Ab Initio Study of the Role of Lysine 16 for the Molecular Switching Mechanism of Ras Protein p21

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ABSTRACT Quantum chemical computations using the ab initio molecular orbital (MO) method have been performed to investigate the molecular switching mechanism of Ras protein p21, which has an important role in intracellular signal cascades. Lys^{16} was demonstrated to be crucial to the function of Ras p21, and the hydrolysis of GTP to GDP was found to be an one-step reaction. The potential energy barrier of this hydrolysis reaction from GTP to (GDP + P) was calculated to be ~42 kcal/mol. The role of GAP (GTPase-activating protein) was also discussed in terms of the delivery of the water molecules required for the hydrolysis.

INTRODUCTION

Ras protein p21, coded by the c-Ha-*ras* oncogene and first separated by Weinbarg at MIT in 1979 (Shih et al., 1979), has an important role in intracellular signal cascades in mammals. Ras p21 is a kind of G protein and is active with GTP but inactive with GDP. This mechanism is called the molecular switching mechanism of Ras protein p21 (Barbacid, 1987).

The mutation of amino acids of Ras p21 induces a carcinogenesis, and this carcinogenesis is dominated by a decrease in the GTPase activity in Ras p21. Close inspection of the structures reported by x-ray crystallography (Abola et al., 1987, 1996; Bernstein et al., 1977) of wild-type (Tong et al., 1989) and mutant (Krengel et al., 1990; Franken et al., 1993) Ras p21 revealed that the mutated amino acids were not involved in GTPase activity. That is, neither the Gly^{12} in the wild-type nor the Pro^{12} and the Val^{12} in the mutant are located near the γ-phosphate of GTP. The distances between the carbon atoms of the side chains of Gly^{12}, Pro^{12}, and Val^{12} and the oxygen atom of γ-phosphate are 3.54 Å, 3.37 Å, and 3.61 Å, respectively.

From an inspection of the PDB (Protein Data Bank) (Abola et al., 1987) data on the wild-type and mutant Ras p21, the nitrogen atom of the side chain of Lys^{16} (Lys^{16}-N) was revealed to keep hydrogen bonds with both the γ-phosphate and β-phosphate of GTP (Table 1). Therefore, we suspect that this Lys^{16} plays an important role in GTP activity.

The purpose of this study was therefore to clarify the role of Lys^{16}. Some research on the relationship between the GTPase of Ras p21 and Lys^{16} has already been reported (Pai et al., 1990; Milburn et al., 1990; Chung et al., 1993), but the reaction mechanism of GTP→GDP hydrolysis has not yet been clarified at the atomic level.

In our previous work (Futatsugi et al., 1997), the mechanism of the reaction of the active site of Ras p21 was investigated using the semiempirical molecular orbital method (PM3 method), and γ-phosphate was found to be dissociated from GTP by the proton relay via Lys^{16}-N. The present study makes clear the details of the GTP→GDP hydrolysis by using the ab initio MO method, which is expected to provide further new findings. We will also discuss why the presence of a GAP (GTPase-activating protein) dramatically increases the GTPase activity of Ras p21, as measured in previous studies (Bollag and McCormick, 1991; McCormick, 1992; Gibbs et al., 1988; Eccleston et al., 1993).

METHODS

Model reaction system

The conformation of the model reaction system was determined by molecular mechanics calculation. The mechanics calculation was performed with an AMBER software package (version 4.1, University of California) (Pearlman et al., 1995). The wild-type Ras p21 (PDB code: 121p) was employed for the initial structure, and ~2800 water molecules for solvent were created using the Monte Carlo method. The parameter of the water molecules is TIP3P. Because Lys^{16} is assumed to be of significant importance to the reaction, the all-atom model (Weiner et al., 1986) was applied for Lys^{16} and the united atom model (Weiner et al., 1984) for the rest of the residues. Energy minimization by molecular mechanics was first performed with the steepest descent method. Next, the algorithm was switched to the conjugate gradient method.

The model reaction system for MO calculations was constructed by extracting the structure of the active site from the above-obtained Ras p21. Mg^{2+} is positioned at the center of the active site of the six-coordinated formation with OH bases of Ser^{17} and Thr^{35}, two H_2O molecules, and the oxygen atoms of γ-phosphate and β-phosphate. In the

Received for publication 28 September 1998 and in final form 23 August 1999.

