Dissection of the GTPase Mechanism of Ras Protein by MD Analysis of Ras Mutants

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ABSTRACT **Controlling the hydrolysis rate of GTP bound to the p21^{ras} protein is crucial for the delicate timing of many biological processes. A few mechanisms were suggested for the hydrolysis of GTP. To gain more insight into the individual elementary events of GTP hydrolysis, we carried out molecular dynamic analysis of wild-type p21^{ras} and some of its mutants. It was recently shown that Ras-related proteins and mutants generally follow a linear free energy relationship (LFER) relating the rate of reaction to the pK$_a$ of the γ-phosphate group of the bound GTP, indicating that proton transfer from the attacking water to the GTP is the first elementary event in the GTPase mechanism. However, some exceptions were observed. Thus, the Gly12 → Aspartic p21^{ras} (G12D) mutant had a very low GTPase activity although its pK$_a$ was very close to that of the wild-type ras. Here we compared the molecular dynamics (MD) of wild-type Ras and G12D, showing that in the mutant the catalytic water molecule is displaced to a position where proton transfer to GTP is unfavorable. These results suggest that the mechanism of GTPase is indeed composed of an initial proton abstraction from water by the GTP, followed by a nucleophilic attack of the hydroxide ion on the γ-phosphorus of GTP. Proteins 2005;59:528–533.** © 2005 Wiley-Liss, Inc.

Key words: p21^{ras}; Gly 12 oncogenic mutation; signal transduction; G-proteins; hydrolysis

INTRODUCTION

p21^{ras} plays a central role in signal transduction pathways that regulate cell proliferation, differentiation, and apoptosis.[1–3] Ras functions as a molecular switch by cycling between an active GTP-bound ON form and an inactive GDP-bound OFF form.[4] The duration of each of the ON and OFF states depends on the presence of a guanine–nucleotide exchange factor (GEF), which catalyzes the exchange of protein bound GDP by GTP,[5] on an intrinsic relatively inefficient GTPase activity[6,7] and on the activity of GTPase activating proteins (GAPs) that accelerate the intrinsic GTPase by several orders of magnitude.[7–11] Importantly, oncogenic mutants of Ras are found in 25–30% of human tumors.[1,2,12] These mutations are found mainly in the positions Gly12, Gly13, and Gln61. All of the oncogenic mutants are characterized by a severely impaired GTPase activity. In view of this, it is of pivotal importance to investigate the mechanism of the intrinsic GTPase activity. Different catalytic mechanisms were proposed for the GTPase activity of p21^{ras}. One suggestion was that Gln 61 serves as the general base that activates a water molecule during catalytic hydrolysis of the GTP molecule.[13–15] Single-point mutations at this site impair GTPase activity, prevent GAP-dependent GTPase activation, and are oncogenic.[1,9,16] However, this mechanism is not consistent with many experimental and theoretical data.[17–19] The main reason is the low pK$_a$ value of Gln, which is inconsistent with a base role. A different catalytic mechanism has been suggested in which the GTP itself serves as a base that extracts a proton from the catalytic water molecule.[18–22] A hydroxide ion is generated by the proton transfer which, subsequently, attacks the γ-phosphoryl group of GTP, producing a trigonal bipyramidal transition state. The pentavalent intermediate then separates into inorganic phosphate and GDP.

In an elegant work Warshel and his colleagues[18] have shown an LFER in the intrinsic and GAP-stimulated GTPase. They have shown that there exists a linear relationship between the logarithm of the reaction rate and the pK$_a$ of the γ-phosphate group of wild-type p21^{ras} and many of its mutants. All the mutants that have low pK$_a$, and therefore, low reaction rates are oncogenic, whereas mutants with pK$_a$ similar to that of wild-type p21^{ras} are not oncogenic, with two exceptions. These results are interpreted to indicate that the first elementary event in the GTPase reaction is the extraction of a proton from the catalytic water by GTP itself. Therefore, the lower the pK$_a$ of the γ-phosphate, the lower is its basicity and the slower is the proton transfer rate. In another work Schweins and Warshel[19] show that their results can be rationalized despite the fact that the proton transfer is not the rate-limiting step in the mechanism. As noted above, there were two exceptions to the LFER observed, namely, that the mutants G12D and Q61L have pK$_a$s similar to that of wild-type ras, but nevertheless, have low GTPase activity.

