985), by using transition-state theory with a preexponential factor of 6×10^{12} s^{-1}, which is found to be applicable for reactions in condensed phases with a significant activation barrier [see, e.g., Warshel et al. (1988), Kraut (1988), and Warshel (1991)]. One may still argue that tunneling correction should reduce $\Delta g_1^{\ddagger}$, but such corrections are expected to be similar in the reference reaction and in the protein (Hwang et al., 1991) and $\Delta g_1^{\ddagger}$ is larger than ΔG_{PT}^{p} even with tunneling correction. This is important, since ΔG_{PT}^{p} is already higher than the measured energy for the whole reaction. Furthermore, the activation energy for the second step of the reaction is larger than zero so that the total activation barrier must be higher than ΔG_{PT}^{p}. Thus, the EVB calculations suggest that Gln61 is not the general base, at least in the absence of GAP.

Electrostatic Validation of the Calculated Activation Barrier. The validity of the conclusions of the previous section depends, of course, on the accuracy of our calculations. In the present case it is possible to check the calculations by rather simple but quite reliable electrostatic considerations. One can exploit the fact that not only the activation free energy, $\Delta g_1^{\ddagger}$, but even the free energy of the proton-transfer process, ΔG_{PT}^{p}, is predicted to be larger than the experimentally determined activation free energy. An estimate of this free energy, which is significantly easier to calculate than $\Delta g_1^{\ddagger}$, can be obtained by using the protein dipoles langevin dipoles (PDLD) method. This method, described in detail in Warshel and Russell (1984) and Langen et al. (1992), allows one to determine the differences between the electrostatic stabilization of the reacting groups in the protein active site and in a water reference system. The corresponding differences in electrostatic energies can then be used in conjunction with the thermodynamic cycle of Figure 3 to determine the free energy of the proton-transfer step. That is, using Figure 3 we can write [see, e.g., Warshel (1981, 1991)]

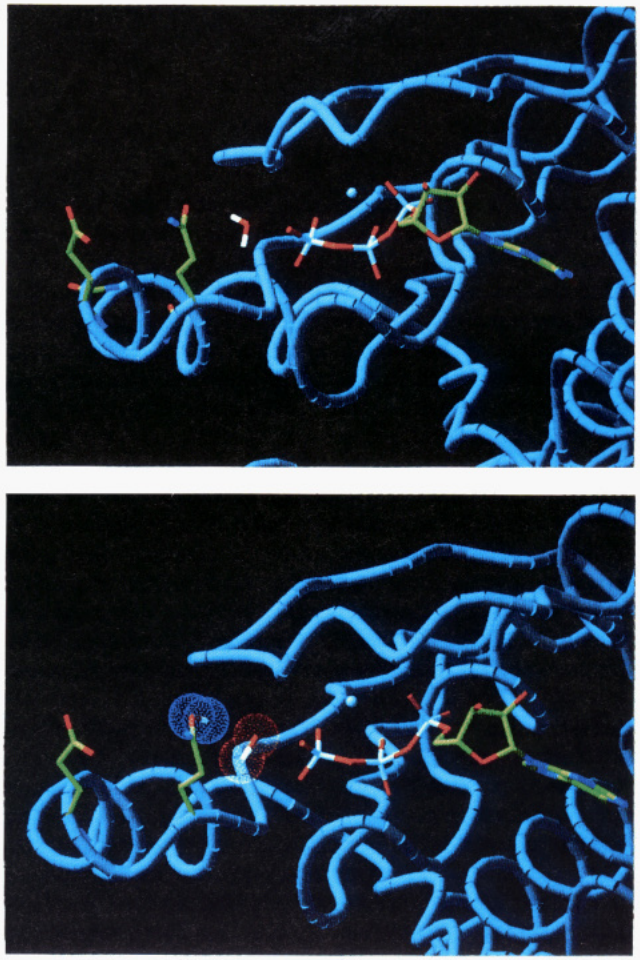

FIGURE 1: (a, top) Structure showing the active site of *ras* p21. Highlighted residues are (from left to right) Glu63, Gln61, water, and GTP. Mg^{2+} is shown as a sphere. (b, bottom) Structure showing the reaction intermediate after the proton-transfer step. Highlighted residues are the same as in (a). The red- and blue-dotted surfaces depict the product of the charge of the given ion and the time-average electrostatic potential from the medium (red indicates positive and blue indicates negative energy contribution). The actual potential energies (relative to the reference water system) are –8 and 17 kcal/mol for the sites of $GlnH^+$ and OH^-. Thus OH^- is less stable in the protein than in the reference solvent cage while the $GlnH^+$ is more stable, but this effect is smaller than the destabilization of the OH^-.