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0006-3495/99/12/3287/06 $2.00

TABLE 1 Distances between Lys^{16} and GTP in crystal structures for the wild-type and the mutant Ras p21 (Å)

Ras p21	Wild-type (GPPCP)	Mutant (G12P)	Mutant (G12V)
PDB code	121p	821p	521p
Oncogenesis	−	−	+
Lys^{16}-N-O-γP*	2.725	2.699	3.108
Lys^{16}-N-O-βP#	3.100	2.886	2.772

Both wild-type and mutant Ras p21 keep hydrogen bonds between the nitrogen atom of the side chain of Lys^{16} and the oxygen atoms of both γ-phosphate and β-phosphate.
*Abbreviation of the oxygen of γ-phosphate.
#Abbreviation of the oxygen of β-phosphate.

computational model, the OH bases of both Ser^{17} and Thr^{35} are replaced by H_2O molecules, and the α-phosphate in GTP is replaced by a hydrogen atom. A water molecule necessary for the hydrolysis was generated to coordinate with the γ-phosphate. Details are shown in Fig. 1. Because this model is a part of the enzyme, the asterisked atoms (*) in Fig. 1 were fixed during the computation to maintain the structure. The degree of freedom of this model system is 101.

Calculation using the *ab initio* MO method

Ab initio MO calculations were performed in the Hartree-Fock level, using a basis functional set, 6–31G**. The structures of the stable states were obtained by geometry optimization, using the energy gradient method. For the determination of the transition state (TS), we first repeated many optimized calculations to obtain the rough atomic geometry of the TS. Next, the exact TS structure was determined using the search algorithm for a transition state. That is, the stable and the transition states are located at the minimum and the saddle points on the 101-dimensional potential energy hypersurface, where a second-derivative matrix shows 101 and 100 positive eigenvalues, respectively. Computation of the vibrational analysis was further performed for the TS structure to check whether the optimized structure was adequate for the saddle point of the hydrolysis reaction. The model molecular system at the saddle point must contain the vibrational mode corresponding to an imaginary frequency. The steepest descent paths from the transition state were calculated in both directions, following the vibrational mode with the imaginary frequency, which provided the lowest energy reaction path connecting a reactant and a product via the saddle point. That is, so-called intrinsic reaction coordinate (IRC) calculations were performed, and structural changes and potential energy changes were presented along the reaction. The software package used for this calculation was Gaussian 94 (Frisch et al., 1995a,b).

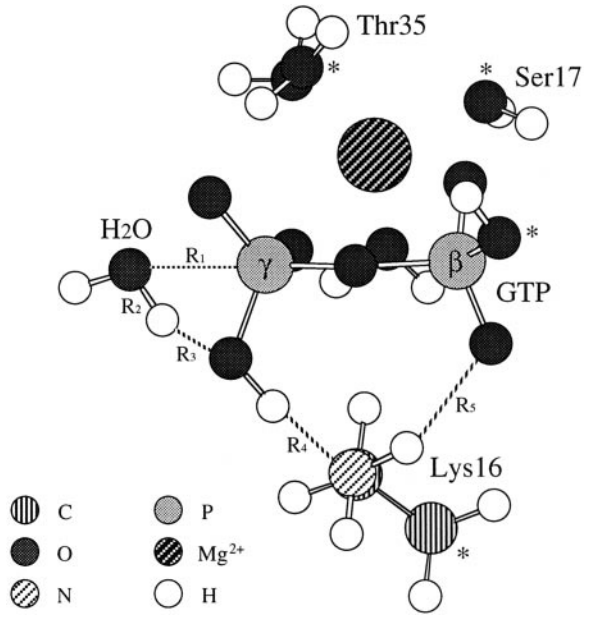

FIGURE 1 Structure of the active site of Ras p21. The conformation of the enzyme was picked out from wild-type Ras p21 (PDB: 121p) and was minimized by molecular mechanics calculation. We coordinate an H_2O molecule to γ-phosphate. Because this reaction occurs in an enzyme, some atoms belonging to the enzyme were fixed, that is, the asterisked atoms were kept at the initial positions during geometry optimization.

RESULTS

The optimized structure for the active site of wild-type Ras p21 is shown in Fig. 2 *a*. It is notable that a proton is attached to the oxygen of γ-phosphate and the structure is maintained in a stable state. Next, an H_2O molecule necessary for the hydrolysis is added. The H_2O molecule is positioned near the phosphorus of γ-phosphate at a distance of 2.1 Å. The optimized structure is obtained by keeping the distance at 2.1 Å, as shown in Fig. 2 *b*. This structure (Fig. 2) is utilized as a preparatory reference in seeking a transition state.

