Protein Engineering vol.10 no.4 pp.381–387, 1997

Comparison of ras-p21 bound to GDP and GTP: differences in protein and ligand dynamics

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This paper documents the first essential dynamics analysis of ras protein ligands and of the protein itself, showing important features of their dynamic properties. Essential dynamics analysis of 300 ps of full solvent molecular dynamics simulations revealed differences in structure and dynamics between GDP- and GTP-bound forms of H-ras-p21. Regions in the protein which exhibited a structural shift correspond to the switch regions described previously. Differences in dynamics between H-ras-p21 GDP and H-ras-p21 GTP may be related to interactions of ras with GAP and its receptor and effector. Molecular dynamics of free GDP (in the absence of protein) were performed in water for 2 ns and analysed using essential dynamics. The conformations of GDP and GTP when bound to the protein were compared with free GDP, revealing that the ligands bind to the protein in an energetically unfavourable conformation. GDP and GTP molecules from various other protein crystal structures were also analysed. These ligands adopt similar conformations to those seen in H-ras-p21.
Keywords: essential dynamics/GDP/GTP/molecular dynamics/ ras protein

Introduction

Mammalian *ras* genes encode proteins of 189 amino acids with an approximate molecular mass of 21 kDa, named p21 proteins. They play an important role in the regulation of cell growth and differentiation and have been implicated in the process of malignant cell transformation (Pincus *et al.*, 1987). Three human genes have been identified and are known as Ha(rvey)-ras, K(irsten)-ras and N(euroblastoma)-ras. The first 165 residues show a high degree of sequence identity, but the rest of their sequences diverge (Kim *et al.*, 1993). Their mechanism of action involves the stimulated exchange of GDP by GTP to generate the active state, which can bind to effectors. After the γ-phosphate of the nucleotide is hydrolysed, the protein returns to its inactive, GDP-bound state. Several proteins interact with ras-p21 proteins to modulate their activity. Some examples are the CDC25-related proteins SDC25C and SOS (which stimulate the guanine–nucleotide exchange reaction) (Zhong *et al.*, 1995), GAP (GTPase activating protein, which promotes GTP hydrolysis) and the putative effector protein Raf (Sprang, 1995).

Analysis of the crystal structures of the GDP- (Tong *et al.*, 1991) and GTP-bound (Pai *et al.*, 1990) forms of H-ras-p21 revealed conformational changes in two main segments of the structure called the switch I and switch II regions (Kim *et al.*, 1993). The switch I region spans residues 30–37, while switch II consists of residues 60–76.

Here, we analyse two different aspects of the GDP- and GTP-bound forms of H-ras-p21, related to the dynamics of the protein. First, the effect of the ligand on the protein was studied, analysing possible differences in dynamics between the two ras protein forms in order to understand its structural flexibility and essential motions. Second, we analysed the effect of the protein on the ligand. We have simulated the molecular dynamics (MD) of ras-p21 bound to GDP and to GTP and the free GDP molecule itself. The dynamic properties of these MD runs were analysed and compared by the essential dynamics approach. This method is able to extract large concerted atomic motions from MD trajectories (Amadei *et al.*, 1993), giving insight into the dynamics of the ras-p21 structure.

Material and methods

Protein analysis

The molecular dynamics (MD) calculations were carried out using the crystal structures of H-ras-p21 complexed with GDP (Tong *et al.*, 1991) and with GTP (Pai *et al.*, 1990) (ras-GDP and ras-GTP, respectively). The calculations were performed with GROMOS (Van Gunsteren and Berendsen, 1987) using the force field that is specific for proteins in a water environment. A truncated octahedral box of water with sides of 60.61 Å filled with 2762 water molecules was used for the GDP form and 74.60 Å wide with 5740 water molecules for the GTP form (the difference in box size and consequently the number of water molecules indicates that the GDP form is more compact than the GTP form). A short energy minimization (EM) using a steepest descent algorithm was used until no significant energy changes could be detected. The EM was followed by a 25 ps HEATUP period, in which the temperature was slowly increased from 0 to 300 K, the time step from 1.6 to 2.0 fs, non-bonded cut-off radius from 7 to 8 Å, long range Coulomb cut-off radius from 7 to 10 Å, the temperature coupling constant from 0.01 to 0.1 ps and the pressure coupling constant from 0.05 to 0.5 ps (Van Aalten *et al.*, 1996a). The SHAKE procedure was applied in the MD simulations (Ryckaert *et al.*, 1977). After the MD runs reached equilibrium, as assessed by evaluation of global geometrical properties (see below), they were continued for a further 300 ps using the final parameters of the HEATUP procedure. The resulting trajectories were used for the ED analyses.

The analyses of the secondary structure, hydrogen bonds and radius of gyration along the MD simulations were performed using DSSP (Kabsch and Sander, 1983) and WHAT IF (WHAT IF trajectory checking menu) (Vriend, 1990) in order to check the quality of the MD simulations.