$$\Delta G_{PT}^{p} = \Delta G_{PT}^{w} + \Delta G_{sol,2}^{w\rightarrow p} - \Delta G_{sol,1}^{w\rightarrow p}$$
$$= \Delta G_{PT}^{w} + \Delta\Delta G_{sol}^{w\rightarrow p} \quad (2)$$

where $\Delta G_{sol}^{w\rightarrow p}$ designates the change in the corresponding "solvation energy" or electrostatic energy upon moving the reacting species in a given configuration from water to the protein active site. To evaluate eq 2, it is necessary to know the free energy of the proton-transfer step in solution. This free energy can be obtained to a very good approximation from the difference between the pK_a's of the donor and acceptor groups [e.g., Warshel (1981, 1991)], using

$$\Delta G_{PT}^{w} \simeq -2.3RT[pK_a(GlnH^+) - pK_a(H_2O)]$$
$$\simeq 23 \text{ kcal/mol} \quad (3)$$

This energy is already as large as $\Delta g_{obs}^{\ddagger}$, reflecting the fact

Table I: Parameters Used in the Calculations[a]

Charges:[b] $V_{qq} = 332q_iq_j/r_{ij}$	
$(HO–C–NH_2)^+$ (ψ_2,ψ_3)	$q_C = 0.301$, $q_O = -0.101$, $q_N = -0.202$, $q_{H1} = 0.366$, $q_{H2} = q_{H3} = 0.318$
$(–O–POO_2)^{2-}$ (ψ_1,ψ_2)	$q_{O1} = -0.48$, $q_{O2} = q_{O3} = q_{O4} = -0.82$, $q_P = 0.94$
$(–O(OH)–POO_2)^{3-}$ (ψ_3)	$q_{O1} = q_{O2} = -0.5$, $q_{O3} = q_{O4} = q_{O5} = -1.0$, $q_P = 1.0$
Nonbonded:[b] $V_{nb} = A_iA_j/r^{12} - B_iB_j/r^6$	
$A_{Mg^{2+}} = 96$	$B_{Mg^{2+}} = 32$

Charged Residues[c]

Asp38, 33, 57	$q_{Asp} = -1$	Mg	$q_{Mg} = +2$
Lys16, 117, 88	$q_{Lys} = +1$	GTP (ψ_1,ψ_2)	$q_{GTP} = -4$
Arg68	$q_{Arg} = +1$	GTP-OH (ψ_3)	$q_{GTP-OH} = -5$
Glu62,63	$q_{Glu} = -1$		

[a] Energies are in kcal/mol, distances in Å, and atomic charges in atomic units. Parameters not listed are the same as in Warshel et al. (1988) and Aquist and Warshel (1989). [b] The sensitivity of the final results to the parameters used was checked by repeating the calculations with modified parameters (e.g., changing the residual charges by 15%). The effect on the final results was minor since similar changes occurred in the energies of the reaction in the protein and in solution. [c] The effect of distant ionized groups was estimated using the macroscopic Coulomb law and a high dielectric constant (Warshel & Russell, 1984). The corresponding effect on the final result is rather small.

that Gln is a weak base in water. Thus, if the free energy for proton transfer in the protein, ΔG^p_{PT}, is similar to ΔG^w_{PT}, then Δg_1^* in the protein is larger than Δg_{obs}^*. Of course, the protein groups can change ΔG_{PT}; this protein effect is given by $\Delta\Delta G^{w\rightarrow p}_{sol}$ of eq 2, which is the change in electrostatic energy of the reacting system upon moving from water to the protein active site. In particular, if the protein microenvironment destabilizes the $GlnH^+$ OH^- ion pair (relative to water), then $\Delta\Delta G^{w\rightarrow p}_{sol}$ is positive and ΔG^p_{PT} is larger than ΔG^w_{PT}. The PDLD calculations (Table II) indicate that the protein destabilizes the ion pair and that ΔG^p_{PT} is ~25 kcal/mol, which again is larger than the experimentally determined activation free energy for the whole reaction.