In this work we present molecular dynamics data relating to the orientation of the catalytic water molecule and show that the G12D mutant is unique in the sense that the

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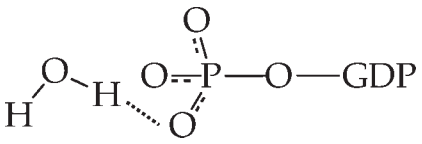

Fig. 1. The ideal positioning of the water molecule for proton transfer to the γ-oxygen of the GTP. Three parameters have to be matched to make possible the proton transfer from the catalytic water molecule to any of the γ-oxygens of GTP: 1. The distance of the transferred proton to the accepting oxygen has to be small enough. 2. The angle Pγ–Oγ–H has to be near tetrahedral. 3. The angle Oγ–H–O(water) has to be near linear.

catalytic water molecule is completely displaced from its original position, and the newly adopted orientations do not allow proton transfer to the γ-phosphoryl group of GTP, thus providing an explanation for the low GTPase activity and oncogenicity of the G12D mutant despite its normal pK_a. A possible reason for these observations is discussed in terms of the changes that take place in the structure of the active site of the mutant.

METHODS

Crystal structure of $p21^{ras}$ was obtained from the Protein Data Bank entry 1qra. Hydrogens were added by using the biopolymer module of the Insight II program. The mutants were produced by changing the designated amino acids using the Insight II program. Two hundred thirty-two water molecules were from the original PDB structure (numbered from 1001–1232), and 966 water molecules were added to the simulation by soaking the structure (by Insight II), forming a 5 Å-wide layer of water molecules around the protein. This resulted in the addition of 961 water molecules (numbered 1–961). The number of water molecules before and after the simulations was constant. This suggested that the density of water molecules does not change during the simulations. Therefore, we considered it unnecessary to perform the simulations under the more rigorous conditions of a box of water under constant pressure to keep the density of the water molecules constant. MD runs were performed with the CHARMm program using the CHARMm 27 force field. The initial structure was minimized by the steepest descents method using 500 steps of 0.02 ps each. The system was heated from 240 to 300 K in 1000 steps of 0.001 ps each, and equilibrated in 3000 steps of 0.001 ps each. The run was carried out for 2 ns in 2,000,000 steps of 0.001 ps each that were divided into 2000 frames. Runs were analyzed with the Decipher module of Insight II.

RESULTS AND DISCUSSION

Identification of the Catalytic Water Molecule of Wild-Type ras

Because the primary elementary event in the GTPase mechanism is supposed to be the proton transfer from the catalytic water molecule to the γ-phosphate group of the GTP moiety, the configurations of the MD runs were analyzed to find out if they fulfill the basic requirements for proton transfer (PT). The ideal positioning of the water molecule relative to the γ-phosphate is depicted in Figure 1. Three criteria have to be met to make possible a proton transfer: (1) the distance between the transferred proton and the accepting γ-oxygen must not exceed 3.2 Å, which is the maximal distance of hydrogen bond formation. (2) The angle Pγ–Oγ–H(water) has to be a near tetrahedral one. (3) The angle Oγ–H(water)–O(water) has to be a near linear one. A conformation that meets these three conditions can be considered as proton transfer competent.