The essential dynamics (ED) method has been described by Amadei *et al.* (1993). Using ED, it is possible to separate 'essential' from 'non-essential' motions in a molecular dynamics trajectory. The ED method is based on the diagonalization of the covariance matrix (Ichiye and Karplus, 1991)

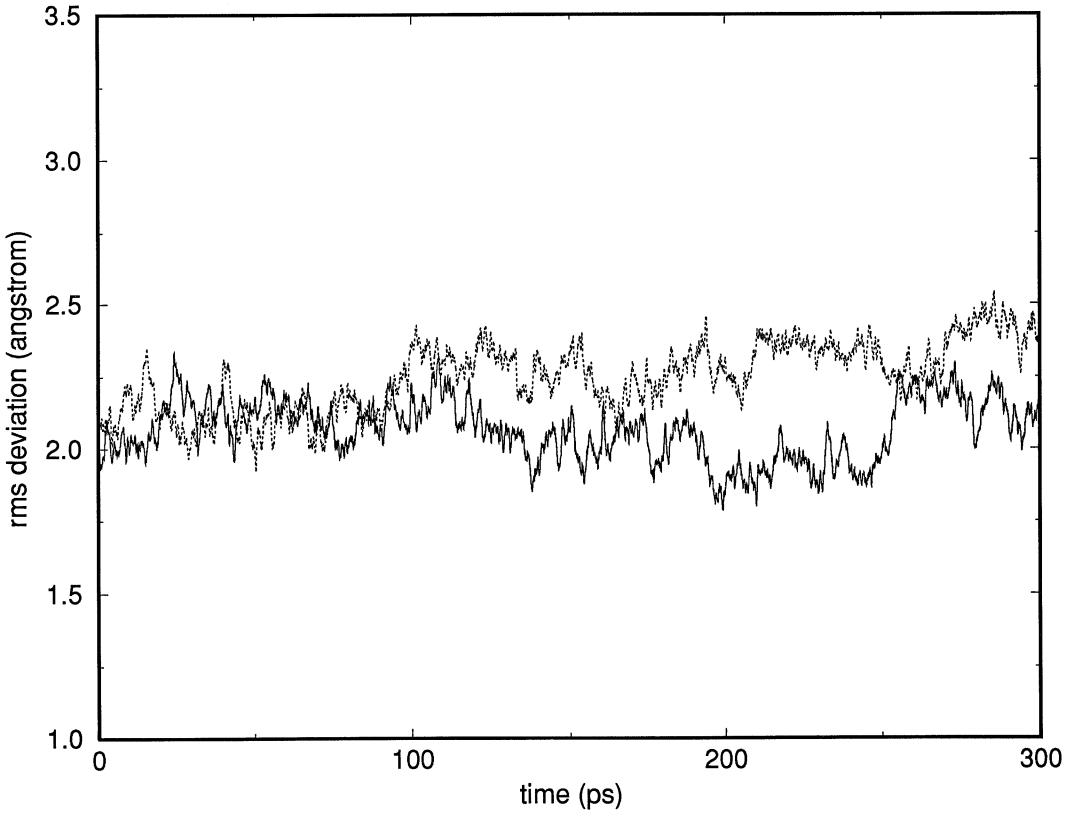

Fig. 1. R.m.s. deviation of structures during the MD runs with respect to their crystal structures (C_α atoms only). Solid line, ras-GDP; dotted line, ras-GTP.

constructed from the alpha-carbon (C_α) coordinates in an MD trajectory:

$$C_{ij} = <(x_i - x_{i,0})(x_j - x_{j,0})>$$

in which x_i and x_j are the separate x,y,z coordinates of the C_α atoms and x_0 are their average positions. A set of eigenvalues and eigenvectors is obtained by diagonalizing this matrix. The eigenvectors represent concerted motions of atoms in Cartesian space (dimension $3N$, where N is the number of C_α atoms) and the eigenvalues give the amplitude of the motion. The central hypothesis of the ED method is that only the motions along the eigenvectors with large eigenvalues are important for describing the functionally significant motions in the protein. The motion along any desired eigenvector can be inspected by projecting the frames from the MD trajectory onto that eigenvector. From this projection a new trajectory can be generated which, upon visual inspection, reveals large concerted motions of atoms. In contrast to normal mode analysis (Brooks and Karplus, 1983; Levit *et al.*, 1985), the ED method does not assume harmonicity of the motions and can be applied to any subset of coordinates (here only C_α atoms are used).

To compare ED properties of two simulations, an ED analysis can be performed on a combined trajectory constructed by concatenating individual trajectories of similar systems (Van Aalten *et al.*, 1995b). This method is a powerful tool for evaluating similarities and differences between the essential motions in different trajectories of the same protein. If the motions are similar, then the eigenvectors (and eigenvalues) coming from the separate trajectories and the combined trajectory should be similar. By projecting the single trajectories on to the eigenvector set calculated from the combined trajectory, information can be obtained about differences in fluctuations and equilibrium structures of the single trajectories along eigenvectors calculated from the combined trajectory. If the average projections of the two separate trajectories on a specific eigenvector from the eigenvector set calculated from the combined trajectory are different, then this indicates that the equilibrium structures along that direction are different. This is called 'static shift'. In addition, the trajectories can show different degrees of fluctuation in the direction of an eigenvector derived from the combined analysis. Hence, ED analysis yields information on differences in both structural and dynamic properties. A combined trajectory (COMB) was constructed by concatenating the two 300 ps trajectories of ras-GDP and ras-GTP. A C_α covariance matrix was built and diagonalized. The resulting eigenvectors were studied for possible differences in structure and dynamics between the two simulations.