The present results might appear somewhat unexpected, given the fact that the nearby Glu63 could in principle greatly stabilize the protonated form of Gln61. This issue can also be examined by the PDLD calculations, as summarized in Figure 4. As seen from the figure, the overall effect of the negatively charged Glu63 on the protonated Gln61 is only ~2 kcal/mol. The reason for this unexpectedly small stabilization is the remarkable compensation between charge–charge interactions and solvation effects (Warshel & Russell, 1984; Warshel & Åqvist, 1991). That is, while the "gas-phase" interaction between Glu63 and the protonated Gln61 is very large (~65 kcal/mol), this interaction is compensated in the protein by an almost equal contribution (~63 kcal/mol) from the protein permanent and induced dipoles and the surrounding water molecules. In other words, the field of Glu63 is shielded quite effectively by the surrounding medium. Such an effect, which is equivalent to a local, high dielectric constant, is quite common for surface groups (Warshel & Åqvist, 1991) and has been analyzed in detail in related cases [see, e.g., Churg and Warshel (1986)].

The conclusion that the proton transfer to Gln61 is not facilitated by the protein can also be deduced by considering the relative electrostatic potentials of the $GlnH^+$ OH^- ion pair in solution and in p21. These potentials, described in Figure 1 in terms of the corresponding electrostatic energies, demonstrate that the OH^- ion is destabilized by *ras* p21 relative to water. Apparently, the $GlnH^+$ is stabilized by the protein, but this effect is smaller than the destabilization of the OH^- ion, so that overall the ion pair is less stable in the protein. The relative destabilization of the OH^- ion is due to the negative charges of the γ-phosphate whose field is shielded in water more effectively than in the protein. This is true despite the fact that the Mg^{2+} ion and other positively charged residues (e.g., Lys16) decrease the negative potential of the γ-phosphate in *ras* p21.

DISCUSSION

The present simulation studies indicate that Gln61 is unlikely to be the base in the intrinsic reaction of p21[1] This finding is consistent with the experimental observation of Der et al. (1986), who showed that all mutations at this position lead to an 8–10-fold reduction of the reaction rate. These are quite small changes, as compared to the effects of mutations in proteins where a residue is directly involved in the catalytic process [see, e.g., Wilkinson et al. (1984) and Sepersu et al. (1987)]. Such mutations usually lead to a reduction of the reaction rate by several orders of magnitude as in the case of staphylococcal nuclease, where the corresponding effect (Sepersu et al., 1987) was quantitatively reproduced by EVB calculations (Åqvist & Warshel, 1989). Furthermore, mutation of Gln to residues which are far better bases, e.g., Glu or His, would have been expected to accelerate the reaction, but such an effect has not been observed.

The X-ray structures of Milburn et al. (1990) and Pai et al. (1989) are not identical, and our simulation started from the coordinates of Milburn et al. (1990). Thus one may wonder about the generality of the present results. Our FEP approach uses the X-ray structure only as its starting point and then explores many configurations, including those of the transition-state region which are not identical to the X-ray structure. Similarly, our approach can generate the position of the water molecules rather than using those observed in the X-ray structure (usually we find water molecules at the X-ray positions). With this procedure we obtain similar energies, to within 4 kcal/mol, for simulations with significantly different initial coordinates. Thus we believe that similar results would have been obtained with the coordinates of Pai et al. (1989).

The mechanism proposed by Pai et al. (1990) emphasizes the possible role of the main-chain carbonyl of Thr35 which is hydrogen bonded to the nucleophilic water molecule. Our calculations include this group, as well as any other group of the protein, and found that its catalytic role is not so obvious. Although the main-chain carbonyl group of Thr35 polarizes the catalytic water, it does not help the overall proton-transfer process, since it destabilizes the generated OH^- ion. Nevertheless, the carbonyl dipole of Thr35 can reorient itself and stabilize the protonated Gln61. The resulting stabilization is small because the energy gain through this movement is partly compensated by reorganization energy effects, an effect frequently encountered in group reorientation.