To roughly determine the atomic geometry of the transition state, we adopted the five distances R_1, R_2, . . . , R_5 in Fig. 1 as parameters. Geometry optimizations were performed while keeping each distance fixed, and calculations were repeated while changing only one of the five parameters by increments of 0.1–0.5 Å. Judging from the potential energy change obtained by the above trials, we deduced the structure of the transition state. Then, geometry optimization was again performed, starting from the deduced structure, using the TS search algorithm. An accurate TS structure was obtained by the optimization calculation, and vibrational analysis confirmed that the optimized structure was appropriate for the saddle point.

Furthermore, we performed IRC calculations starting from the TS structure and obtained the reaction path shown in Fig. 3. The abscissa indicates the IRC distance measured from the TS, and the ordinate indicates the potential energy. Four important structures, including the TS structure on this reaction path, are shown in Fig. 4.

Fig. 4 *B* represents the optimized TS structure. Fig. 4, *A* and *C*, indicates, respectively, the structures of the reactant

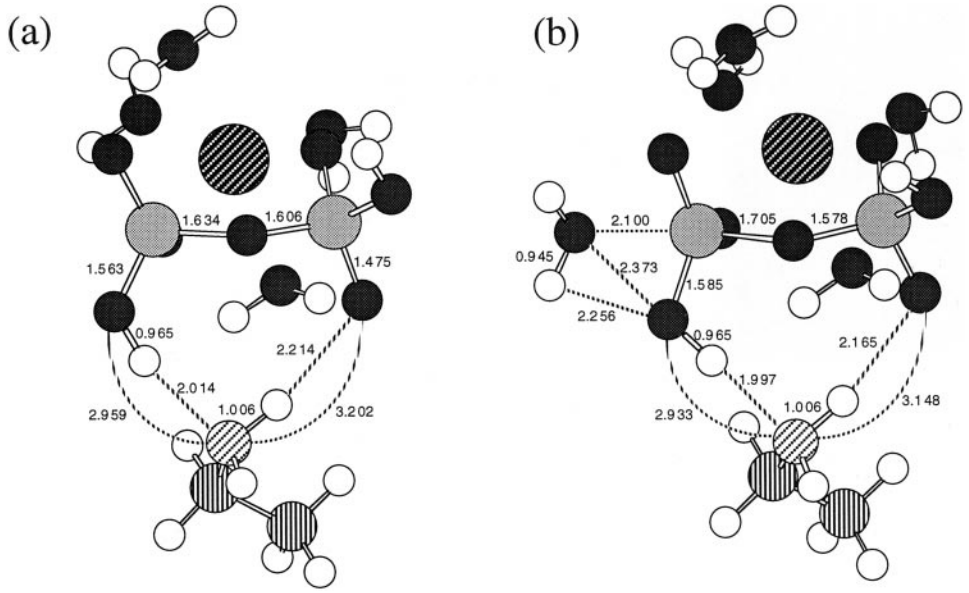

FIGURE 2 Optimized structures of the active site of Ras p21. (*a*) The wild-type Ras p21. (*b*) The structure with an H_2O molecule positioned near γ-phosphate.

and the product obtained by IRC calculations from Fig. 4 *B*. Geometry optimization with the energy gradient method from Fig. 4 *C* gave a more stable structure Fig. 4 *D*, in which γ-phosphate is completely dissociated from GTP.

It is not surprising that structure *A* in Fig. 4 is different from that in Fig. 2 *b*, because the position of the H_2O molecule is restricted in Fig. 2 *b*. Our calculation naturally suggested that structure *A* in Fig. 4 was energetically more favorable by 20.4 kcal/mol.

The proton of Lys^{16}-N does not transfer to the oxygen of β-phosphate in structure *D* in Fig. 4. Therefore, we performed additional calculations, using the model taken from PDB data (1q21) of GDP-binding Ras p21 in Fig. 5 *A*. In this state, the γ-phosphate is already released from Ras p21. The geometry optimization was also executed using HF/6–31G**. From the results shown in Fig. 5 *B*, it was confirmed that the proton of Lys^{16}-N transferred to the oxygen of β-phosphate.