Ligand analysis

The molecular dynamics of GDP in the absence of protein (free GDP) were performed using the same force field (GROMOS) as used in the protein simulations. A truncated octahedral box of water with sides of 2.77 Å filled with 324 water molecules was used. The MD simulation was preceded by a short EM procedure using a conjugate gradient algorithm with SHAKE. The MD simulation was then initiated by taking atomic velocities from a Maxwellian distribution at 300 K. The simulation was then continued for 2 ns. ED analyses of the resulting trajectory as well as the trajectories of GDP and GTP in the presence of the protein were carried out.

The first three eigenvectors of the ED of free GDP were used to analyse the position of the MD structures in the

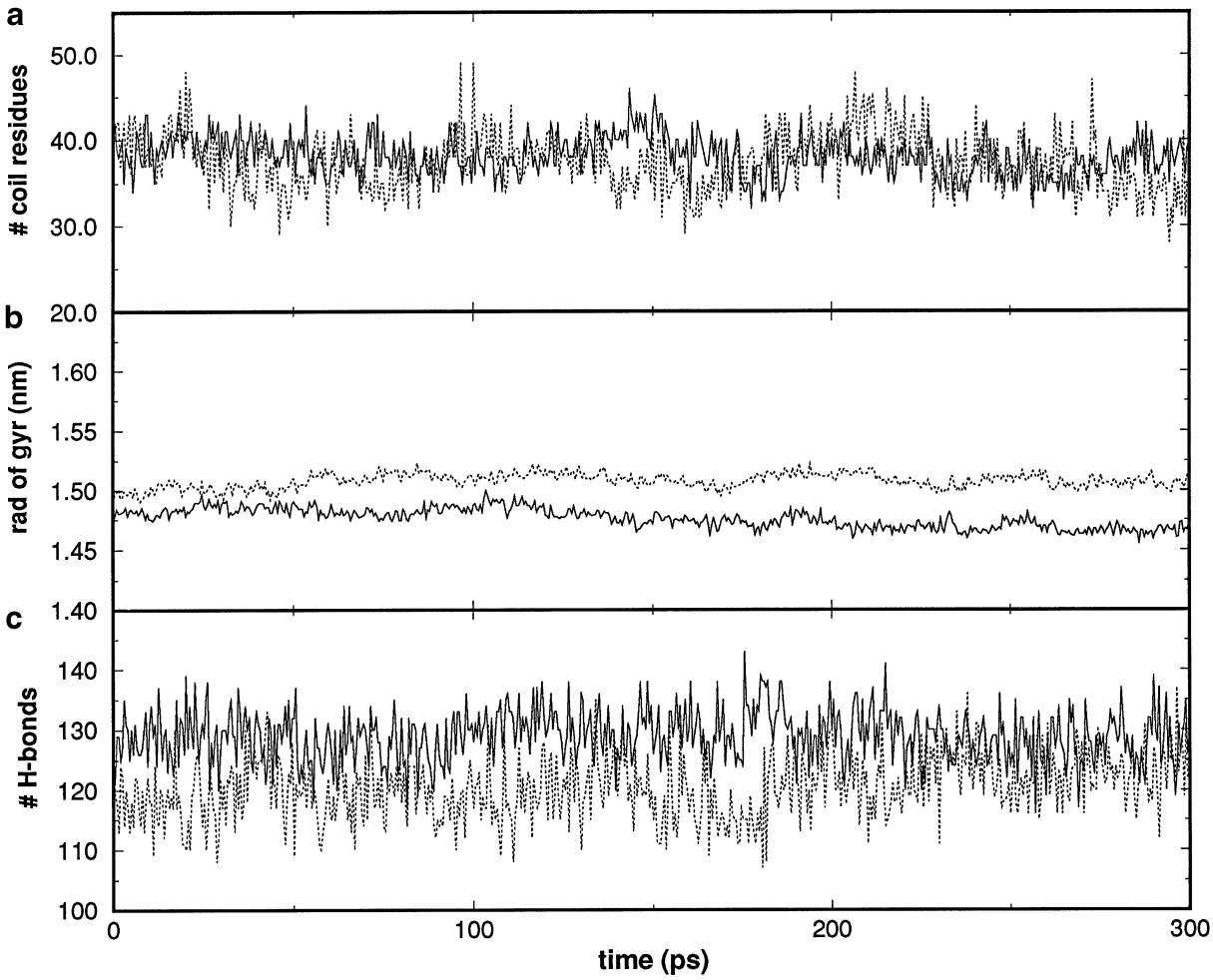

Fig. 2. (**a**) Number of residues in random coil conformation, (**b**) radius of gyration and (**c**) number of backbone–backbone hydrogen bonds in the MD simulation. Solid line, ras-GDP; dashed line, ras-GTP.