The layout of water molecules in the vicinity of the γ-phosphate of the GTP moiety for both the wild-type and the G12D mutant is shown in Figure 2. The figure represents the average structures of the systems. For clarity, the proteins were blanked out, leaving only the GTP moiety and the water molecules. The displayed water molecules include both the crystallographic water molecules and the bulk water molecules derived from the solvation of the systems. The Insight II program was used to locate all the atoms in the system that are within a 3.2-Å distance from any of the γ-phosphate oxygens. In the wild-type ras four such water molecules were located as denoted in Figure 2(A). Only wat1117 (with all its atoms) is within the allowed distance from O1γ. O2γ is within the 3.2-Å distance from both hydrogens of wat1002 (but not its oxygen), and from the H2 hydrogen only of wat1003. However, wat1002 and wat1003 were not further analyzed for proton transfer competence for several reasons. First, water1003 is in a back position relative to the γ-phosphate, as can be seen in Figure 2(A), and such a position is unfavorable for a head-on inline attack on the γ-phosphorus. For the hydroxide ion to perform an efficient nucleophillic attack that leads to the formation of a phosphopentavalent intermediate it has to be in a front position. Therefore, even if there were some proton transfer-competent configurations, the hydroxide ion formed would not be competent for nucleophillic attack. Water molecule 1002 is also in a slightly back position, and in addition, the average distance of the wat1002 oxygen from the γ-phosphorus is 5.11 Å that is too large for a nucleophillic attack. These two requirements for nucleophillic attack competence are also discussed later. O3γ is within the 3.2-Å distance of wat1117 and H2 of wat1004. Both wat1117 and wat1004 are in suitable front position for the subsequent nucleophillic attack. Therefore, wat1117 and wat1004 were further analyzed to determine which configurations were proton transfer competent. It is noteworthy that all the above allocated water molecules belong to the crystallographic water molecules, whereas no molecule of the bulk solvent molecules (numbered from 1–961) met even the first condition of being in the 3.2-Å range. The analysis for proton transfer competence included monitoring of the above-mentioned three parameters. A configuration was considered as proton transfer competent only if the distance of the hydrogen atom from the γ-phosphate oxygen was equal or less than 3.2 Å. The angle Pγ–Oγ–H(water) was considered as tetrahedral if it was more than 70° and less than 140°. The angle Oγ–H(water)–O(water) was considered as linear if it was more than 145°. Typical trajectories for the proton transfer parameters of hydrogens 1 and 2 of wat1117 with O1γ are shown in Figure 3. Trajectories of all other interactions were computed (not

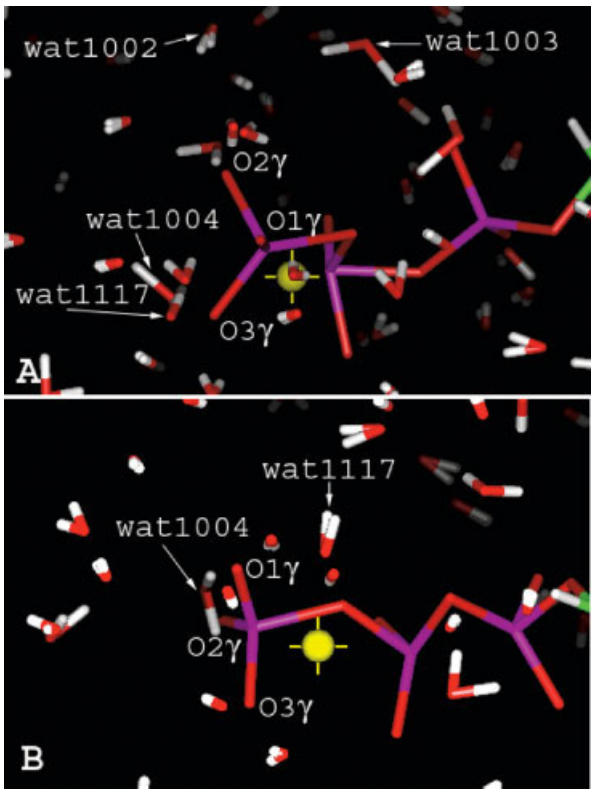

Figure 2.

shown), and the number of competent configurations is summarized in Table I. It is seen that in wild-type ras only wat1117 contributes proton transfer-competent configurations. All proton transfer-competent configurations were associated with O1γ and O3γ, but not with O2γ. The total number of proton transfer-competent configurations was 1055. These results suggest that the wat1117 is the catalytic water molecule in wild-type ras.

Analysis of Proton Transfer-Competent Water Orientations in the G12D Mutant

Because the pK_a for the γ-phosphate of wild-type ras was found to be very close to that of the G12D mutant, and nevertheless, the intrinsic GTPase reaction rate is considerably lower in the mutant,[18] we investigated the proton transfer competence of the relevant water molecules in this mutant. An MD run for the mutant was performed as described under Materials and Methods. The atoms of all water molecules in the mutant, including original crystallographic waters and added solvent molecules, were scanned to locate any atoms that were within the distance of 3.2 Å from any of the γ-phosphate oxygens. The layout of all the water molecules in the vicinity of the γ-phosphate is shown in Figure 2(B). Only the hydrogens of wat1004 were found to interact with O2γ. No interactions were detected with O1γ or O3γ. The number of proton transfer-competent configurations was computed using the same parameters as for the wild-type ras, and was calculated to be 191 (Table I).