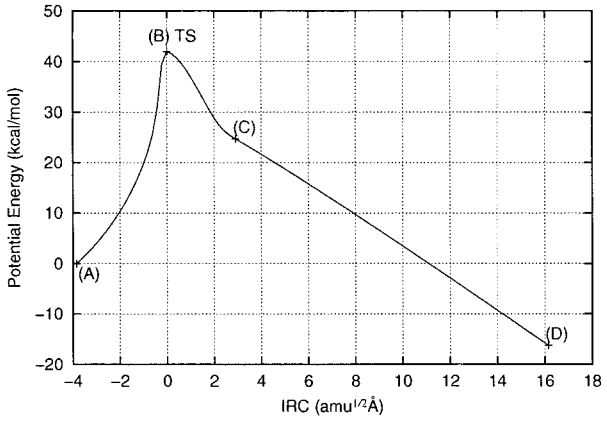

FIGURE 3 Potential energy changes following the structural changes in the active site of Ras p21. The abscissa indicates the intrinsic reaction coordinate (IRC) ($amu^{1/2}$ Å), and the ordinate indicates the potential energy (kcal/mol).

DISCUSSION

Active site structure in GTP-binding form

Before the hydrolysis reaction, the active site of the enzyme holds the structure in which the proton of Lys^{16}-N is attached to the oxygen of γ-phosphate, as shown in Fig. 2 *a*. It is generally accepted that the nitrogen of the side chain of the basic amino acid lysine is NH_3^+ in vital bodies (Stryer, 1975). Hence it would be reasonable to assume that the proton originally bound to Lys^{16}-N transfers to γ-phosphate at the same time that the Ras p21 binds to GTP.

Proton relay reaction via the nitrogen of the side chain of Lys^{16}

Because the active site structure in GTP-binding form (Fig. 2 *a*) was determined, the H_2O molecule was added near the γ-phosphate of this structure, and the preparatory structure (Fig. 2 *b*) was obtained by geometry optimization. We searched the TS structure by referring to the preparatory structure and eventually determined the reaction path of the GTP→GDP hydrolysis reaction by using IRC calculations.

Furthermore, we obtained the structure in which the γ-phosphate is completely dissociated from GTP (Fig. 4 *D*). This structure, *D*, was obtained by the geometry optimization for a stable state, starting from structure *C*, which is located at the last point of the IRC calculation in the forward direction of the hydrolysis reaction. On the other hand, structure *A* is located at the last point of the IRC calculation in the reverse direction. However, optimization of this structure was not able to provide an optimized structure before *A*. The reason for this is that the position of the H_2O molecule