essential space spanned by these eigenvectors, as described by van Aalten *et al.* (1996b). In this method a density plot (2D grid) is generated showing the frequency with which certain areas of the essential space are visited. This is indicative of the most favourable regions during the simulation. Thus, the density distribution in the essential space was studied by calculating the projection of the free GDP trajectories on to a 2D grid formed by a combination of two of the first three eigenvectors. The bound GDP and GTP trajectories were fitted on the same reference structure as used for the free GDP simulations and projected on to the eigenvectors of the free GDP simulation. This was carried out to compare the conformational state of the ligand when bound to the protein with free GDP in solution. The positions of the GDP and GTP structures in the essential space of the three first eigenvectors for the simulations in the protein were compared with the density distribution of free GDP. The same kind of analysis was carried out comparing the free GDP molecule and 17 different GDP and GTP molecules found in crystal structures of ras-p21, Ef-Tu and G protein.

In order to analyse the differences in structure and dynamics between the GDP and the GTP molecules when bound to the protein, the trajectories from 300 ps simulations were concatenated and a combined essential (COMB) dynamics analysis was performed.

All calculations were performed on a Silicon Graphics Indy workstation with an R4000 processor and on a 16 processor R8000 Power Challenge. Visual inspections were carried out with WHAT IF (Vriend, 1990) and MolScript (Kraulis, 1991).

Results

Protein analysis

The root mean square (r.m.s.) deviations of the C_α atoms during the last 300 ps of the MD simulations of ras-GDP and ras-GTP compared with their respective crystal structures are shown in Figure 1. The plot shows that both simulations are stable. Figure 2a, b and c illustrate the number of residues in a random coil conformation, the radius of gyration and the number of backbone–backbone hydrogen bonds, respectively, for the structures in the MD simulations. There are no significant differences in the number of residues in a random coil conformation, indicating that the global secondary structure is preserved. Similarly, the radius of gyration and the number of backbone–backbone hydrogen-bonds indicate that the tertiary structures of ras-GDP and ras-GTP do not change significantly. In short, we have produced two stable trajectories, which may be used for further analyses.

The average projections and the mean square fluctuations in these projections of the separate ras-GDP and ras-GTP trajectories on to the eigenvectors calculated from the combined (COMB) trajectory are shown in Figure 3a and b, respectively.

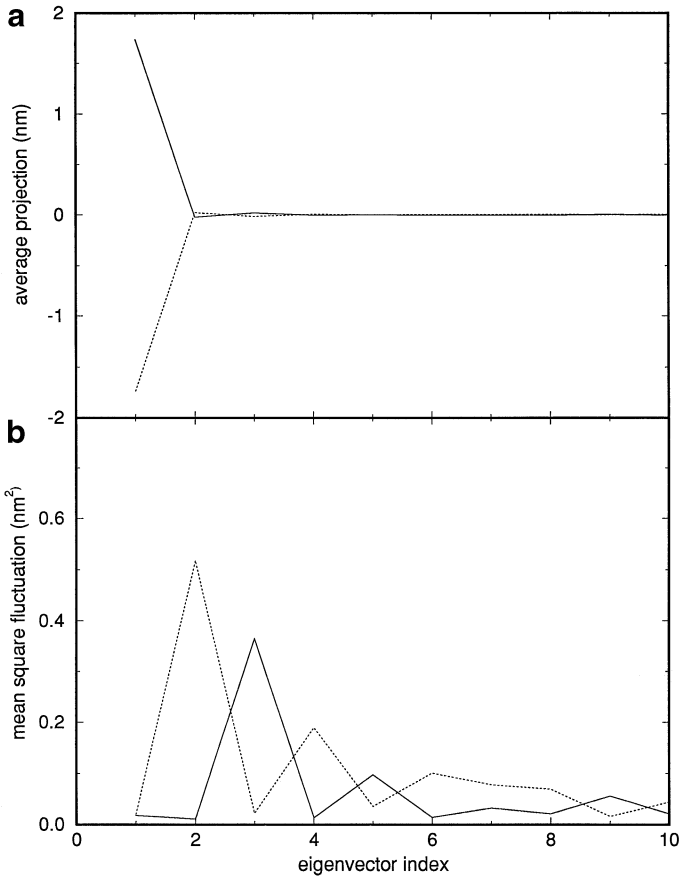

Fig. 3. (**a**) Average projection and (**b**) mean square fluctuation of ras-GDP (solid line) and ras-GTP (dotted line) trajectories projected on to the COMB eigenvector set.