If Gln61 is not the general base for the intrinsic GTPase activity of p21, then we are left with only few other proton acceptors. In fact, the X-ray structure does not show an obvious residue in an appropriate position, and no candidate base has yet been identified by site-specific mutagenesis.

[1] After completing the main part of this work (Schweins, 1991), we became aware of a related proposal (Prive et al., 1992) that interprets structural studies of mutations at position 61 and other considerations as evidence against the GB61 mechanism. However, this insightful work did not use energy considerations to correlate the structural information with the energetics of the GB61 mechanism.

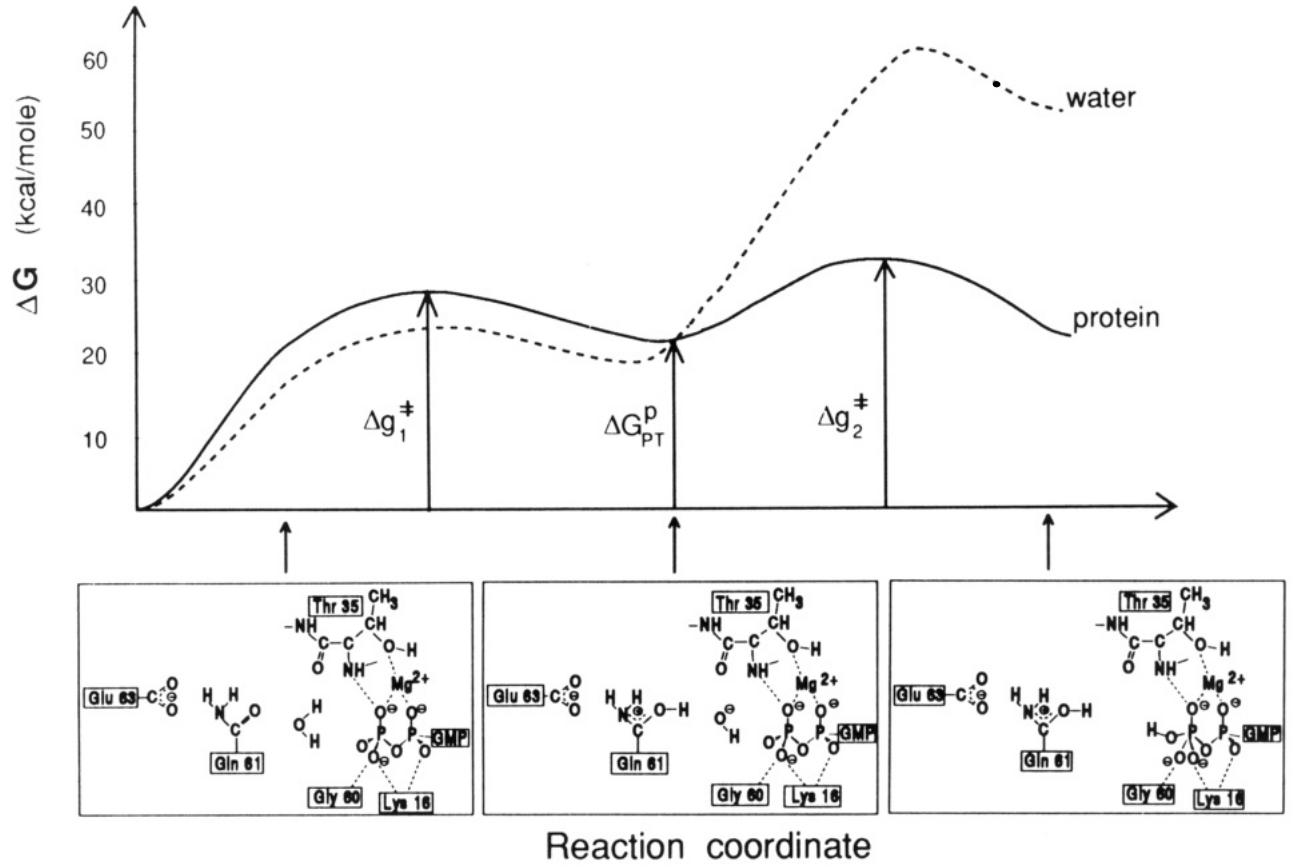

FIGURE 2: Calculated EVB/FEP reaction profiles of the GB61 mechanism in p21 and in a reference solvent cage. $\Delta g_1^{\ddagger}$ and ΔG_{PT} are respectively the activation barrier and the free energy for proton transfer from a water molecule to Gln61. Superscripts p and w designate protein and water. The lower part of the figure schematically describes representative configurations along the reaction coordinate.