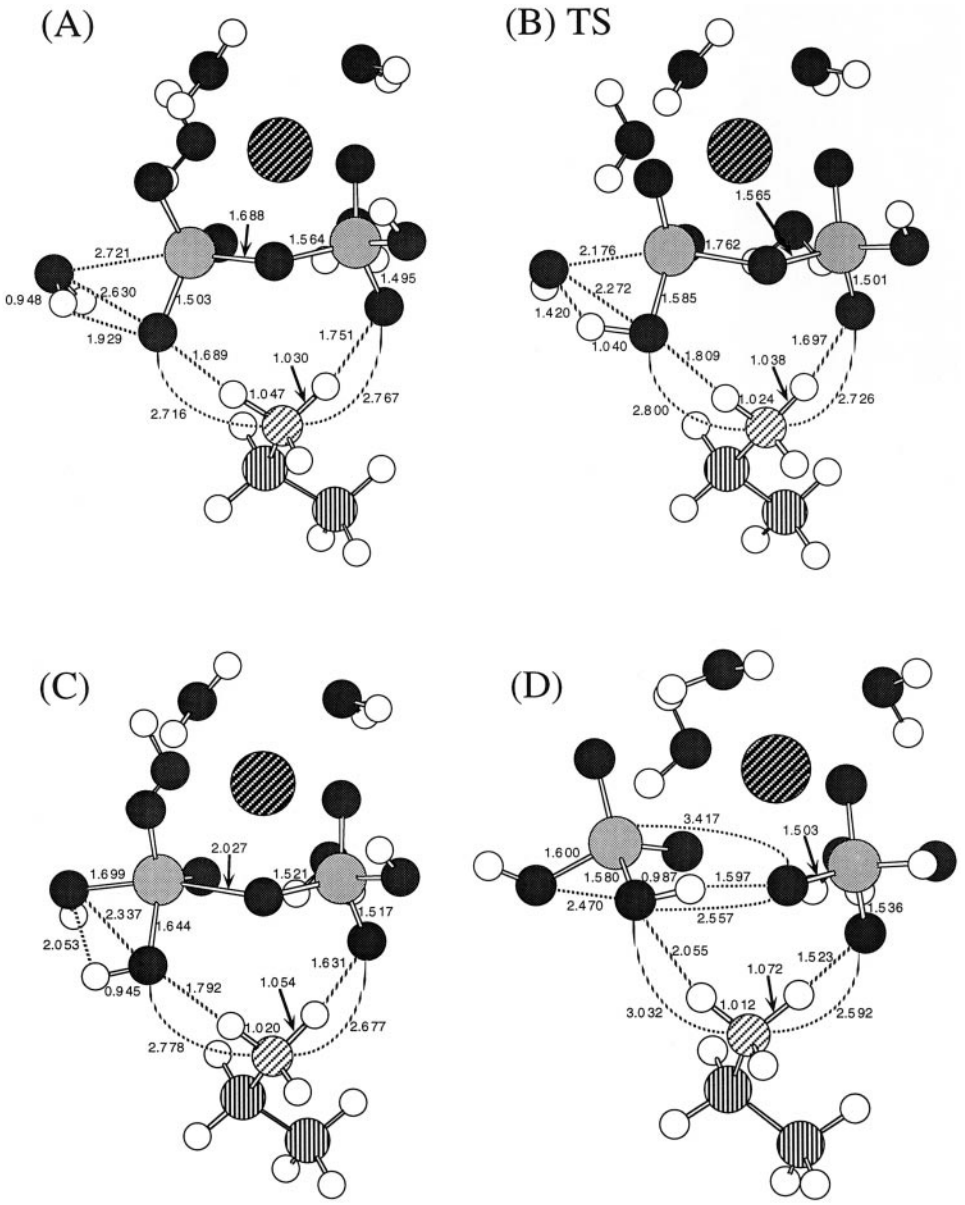

FIGURE 4 Structures in the hydrolysis reaction. (*A*) The initial structure. (*B*) The transition state. (*C*) The structure by IRC calculation for the direction of the dissociation of γ-phosphate. (*D*) The separation of γ-phosphate from GTP.

changes markedly because of the lack of surrounding residues in the model. It is expected that the H_2O molecule is usually held by Gln^{61} and GAP in the enzyme.

The potential energy change along this reaction path is presented in Fig. 3. First of all, structure *A* of Fig. 4 is an initial structure of the GTP→GDP hydrolysis reaction. The proton bound to the oxygen of γ-phosphate moved to Lys^{16}-N, and Lys^{16}-N became NH_3^+ as a consequence of the supply of an H_2O molecule to γ-phosphate. That is, it is understood that Lys^{16}-NH_3^+ is formed at the starting point of the hydrolysis reaction. It is expected that the transfer of the proton of the H_2O molecule to the oxygen of this γ-phosphate is enhanced by this proton relay from the oxygen of γ-phosphate to Lys^{16}-N.

Next the proton of the H_2O molecule is moving to the oxygen of γ-phosphate in structure *B*. This structure is the TS, and the energy difference from the initial structure was ~42 kcal/mol.

This value seems high for the activation energy of an enzyme reaction. However, the H_2O molecule would be held by the Gln^{61} and GAP before the hydrolysis reaction, which may lower the activation energy of the reaction if the potential energy of the holding state is high.

Structure *C* is a result of the IRC calculation in the forward direction of the hydrolysis reaction. The distance between γ-phosphate and β-phosphate expands considerably to 2.027 Å, which suggests the dissociation of γ- and β-phosphates.

In *D*, the distance between γ-phosphate and β-phosphate is 3.417 Å and γ-phosphate is completely dissociated from GTP. The distance between Lys^{16}-N and the proton on the side of β-phosphate is 1.072 Å, and the proton is slightly drawn to β-phosphate.