Table I. (**a**) Switch regions of p21ras; (b) conserved regions among ras proteins (G region) and their binding function (conserved residues are in bold; X = any residue); (**c**) receptor binding in ras proteins; (**d**) effector binding in ras protein; (**e**) GAP and GTP hydrolysis residues interactions

(a) Switch regions		
Switch I	30 EYDP**T**IED 38	
Switch II	60 DVG**GQ**EEYSAMRDQYMRT**G**Q 76	
(b) G regions		
G1	10 GXXXXGKS(T) 18	β-Phosphate
G2	32 **R**XX**T**XGI 38	γ-Phosphate
G3	56 DXX**G** 60	γ-Phosphate
G4	116 NKXD 119	Guanine ring
G5	145 TSAKXT 147	Guanine ring
Mg^{2+}	17 S 35 T	Magnesium
(c) Receptor binding		
G4 region	Mutations of residues in this region alter receptor binding (Bourne *et al.*, 1991)	
(d) Effector binding		
G2 region (switch I)	Mutational experiments determining effector specificity (Marshall, 1993) Crystal structure: Rap–Raf complex (Nassar *et al.*, 1995)	
(e) GAP and GTP hydrolysis interactions		

Experiments and their conclusions:
Tyr 64 replacement with any non-hydrophobic amino acid or tryptophan completely blocked activation of ras mutant by GAP: Switch II region and loop 4 are involved in GAP activation (Nur-E-Kamal *et al.*, 1992).
Somatic mutations that replace Q61 reduce GTPase activity and contribute to generation of human tumours (Barbacid, 1987): Glu61 side chain is involved in GTP hydrolysis.
Crystal structures of H61 and L61 mutants show these residues could not activate Wat175 near the γ-phosphate of GTP. Similarly, crystals of the R12 and V12 mutants show how these mutations prevent access of Q61 to Wat175 (Krengel *et al.*, 1990): Glutamine is conserved at that position 61 because of its specific capacity to activate nucleophilic attack by a catalytic water molecule.
Obs. GTPase activity is increased by a factor of 10^5 in the presence of GAP (Goody *et al.*, 1992).

The main differences between the two ras-forms are described by eigenvectors 1, 2 and 3. The difference in average projection observed in eigenvector 1 (Figure 3a) is caused by differences in the crystal structures used for the simulation. The superposition of the minimum and the maximum projection structures of this eigenvector reveals regions with a conformational difference, which correspond to the switch regions I and II described by Kim *et al.* (1993).

Figure 3b indicates that in addition to the static shift there are differences in fluctuation between the two structures, described mainly by eigenvectors 2 and 3. The motions of these eigenvectors can be related to the interaction of ras with GAP and its receptor and effector, respectively, as shown below. There is a relationship between the mobility of particular protein segments and their ability to undergo large structural changes.

Table I documents (a) the switch regions of H-ras-p21, (b) the conserved sequence motifs (G regions) found in guanine nucleotide-binding proteins with their associated binding functions, (c) the receptor binding site, (d) the sites of interaction with effector proteins and (e) the residues involved in interaction with GAP and in GTPase activity.

Regions involved in the motion described by the second eigenvector are mainly residues 61–70, 86–108 and 129–138. This motion is observed in the GTP form, but constrained in the GDP form (Figure 3b). Figure 4 shows the kind of motion described by this eigenvector. The first set of residues (61–70) lies in the switch II region. Residues 61–65, specifically Glu61, are involved in the binding of GAP, which activates GTP hydrolysis (Marshall, 1993). More flexibility around this residue was observed in the GTP form than in the GDP form. Mutations that replace this residue reduce GTPase activity in ras and contribute to the generation of malignant human tumors (Barbacid, 1987).

Residues 30–33, 65–69 and 117–138 are involved in the motion described by eigenvector 3 (Figure 5). Residues 30–33 are part of the switch I region. The fact that this region shows more flexibility in the GDP-form than in the GTP-form is consistent with it being a site of interaction with the γ-phosphate in the GTP-form. This region is also an effector binding site. Marshall (1993) pointed to the importance of individual residues in effector recognition. Substitution of residues 31–42 of Rap1A (a member of the ras superfamily) by the sequence present in ras-p21 altered the specificity of interaction to ras-p21 effectors. It was also shown that residues outside this region are required for effector activation. McCormick (1996) suggested two different models for ras proteins and their effectors interaction and also demonstrated that residues additional to those in the switch I region are involved in the activation process.

Residues 65–69 are part of the switch II region and also appear to be more restrained in the GTP form. This may be