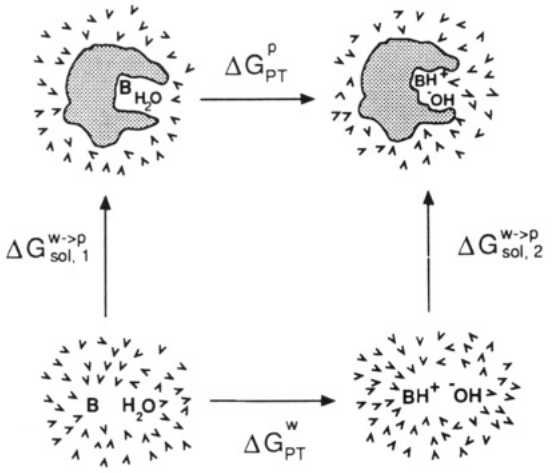

FIGURE 3: Thermodynamic cycle relating the free energies of the proton-transfer step in solution to the corresponding energetics in the protein's active site.

Table II: Energetics of the Proton-Transfer Step from Water to Gln61[a]

		energy in protein		energy in water	
method	process	ΔG_{PT}^{p}	$(\Delta g_1^{\ddagger})^p$	ΔG_{PT}^{w}	$(\Delta g_1^{\ddagger})^w$
EVB/FEP	$B + H_2O \rightarrow BH^+ + OH^-$	25	30 ± 4	22	26 ± 3
PDLD	$B + H_2O \rightarrow BH^+ + OH^-$	23		22	

[a] Energies in kcal/mol. The EVB/FEP simulations were carried out as described by Warshel et al. (1989) and with the recent modifications of Lee et al. (1992), using the ENZYMIX program (Warshel & Creighton, 1989). The simulation involves 11 mapping steps of 2 ps each with 2-fs time steps at 300 K. Cutoff radii of 15, 20, and 18 Å were used for the water sphere, the explicit protein region, and the surrounding grid of Langevin dipoles [see also Lee et al. (1992)]. The PDLD calculations were done with the POLARIS program (Warshel & Creighton, 1989), averaging the calculations over eight protein configurations. The estimated error range reflects the average results of several simulations and the uncertainty in the exact activation barrier in solution.

Another nearby water molecule could act as an alternative base. Of course, nucleophilic attack might also precede the proton-transfer step, or both steps may be concerted. It is possible that Asp57 is a general base for a water molecule that is bound to the Mg^{2+} ion. However, the nucleophilic attack of this water seems to be inconsistent with the observation of in-line attack and inversion of the γ-phosphate center (Feuerstein et al., 1989).

The γ-phosphate itself might also serve as a proton acceptor since the phosphate anion could be a stronger base than water. This depends, however, on the effect of the Mg^{2+} ion. Gln61 could play a major role in this mechanism by stabilizing the generated negative charge rather than by serving as base. For example, the amide group can provide hydrogen bond stabilization to the OH^- nucleophile. In fact, as was shown in the related studies of serine proteases [see, e.g., Hwang and Warshel (1987) and Warshel et al. (1988)], such a stabilization can reduce the activation barrier by as much as 5 kcal/mol. This maximum contribution can be obtained only if the hydrogen bond donor is already prealigned in the optimal direction toward the negative charge (Hwang & Warshel, 1987; Warshel et al., 1988).

A recent work (Prive et al., 1992) has proposed that Gln61 could, in principle, contribute to catalysis by stabilizing the γ-phosphate in the transition-state conformation. However, one has to keep in mind that the distance between the amide group and the γ-phosphate is about 6 Å. Moving Gln61 from