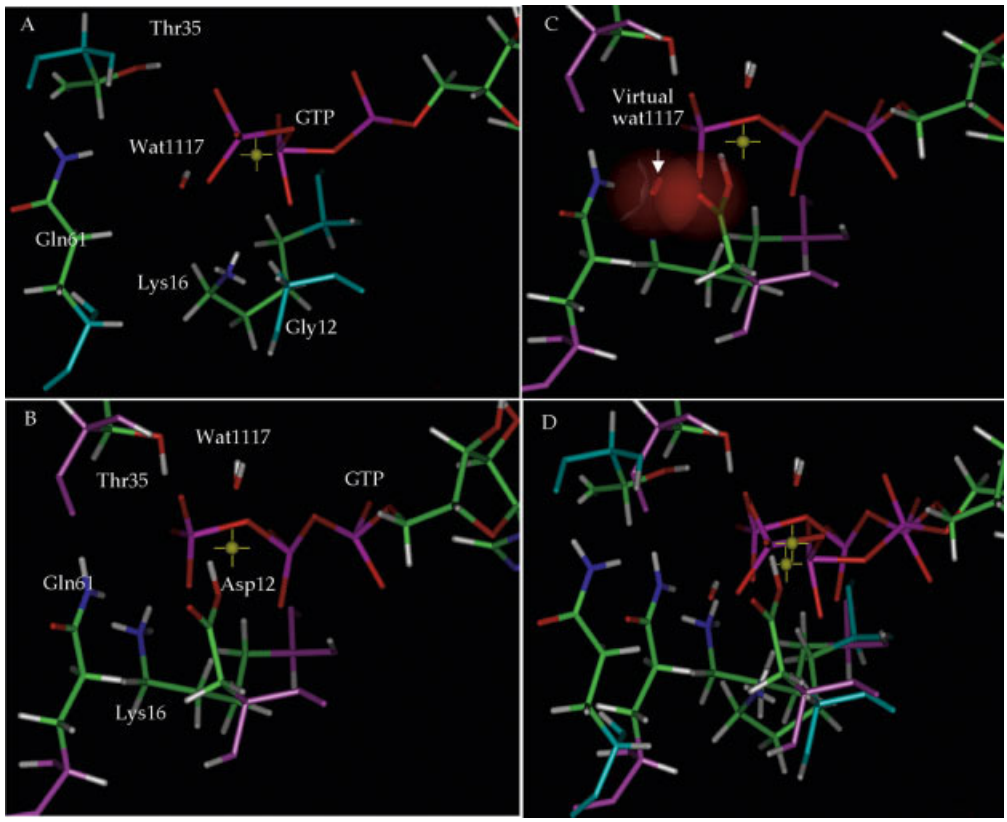

Figure 6.