γ-Phosphate is dissociated finally from GTP. The structure of Fig. 5 *B* was obtained by geometry optimization of the active site of GDP-binding type Ras p21 (Fig. 5 *A*). The

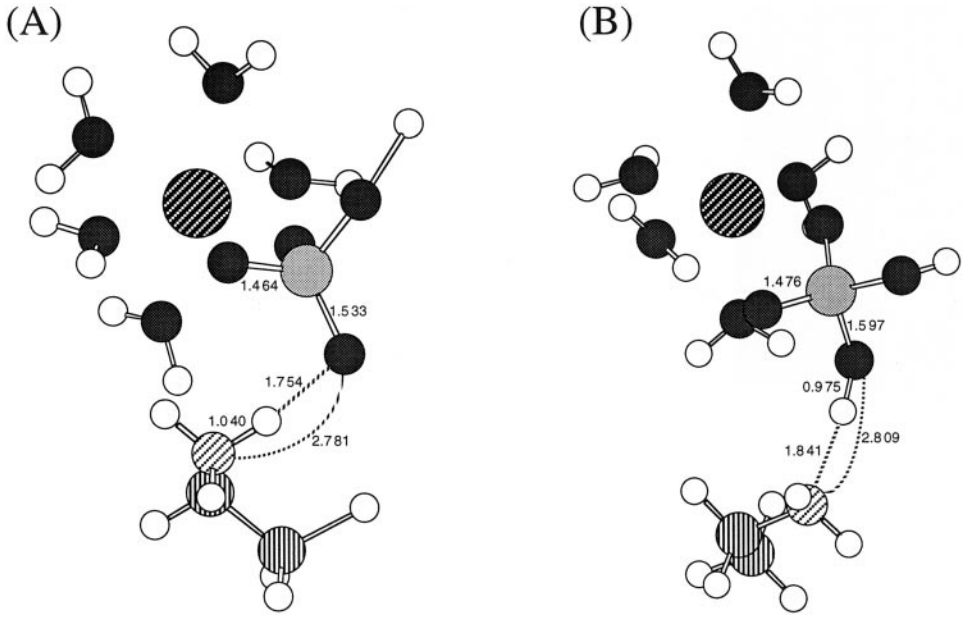

FIGURE 5 The active site of GDP binding Ras p21. (*A*) The structure in PDB data (1q21) of GDP binding form (inactivating form). (*B*) The optimized structure.

proton of Lys^{16}-N moves to the oxygen of β-phosphate. Hence the following behavior is recognized for the proton. A proton of Lys^{16}-N transfers to the oxygen of γ-phosphate when GTP is combined with Ras p21. When the GTP→GDP hydrolysis reaction starts, the proton returns to Lys^{16}-N, and the nitrogen becomes NH_3^+. The proton of Lys^{16}-N transfers to β-phosphate when γ-phosphate is dissociated from GTP. Therefore, it is concluded that a series of proton relay reactions plays an important role in the GTP→GDP hydrolysis reaction.

Role of Gln^{61} GAP in the delivery of water molecules

The reaction starts when an H_2O molecule approaches γ-phosphate in the initial structure of the hydrolysis reaction. It has been considered that the H_2O molecule is divided into H^+ and OH^- by Gln^{61}, and the phosphorus of γ-phosphate is attacked by OH^- (Langen et al., 1992; Goody et al., 1992; Maegley et al., 1996). However, it is not plausible to suggest that the decomposition of the H_2O molecule is caused only by a mere amino acid residue. Moreover, Gln^{61} was observed to be largely apart from GTP in the structural model of Ras p21 obtained from x-ray crystal analysis.

According to the present results, the hydrolysis reaction reasonably proceeds when the H_2O molecule is coordinated with the γ-phosphate of GTP combined into Ras p21. Hence it is thought that the supply of the water molecule to γ-phosphate is another important factor in determining the reaction rate. The GTP→GDP hydrolysis reaction was found by experiment to be inhibited in the absence of GAP (Bollag and McCormick, 1991). This strongly suggests that GAP has a significant role in the supply of H_2O molecules. Hence we speculate that GAP brings Gln^{61} to the vicinity of GTP and Gln^{61} helps a water molecule to be coordinated with the γ-phosphate.

The x-ray crystal analysis of Ras p21 combining with GAP was reported recently (Scheffzek et al., 1997). The report suggested that the structure of the active site was very similar to that of the G protein $G_{i\alpha1}$, which has an intrinsic GAP. When the active site structures of Ras p21 without GAP and that of $G_{i\alpha1}$ are compared, there is a clear difference in the distance between Gln^{61} and γ-phosphate.