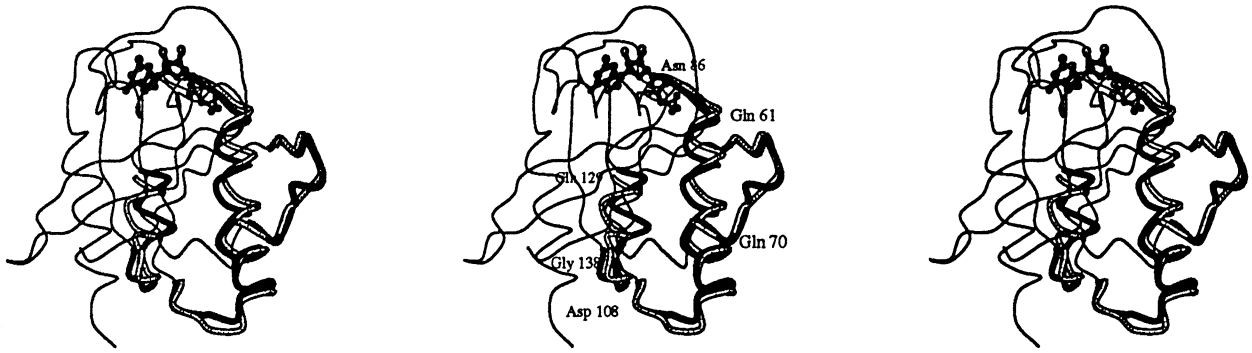

Fig. 4. Three-dimensional representation of the superposition of the minimum and maximum projection structures, showing the kind of motion described by eigenvector 2. The regions described by residues 6170 (switch I) and 86–108 move down relative to the GDP molecule, while the segment 129–138 residues move in the opposite direction. The average structure (thin black line) and the two extremes of the eigenvector motion are shown (thick black and white lines). GDP molecule in ball-and-stick form.

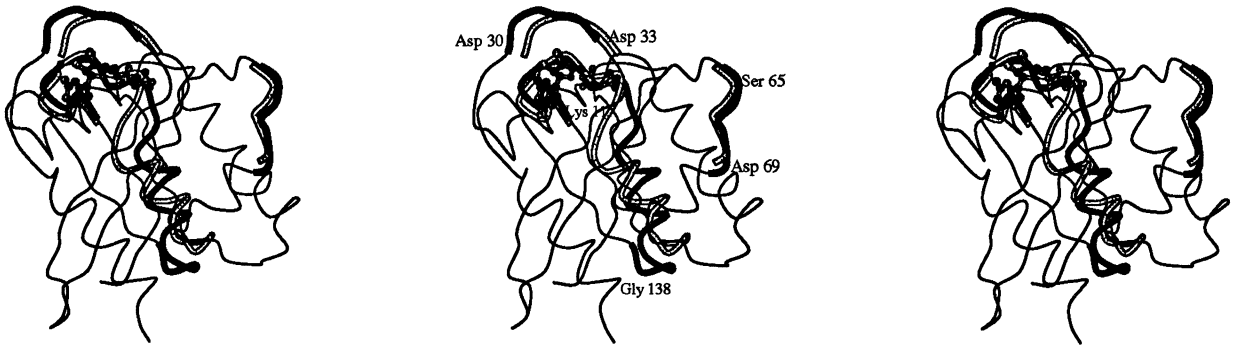

Fig. 5. Three-dimensional representation of the superposition of the minimum and maximum projection structures, showing the kind of motion described by eigenvector 3. Residues 30–33 (switch I), 65–69 (switch II) and 117–138 move relative to each other towards to the ligand. The average structure (thin black line) and the two extremes of the eigenvector motion are shown (thick black and white lines). GDP molecule in ball-and-stick form.

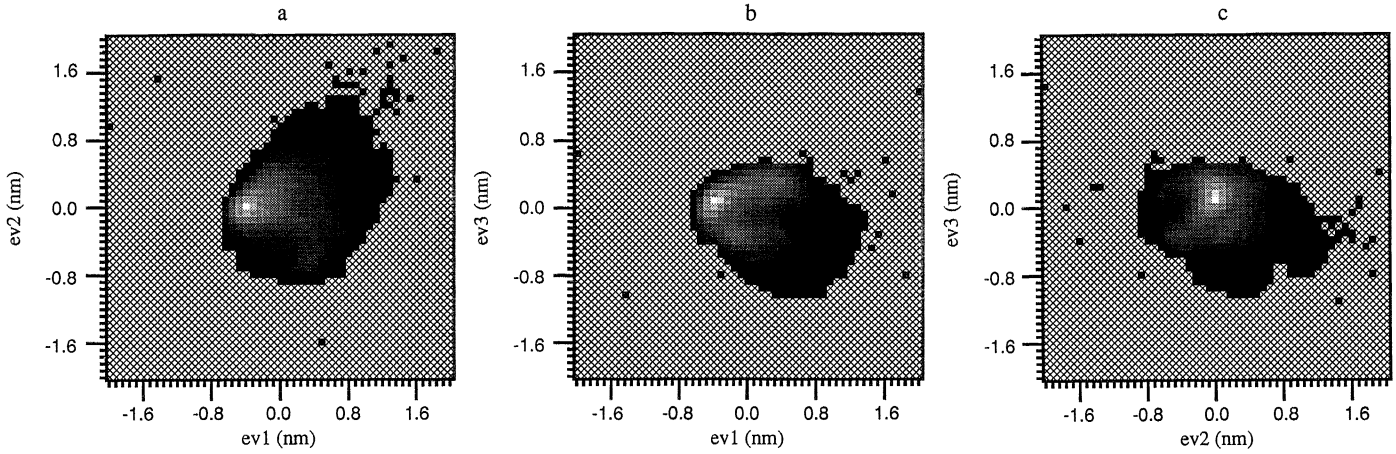

Fig. 6. Density plot of free GDP in two-dimensional planes spanned by the combination of eigenvectors 1, 2 and 3. Black region, low density (i.e. almost never visited), and white region, high density (often visited) of the ligand in the essential space. (**a**) Eigenvectors 1 and 2; (**b**) eigenvectors 1 and 3; (**c**) eigenvectors 2 and 3.