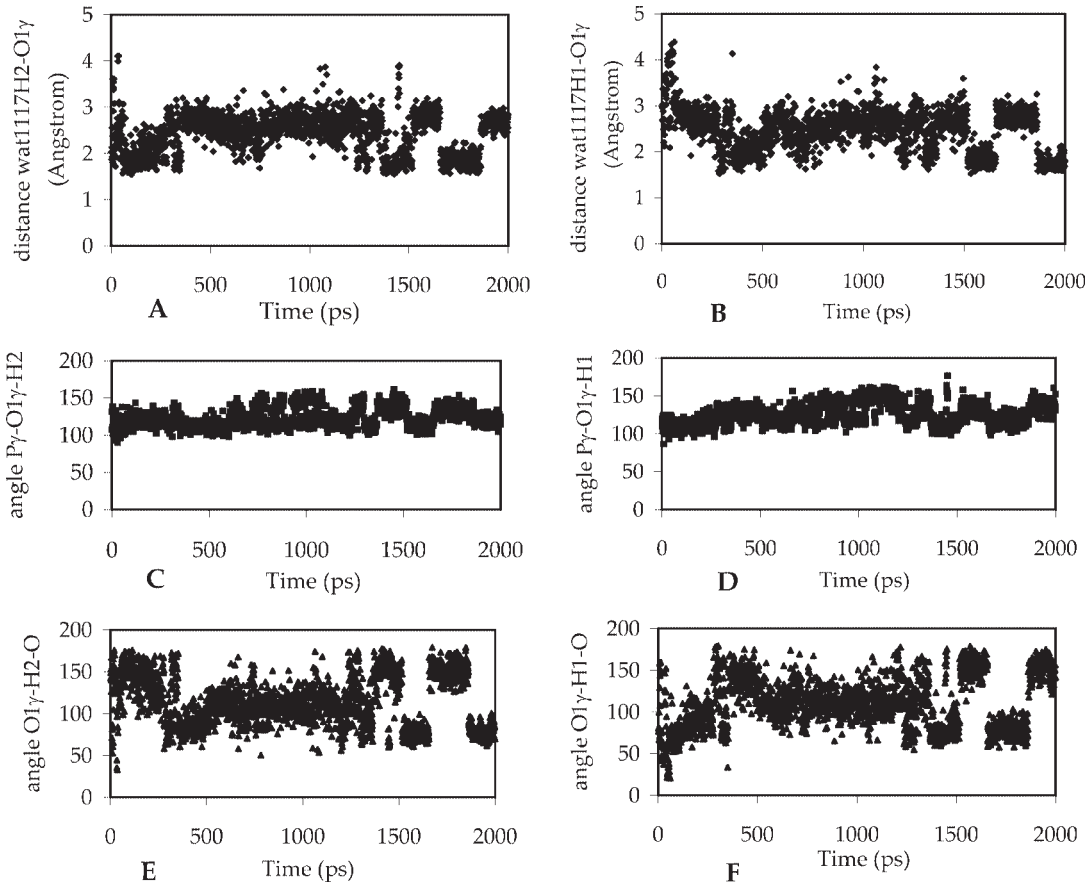

Fig. 3. Monitoring of proton transfer competence parameters. The three parameters for proton transfer competence of the interaction between wat1117 and O1γ were monitored. (**A**, **B**) The distance between hydrogens 1 and 2 to O1γ, respectively. (**C**, **D**) The angle Pγ–O1γ–wat1117 hydrogen H1 and H2, respectively. (**E**, **F**) The angle O1γ–wat1117 hydrogen H1 and H3–Oxygen of wat1117, respectively.

Therefore, it turns out that wat1117 is no more the catalytic water in G12D, and wat1004 partially takes over the task. It should be stressed out that wat1004 is a crystallographic water molecule and that not even one bulk water molecule approached the γ-phosphate oxygens to a distance of 3.2 Å or less.

Because the ratio of proton transfer-competent configurations in wild-type ras versus G12D mutant is 1055/191 = 5.5, these results predict at least a 5.5-fold lower GTPase activity of the mutant. However, further refinement of the calculations to account for nucleophillic attack competence were carried out as described below.

TABLE I. Transfer Competent Configurations of Wat1117 and Wat1004 in Wild-Type Ras

	Transfer competent configurations			
	Wild-type Ras		G12D	
Hydrogen donor–oxygen acceptor	Wat 1117	Wat 1004	Wat 1117	Wat 1004
H1–O1	284	0	0	0
H2–O1	368	0	0	0
H1–O2	0	0	0	117
H2–O2	0	0	0	74
H1–O3	137	0	0	0
H2–O3	266	0	0	0

The MD runs of wild-type ras were analyzed for Wat 1117 and Wat 1004. A configuration was considered as transfer competent upon fulfillment of three conditions: (1) the distance between the transferred hydrogen and accepting oxygen is equal or less than 3.2 Å. (2) The angle Pγ–Oγ–H(water) is more than 70° and less than 140°. (3) The angle Oγ–H(water)–O(water) is more than 145°.

Fig. 2. Water molecules layout in the wild-type and G12D ras proteins. The average configuration of the MD of the wild-type ras (**A**) and G12D mutant (**B**) was computed by the Decipher module of the Insight II, and the proteins were blanked out, leaving the GTP, Mg^{2+} ions (yellow balls) and water molecules on the screen. The program was used to locate the water molecules that were within the 3.2-Å distance from any of the γ-phosphate oxygens. The located water molecules are marked.