Fig. 6 shows the comparison between Ras p21 and $G_{i\alpha1}$. The structure shown in white is Ras p21 and that in black is $G_{i\alpha1}$. Gln^{204} of $G_{i\alpha1}$ corresponds to Gln^{61} of Ras p21. In Ras p21, the distance between Gln^{61} and γ-phosphate is large,

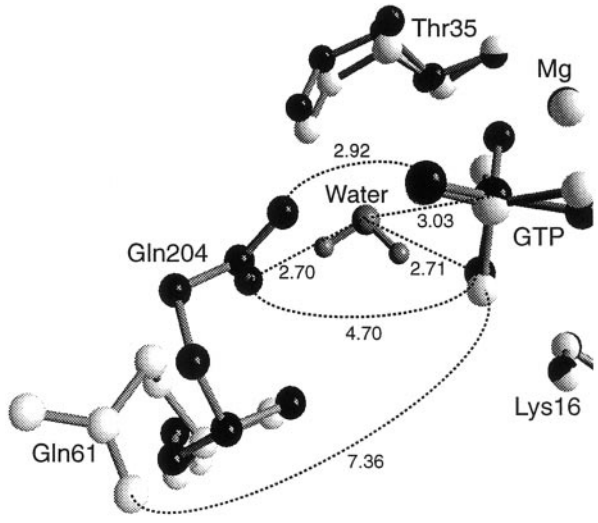

FIGURE 6 Comparison between Ras p21 and $G_{i\alpha1}$. The structure shown in white is Ras p21 and that in black is $G_{i\alpha1}$. Incidentally Gln^{204} of $G_{i\alpha1}$ corresponds to Gln^{61} of Ras p21.

and they cannot maintain an interaction. However, Gln^{204} and γ-phosphate can interact with each other when an H_2O molecule is present in $G_{i\alpha 1}$. This supports the notion that the role of GAP is to deliver an H_2O molecule by supporting the movement of the Gln residue.

CONCLUSIONS

We conclude the following from the present theoretical research:

1. In Ras p21, Lys^{16} initiates the GTP→GDP hydrolysis reaction through the proton relay mechanism. This reaction is a one-step reaction, and a GTP is dissociated into GDP and a phosphate.

2. In the GTP→GDP hydrolysis reaction, GAP has a role in supplying an H_2O molecule to γ-phosphate via Gln^{61}, and, consequently, its presence increases GTPase activity.

REFERENCES

Abola, E. E., F. C. Bernstein, S. H. Bryant, T. F. Koetzle, and J. Weng. 1987. Protein Data Bank. *In* Crystallographic Databases: Information Content, Software Systems, Scientific Applications. Data Commission of the International Union of Crystallography, Bonn, Cambridge, and Chester. 107–132.

Abola, E. E., N. O. Manning, J. Prilusky, D. R. Stampf, and J. L. Sussman. 1996. The Protein Data Bank: current status and future challenges. *J. Res. Natl. Inst. Stand. Technol.* 101:231–241.

Barbacid, M. 1987. *ras* genes. *Annu. Rev. Biochem.* 56:779–827.

Bernstein, F. C., T. F. Koetzle, G. J. B. Williams, E. F. Meyer, M. D. Brice, Jr., J. R. Rodgers, O. Kennard, T. Shimanouchi, and M. Tasumi. 1977. The Protein Data Bank: a computer-based archival file for macromolecular structures. *J. Mol. Biol.* 112:535–542.

Bollag, G., and F. McCormick. 1991. Differential regulation of rasGAP and neurofibromatosis gene product activities. *Nature.* 351:576–579.

Chung, H.-H., D. R. Benson, and P. G. Schults. 1993. Probing the role of lysine 16 in Ras p^{21} protein with unnatural amino acids. *J. Am. Chem. Soc.* 115:6414–6415.

Eccleston, J. F., K. J. M. Moore, L. Morgan, R. H. Skinner, and P. N. Lowe. 1993. Kinetics of interaction between normal and proline 12 Ras and the GTPase-activating proteins, p120-GAP and neurofibromin. *J. Biol. Chem.* 268:27012–27019.

Franken, S. M., A. J. Scheidig, U. Krengel, H. Rensland, A. Lautwein, M. Geyer, K. Scheffzek, R. S. Goody, H. R. Kablitzer, E. F. Pai, and A. Wittinghofer. 1993. Three-dimensional structures and properties of a transforming and a nontransforming glycine-12 mutant of $p21^{H\text{-}ras}$. *Biochemistry.* 32:8411–8420.