because this region is involved in binding to the γ-phosphate. These interactions restrain the motion described by this eigenvector, but not the motion present in eigenvector 2, thereby allowing interaction with GAP. The motion described by the third eigenvector involves switch regions I and II, which move with respect to one another and both in the direction of the γ-phosphate of the GTP molecule. In the motion described by the second eigenvector, the switch II motion is in a different direction, while switch I does not display any significant motion (Figures 4 and 5). Residues 117–138 include part of the binding site for the receptor (residues 116–119). This region is also more flexible in the GDP form (see Figure 3b), possibly because the protein only binds to the receptor when complexed with GDP. The region involved in this third eigenvector motion is essential for the function of the ras protein. The motion of regions with no correlation with

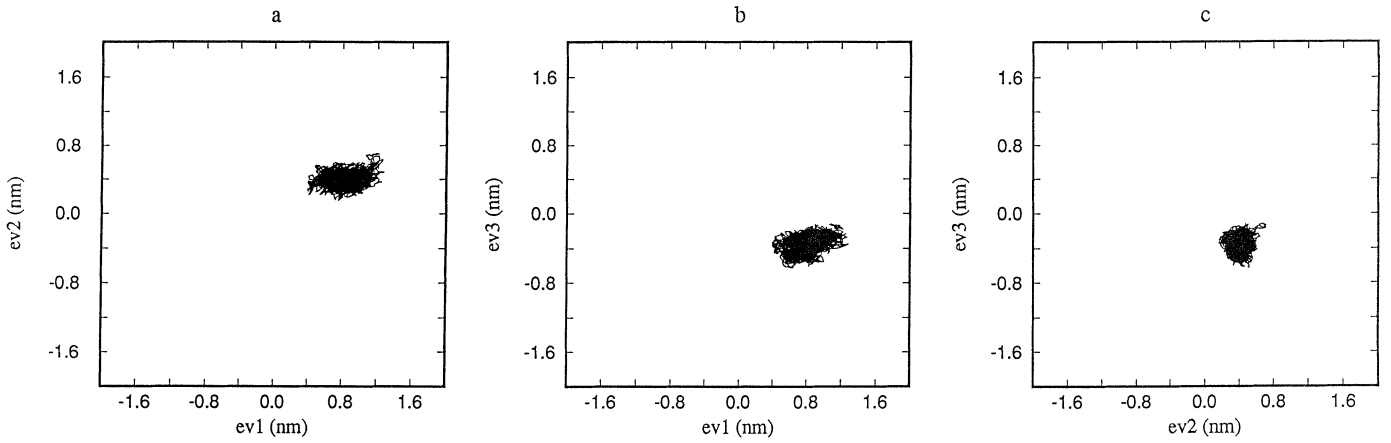

Fig. 7. Projection of the trajectory of GDP bound to ras protein on to the eigenvectors of the free GDP simulations. (**a**) Eigenvectors 1 and 2; (**b**) eigenvectors 1 and 3; (**c**) eigenvectors 2 and 3.