Fig. 6. Active-site structures of the wild-type and G12D mutant. The average configurations of wild-type ras (**A**), G12D (**B**, **C**), and superimposed structures of both proteins (**D**) are shown. In (**C**), a virtual water molecule is placed in the mutant in the coordintes originally occupied by wat1117 in the wild type, and vdw surfaces are drawn around the hydrogen HE22 of Gln61, the oxygen atom of the virtual oxygen of wat1117 and OD1 of Asp12. In (**D**), both structures are superimposed by using all atoms of the proteins (except Gly12 of wild type and Asp12 of the mutant), GTP, and Mg^{2+} (yellow) with Insight II. Backbone atoms are in light blue in wild-type ras, and light violet in G12D mutants.

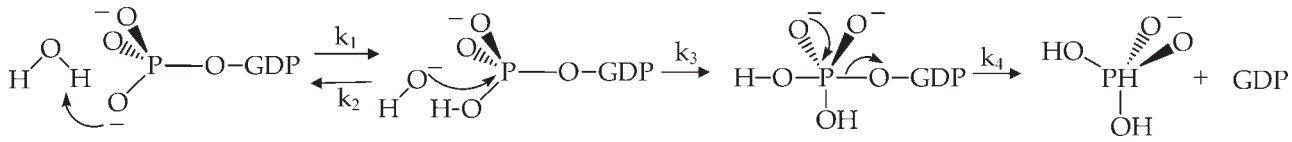

Fig. 4. The mechanism of GTPase in ras. The first step is the abstraction of a proton from water by the γ-phosphate of GTP. The second step is a nucleophilic attack of the hydroxide ion formed on the γ-phosphorus atom, which yields a pentavalent intermediate. The third step is the dissociation of GDP with the formation of P_i.

Analysis of Nucleophilic Attack Competent Configurations

As seen in Figure 4, the second step of the GTPase reactions is the nucleophilic attack of the hydroxide ion that is formed on the γ-phosphorus to yield the pentavalent intermediate. There are two conditions that have to be met by a hydroxide ion to perform the correct nucleophillic attack, as already mentioned above. First, the hydroxide ion has to attack from left to right in a front attack, as depicted in Figure 4. If the hydroxide ion is on the back side, namely, to the right of the γ-phosphorus, no pentavalent intermediate can be formed. It is seen in Figure 2 that the catalytic water molecule wat1117 is displaced in the mutant from a front to a back position. This is another reason why wat1117 cannot function as a catalytic water, besides the fact that it does not meet the initial requirements for proton transfer competence. The second condition for proper nucleophillic attack is that the hydroxide ion must not be too far from the γ-phosphorus. The distances of the oxygen of wat1117 in wild-type ras, and of wat1004 in G12D were computed, and are shown in Figure 5. Every proton transfer-competent configuration was reevaluated by imposing the very permissive condition that the oxygen of the catalytic water molecule not be larger than 5 Å. All the proton transfer-competent configurations of wild-type ras satisfied this condition, whereas only 108 out of the 191 proton transfer-competent configurations of G12D met this requirement. Therefore, the ratio of the configurations that are both proton transfer competent and nucleophillic attack competent of the wild-type ras and G12D is 1055/108 = 9.8. This ratio is close to the 15-fold decrease of GTPase activity of the G12D mutant compared to the wild type.[18] The real ratio is most probably still higher, because the average distance of the wat1117 oxygen from the γ-phosphorus is 3.99 Å, compared with 4.81 Å for G12D (Fig. 5). Thus, under less permissive distance for nucleophillic attack competence the ratio would be higher, for instance, if the distance is required to be equal or less than 4.5 Å, the ratio is 1047/14 = 75.

Interestingly, the bulk water molecules do not approach the γ-phosphorus significantly. The nearest solvent water molecules oxygens have the following average distance from the ϕ-phosphorus: wat798: 7 Å, wat837: 9.3 Å, wat805: 10.5 Å, wat829: 13.36 Å, wat844: 14.01 Å, wat734: 17 Å.