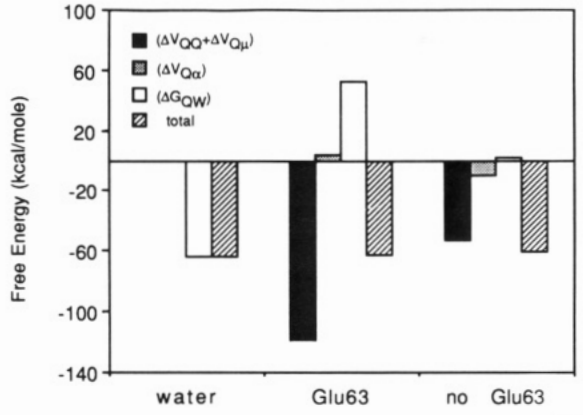

FIGURE 4: Effects of Glu63 on the energy contributions involved in the interaction between OH^- and Gln61. ΔV_{QQ} is the Coulombic interaction between $GlnH^+$ and OH^-. $\Delta V_{Q\mu}$ is the interaction of these groups with all the other protein charges. $\Delta V_{Q\alpha}$ is the interaction of $GlnH^+$ and OH^- with induced dipoles in the protein. ΔG_{QW} is the interaction with the solvent. This figure illustrates how the very large charge–charge interaction between $GlnH^+$ and Glu63 is almost completely compensated for by the change in solvation energy, such that the total energy remains almost constant.

its equilibrium position toward the γ-phosphate may cost a significant amount of energy. The contribution of this residue in stabilizing the pentacoordinated phosphate transition state might be less than the corresponding contribution of nearby water molecules, which would stabilize the transition state when Gln61 is kept at its original place. Here again, one has to consider the concept of compensation through reorganization energy (Warshel, 1991). A significant catalytic effect is hard to obtain when the relevant residue is not at the correct position and orientation in the ground state. The examination of the effectiveness of such a mechanism in isolated p21 will be checked by the EVB/FEP approach.

One of the reasons why the protein is unable to accelerate the GB61 reaction relative to the water reference system appears to be the large compensation for the Glu63–Gln61 interaction. This compensation is mainly due to surrounding water molecules, which shield the field of Glu63 very effectively. Thus, a replacement of these solvent molecules by a medium with a lower local dielectric constant may decrease the free energy and the activation free energy of the proton-transfer reaction. It is possible that the binding of GAP to *ras* involves removal of the water molecules around Glu63 and creation of an environment that leads to a stronger electrostatic interaction between Glu63 and Gln61. Therefore, for the GAP-induced GTPase reaction a mechanism with Gln61 as a base cannot be ruled out. In fact, such a model could explain why all mutations at position 61 make it impossible for *ras* to be activated by GAP, while the effect of these mutations on the intrinsic GAP-independent GTPase activity is relatively small. Such a model may also explain why mutations at position 63 lead to transforming activity (Fasano et al., 1984). Unfortunately, we still do not have any kinetic data on the hydrolysis with and without GAP for these mutants.

Another way by which GAP could help p21 to catalyze the GTP hydrolysis is by controlling the orientation of Gln61 in the protein. As the X-ray studies reveal (Pai et al., 1990; Krengel et al., 1990), both Gln61 and Glu63 are located in a flexible loop which has a higher thermal mobility. GAP could "freeze" this loop in a catalytic conformation so that Gln61 becomes oriented in a way that requires a small reorganization for stabilization of the transition state (probably by interacting with the OH^- nucleophile).

An instructive experiment that should be very informative is the examination of the actual effect of mutation of Glu63 on the GTP hydrolysis reaction of p21, in the absence of GAP. The effect of Glu63 on the GB61 mechanism can be estimated quite accurately; since this group is well solvated, its effect can be evaluated in a reliable way by using Coulomb's law with a dielectric constant between 40 and 80 (Warshel & Russell, 1984; Warshel & Åqvist, 1991) as well as by the PDLD method. Both approaches give a stabilization effect of approximately 1–2 kcal/mol, which corresponds to 1 order of magnitude change in the rate constant. Therefore, although the predicted change in energy is rather small, the corresponding change in rate constant should be reproducible in a mutation experiment. Thus, mutation of Glu63 should change the rate by more than a factor of two if the GB61 mechanism is operative. If such effect is not detected, then the GB61 mechanism can be excluded.

While elucidation of the actual mechanism of p21 and GAP/p21 may still take some time, we have demonstrated here how one can use computer modeling approaches to examine an assumed mechanism. A combination of structural studies, site-specific mutagenesis, and computer modeling approaches may help in the establishment of the catalytic reaction of p21. A good example is the above prediction of the effect of Glu63 in the GB61 mechanism.

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