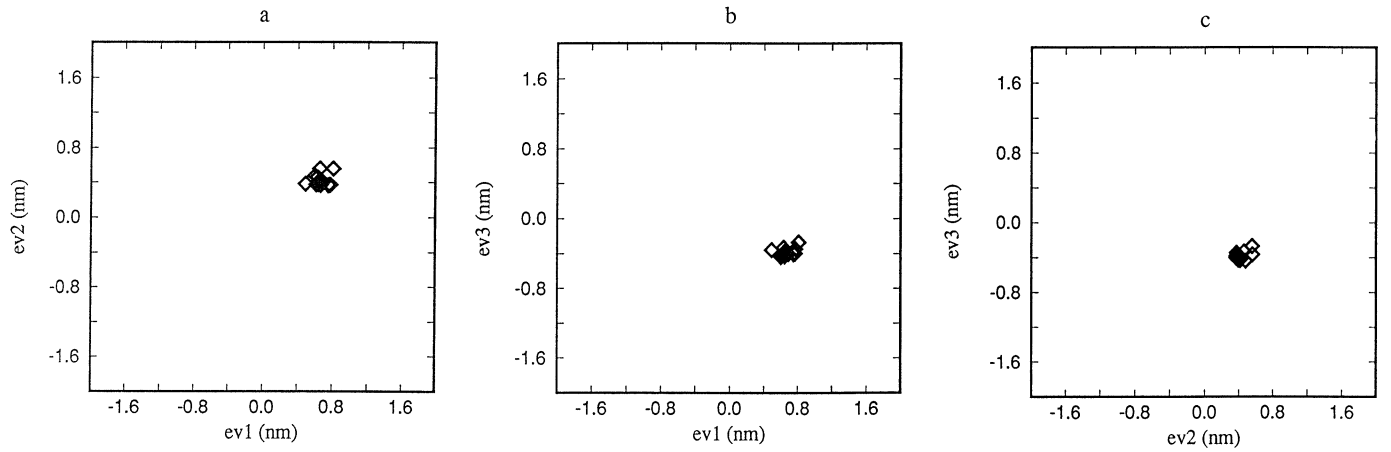

Fig. 8. Projection of the ras-p21, Ef-Tu and G protein crystal structures on to the eigenvectors of the free GDP simulation. (**a**) Eigenvectors 1 and 2; (**b**) eigenvectors 1 and 3; (**c**) eigenvectors 2 and 3.

biological function may be a consequence of the other motions, or could be indicative of functions still unknown.

Ligand analysis

The density plots constructed from the simulation of free GDP in 2-D planes spanned by the combination of eigenvectors 1, 2 and 3 are shown in Figure 6. Only the three first eigenvectors were used in this analysis since they represent 73% of the total motion (result not shown). The white spots represent the most frequently visited (low-energy) regions in the essential space. The projection of the trajectories extracted from the simulations of GDP and GTP complexed to the protein on to those from the free GDP simulation reveal that the molecules are in energetically unfavourable regions for eigenvectors 1, 2 and 3 (Figure 7). The results for GTP are not shown since they were indistinguishable from those obtained for GDP. The projection of GDP and GTP molecules in the Ef-tu, ras and G protein crystal structures on to the eigenvectors of the free GDP simulation show that the GDP and GTP structures are also in energetically unfavorable regions (Figure 8). The ligands in the MD simulation are found in the same conformational state as in the crystal structures, showing that the GDP and GTP structures are properly treated by the GROMOS force field. They also indicate that when the ligand is bound to the protein, it assumes a different conformation from that which exists in solution. The combined analyses of the bound forms of GDP and GTP show strong similarity in structure and dynamics, agreeing with the results of the direct eigenvector projection analysis, which revealed that both ligands occupy the same essential sub-space defined by the eigenvectors.

Discussion

The essential dynamics analyses of the simulations of ras-GDP and ras-GTP in water show that most of the differences between the two structures are described by three eigenvectors. Eigenvector 1 describes a structural shift which corresponds mainly to structural differences in the switch regions, as seen in the X-ray structures. Eigenvectors 2 and 3 reveal differences in dynamics, which could be related to the biological function of the protein. Parts of these flexible regions show some overlap. However, as seen in Figure 4a and b, the dynamics described by these eigenvectors are different. The motion described by eigenvector 2 is only observed in the GTP form but restrained in the GDP form. The opposite was found for eigenvector 3, where motion is only observed in the GDP form. Although almost the same residues are involved in these motions, ligand exchange appears to induce a different kind of motion in these regions, presumably thereby modulating the function of the protein. The observations described in this work are consistent with the molecular dynamics studies of ras bound with GDP or with GTP described by Dykes *et al.*

(1993), Foley *et al.* (1993) and Diaz *et al.* (1995). In this paper, we have extended those first analyses by showing changes in the dynamic behaviour of the flexible regions upon ligand exchange, correlating it to the biological function. To investigate these effects, residues involved in these motions will be mutated and the mutant proteins will be assayed. The hypothesis that binding sites are generated from particularly flexible regions of proteins can be supported by the work of van Aalten *et al.* (1995a). Their results demonstrated that apo-CRBP has larger fluctuations in the retinol binding site than holo-CRBP and that this binding site region is more flexible than the rest of the protein, when the retinol is absent.

It is not uncommon for ligands to bind to proteins in conformations other than that which they adopt in solution. For example, the immunosuppressant cyclosporin has different structures in solution and bound to cyclophilins (Fesik *et al.*, 1991). Neri *et al.* (1991) have also shown, by NMR studies, that cyclosporin binds two differents cyclophilins (64% sequence identity), in the same conformation. Van Aalten *et al.* (1996b) have also shown, using calculations similar to those performed here, that retinoid dynamics can be described by just three eigenvectors. By projecting crystal structures on to these eigenvectors, they demonstrated that retinoids are bound to their binding proteins in energetically unfavourable conformations. These general observations are similar to the results described here using crystal structures analysis, where the GDP and GTP molecules have been shown to adopt conformational states similar to those bound to ras protein during the simulation. These states are, however, energetically different from those prefered in free solution.

Acknowledgements

We thank Gert Vriend for helpful discussions during this work and critically reading the manuscript. We thank the EMBL for making available the 16 processor R8000 Power Challenge on which the simulations were performed. This work was supported by grants from the National Centre of Genetic Resources and Biotechnology – Brazilian Company for Agricultural Research (CENARGEN/EMBRAPA), Brazil.

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Received April 10, 1996; revised November 18, 1996; accepted December 30, 1996