Frisch, M. J., Æ. Frisch, and J. B. Foresman. 1995a. Gaussian94 User's Reference. Gaussian, Inc., Carnegie, Pennsylvania.

Frisch, M. J., Æ. Frisch, and J. B. Foresman. 1995b. Gaussian94 Programmer's Reference. Gaussian, Inc., Carnegie, Pennsylvania.

Futatsugi, N., M. Hata, and M. Tsuda. 1997. The importance of the role of lysine 16 and GAP on the molecular switching mechanism of Ras protein p21. *J. Chem. Soc. Jpn.* 7:516–522.

Gibbs, J. B., M. D. Schaber, W. J. Allard, I. S. Sigal, and E. M. Scolnick. 1988. Purification of ras GTPase activating protein from bovine brain. *Proc. Natl. Acad. Sci. USA.* 85:5026–5030.

Goody, R. S., E. F. Pai, I. Schlichting, H. Rensland, A. Scheidig, S. Franken, and A. Wittinghofer. 1992. Studies on the structure and mechanism of H-ras p21. *Philos. Trans. R. Soc. Lond. B.* 336:3–11.

Krengel, U., I. Schlichting, A. Scherer, R. Schumann, M. Frech, J. John, W. Kabsch, E. F. Pai, and A. Wittinghofer. 1990. Three-dimensional structures of H-ras p21 mutants: molecular basis for their inability to function as signal switch molecules. *Cell.* 62:539–548.

Langen, R., T. Schweins, and A. Warshel. 1992. On the mechanism of guanosine triphosphate hydrolysis in *ras* p21 proteins. *Biochemistry.* 31:8691–8696.

Maegley, K. A., S. J. Admiraal, and D. Herschlag. 1996. Ras-catalyzed hydrolysis of GTP: a new perspective from model studies. *Proc. Natl. Acad. Sci. USA.* 93:8160–8166.

McCormick, F. 1992. Coupling of ras p21 signalling and GTP hydrolysis by GTPase activating proteins. *Philos. Trans. R. Soc. Lond. B.* 336: 43–48.

Milburn, M. V., L. Tong, A. M. de Vos, A. Brunger, Z. Yamaizumi, S. Nishimura, and S.-H. Kim. 1990. Molecular switching for signal transduction: structural differences between active and inactive forms of protooncogenic *ras* proteins. *Science.* 247:939–945.

Pai, E. F., U. Krengel, G. A. Petsko, R. S. Goody, W. Kabsch, and A. Wittinghofer. 1990. Refined crystal structure of the triphosphate conformation of H-*ras* p21 at 1.35 Å resolution: implications for the mechanism of GTP hydrolysis. *EMBO J.* 9:2351–2359.

Pearlman, D. A., D. A. Case, J. W. Caldwell, W. S. Ross, T. E. Cheatham, III, D. M. Ferguson, G. L. Seibel, U. C. Singh, and P. A. Kollman. 1995. AMBER Version 4.1. University of California, San Francisco.

Scheffzek, K., M. R. Ahmadian, W. Kabsch, L. Wiesmüller, A. Lautwein, F. Schmitz, and A. Wittinghofer. 1997. The Ras-RasGAP complex: structural basis for GTPase activation and its loss in oncogenic Ras mutants. *Science.* 277:333–338.

Shih, C., B. Shilo, M. P. Goldfarb, A. Dannenberg, and R. A. Weinberg. 1979. Passage of phenotype of chemically transformed cells via transfection of DNA and chromatin. *Proc. Natl. Acad. Sci. USA.* 76: 5714–5718.

Stryer, L. 1975. Biochemistry, 1st Ed. W. H. Freeman, New York.

Tong, L., M. V. Milburn, A. M. de Vos, and S.-H. Kim. 1989. Structure of *ras* protein. *Science.* 245:244.

Weiner, S. J., P. A. Kollman, D. A. Case, U. C. Singh, C. Ghio, G. Alagona, S. Profeta, and P. Weiner. 1984. A new force field for molecular mechanical simulation of nucleic acids and proteins. *J. Am. Chem. Soc.* 106:765–784.

Weiner, S. J., P. A. Kollman, D. T. Nguyen, and D. A. Case. 1986. An all atom force field for simulations of proteins and mucleic acids. *J. Comput. Chem.* 7:230–252.