Structure of the Active Site of Wild-Type ras and G12D

In an attempt to gain insight into the possible reason for the displacement of wat1117 from its original position, the

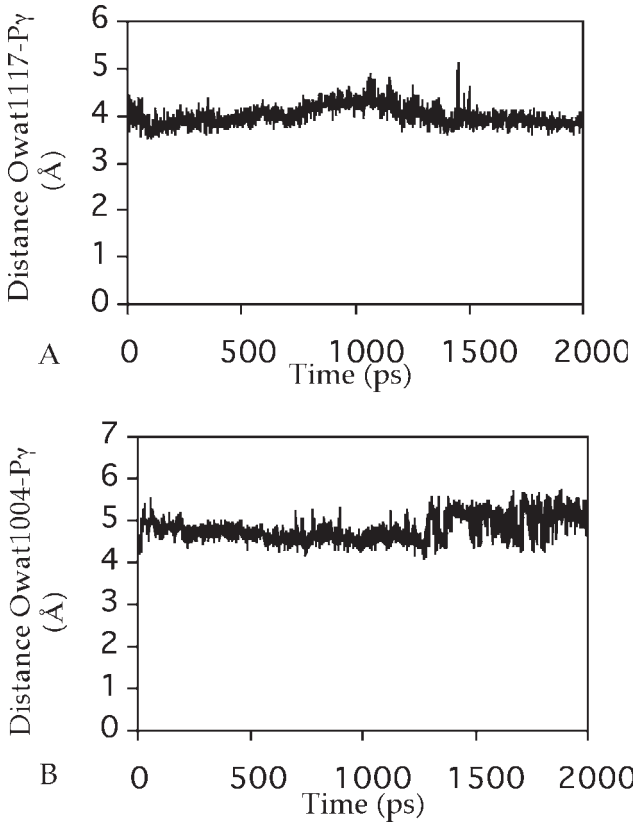

Fig. 5. Distance of catalytic water molecule oxygens from the γ-phosphorus of GTP. The distance of the oxygen of wat1117 of wild-type ras (**A**) and of the oxygen of wat1004 of the G12D mutant (**B**) from the γ-phosphorus of the GTP is computed.

average structures of the active sites of wild-type ras and G12D were compared (Fig. 6). It is seen that Gln61 and the mutated Asp12 come into the proximity of the site that was occupied by wat1117 in the wild-type structure [Fig. 6(C)]. This was calculated by first placing a pseudomolecule of water in the G12D structure in the place that had been occupied by wat1117 in the wild-type structure. The distances measured from the oxygen of the virtual wat1117 to Asp12OD1 and Asp12OD2 were 1.99 and 2.16 Å, respectively, and the distance to Gln61HE22 was 1.79 Å. When both structures are superimposed [Fig. 6(D)], it can be clearly seen that Gln61 comes closer to wat1117, and that Asp12 is also close to that molecule, whereas, Gly12 of the wild type is far away. These results suggest that at least one reason for the displacement of wat1117 from its original position might be the steric clash between it and the nearby residues. Lys16 also adopts a different position; however, this does not bring it to a close proximity to

wat1117, because the average distance from the oxygen of wat1117 and the side-chain amino nitrogen is 6.81 Å.

CONCLUSION

In this work we have provided means for the computational dissection of the GTPase reaction into its two initial steps, namely, the proton transfer and the nucleophilic attack of the formed hydroxide on the γ-phosphorus. By doing so, an explanation is provided for the low reaction rate of the G12D mutant relative to the wild-type ras, despite the similarity in the pK_as of the γ-phosphate group in these two species. The ability to explain the biochemical results by describing the GTPase mechanism as composed of a proton extraction from the catalytic water by the γ-phosphate group of GTP, with a subsequent nucleophilic attack that forms a pentavalent intermediate, lends more probability that this faithfully describes the true mechanism in nature. The water molecule configurational ananlysis used in this study is a novel approach in protein bioinformatics that can be employed to gain a more detailed insight into the mechanisms of hydrolases. Moreover, in every reaction where a transfer of an atom or a group of atoms takes place between a donor and acceptor in the active site of an enzyme, an analysis of the distances of the transfered group and the angles involved may shed light on the enzymatic mechanisms.

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