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Three-Dimensional Structure of p21 in the Active Conformation and Analysis of an Oncogenic Mutant

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The three-dimensional structure of the active guanosine triphosphate (GTP)-analogue-containing complex of the H-*ras*-encoded p21 has been determined. It was necessary to correct the topology of p21 as published earlier. The structure analysis shows all of the interactions between protein and GTP and how the important cofactor Mg^{2+} is bound. From the oncogenic mutants of p21 crystallized, a Gly12 to Arg mutation has been analyzed in detail. It shows that the overall structure of the mutant is not perturbed and that the side chain of Arg12 is coming close to the γ-phosphate for an interaction.

Introduction

The prevalence of mutated forms of the *ras* oncogene in human tumors makes it an attractive target to study the function of the *ras* gene product p21 (*1,2*). These mutations are usually point mutations in two amino acids of the p21 sequence. It may turn out that the malfunctioning of mutated p21 is involved in, if not responsible for, uncontrolled growth of certain human tumors. To understand the role of p21 in these processes and to design therapeutical agents directed toward mutated p21, it is important to understand the structure of this protein and its transforming mutants and the biochemical processes it is involved in. A major focus of our present research is thus to investigate the structure of p21, the structure of oncogenic mutants, and the guanosine triphosphate (GTP)-guanosine diphosphate (GDP) conformational transition, which is the key reaction of all guanine nucleotide binding proteins.

The biochemical properties of p21 are summarized in Table 1. Table 1 shows that p21 binds GDP/GTP with extremely high affinity which is dependent on the presence of Mg^{2+}. It also shows that the tight binding is specific for the guanine base and is very dependent on the presence of the β-phosphate group. The affinity between guanosine monophosphate (GMP) and p21 is six orders of magnitude lower than for GDP/GTP. The GTPase activity of p21 is absolutely dependent on the presence of Mg^{2+}, underscoring the importance of this cofactor.

Table 1. Biochemical properties of Ha-*ras* p21.

Binds GDP / GTP with high affinity
K_a (GDP) = 6.1×10^{11} M^{-1} at 5°C
K_a (GTP) = 1.8×10^{12} M^{-1} at 5°C
Binds GDP / GTP with high specificity
K_a (GTP) / K_a (ITP) ~ 100
K_a (GDP)/K_a h^6GDP ~ 30
GMP affinity is approximately 10^6 times lower
Slow off-rates in the presence of Mg^{2+}
Fast off-rates in the presence of EDTA
Has low intrinsic GTPase, dependent on Mg^{2+}
GTPase is stimulated by GAP

Overall Structure

We have recently crystallized the complex of p21 with GDP and slowly hydrolyzing analogues of GTP: GppNHp, $GppCH_2p$ and GTPγS (*3*). While the crystals of the GppNHp and the $GppCH_2p$ complex are isomorphous, the GTPγS crystals show a different morphology. The $P3_221$ crystals of the GppNHp complex are easy to grow, very stable mechanically, and in the X-ray beam diffract to very high resolution (at least 1.4 Å). The three-dimensional structure has been revealed using heavy-atom isomorphous replacement techniques. The electron density has been calculated at 2.5 Å resolution, and the resulting model of the p21-GppNp complex has recently been published (*4*).

The secondary structure elements are shown in Figure 1, together with the position of some amino acids that are important for GTP binding, GTPase activating

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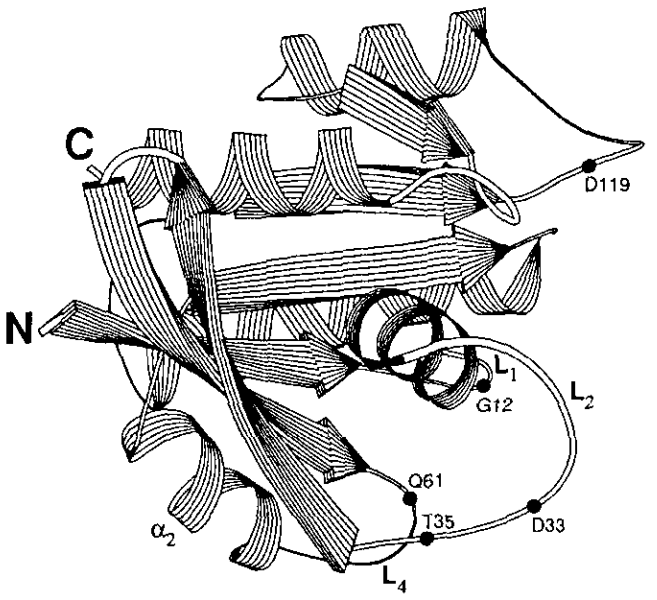

FIGURE 1. Schematic view of the topology of the three-dimensional structure of p21 GppNHp. Some amino acids important for the binding of nucleotide and the oncogenic activation are labeled. Loops L1, L2, and L4 are important for GAP binding and the GTPase activity.

protein (GAP) interaction and oncogenic activation. It has been shown by mutational analysis that residues 32 to 40 are involved in the interaction with GAP. They are located in loop L2 and the second β-sheet. These residues, like Asp-33 and Asp-38, are highly exposed to the solvent and are well defined in the structure. Since loop L4, residues 59 to 64, is also located on the outside of the molecule and very near to L2, we postulate that this loop is also involved in the interaction with GAP. In confirmation of this we have shown that the product of the K-*rev* gene, which has the same sequence in L2 and a different one in L4, is not activated by GAP but binds very tightly to it (*5*).

As a result of our structure analysis, we had to change the topology from the one described by DeVos et al. (*6*) in the report of the structure of the GDP complex of p21 (1–177) to the one shown in Figure 1. This topological arrangement of secondary structure elements is the same as that described for the G-binding domain of EF-Tu by LaCour et al. (*7*) and Jurnak (*8*). The difference between our structure and that of DeVos and coworkers (*6*) is the arrangement of the second and third β-strand and the corresponding loops. The second difference is that we find an additional helix following loop L4. On the basis of our structural model and of sequence alignments, it becomes more and more likely that all guanine nucleotide binding proteins involved in signal communication (not the enzymes like GMP-ATP phosphotransferases) have the same three-dimensional structure in their guanine nucleotide binding domain. We are just completing the high resolution structure at 1.4 Å, which seems to provide us with more fascinating details of the structure of the p21 triphosphate conformation.

Nucleotide Binding Site

The details of the interaction of the guanine nucleotide with the protein are described in Figure 2. It shows all atoms of either protein or water residues within 3.4 Å of any atom of GppNHp along with the corresponding distances. The high affinity for GDP and GTP is reflected in the great number of polar interactions between the protein and the nucleotide GppNHp.

The guanine base of the nucleotide is bound by interaction with the conserved elements NKXD (residues 116–119) and SAK (residues 145–147), which are conserved in the great majority of guanine nucleotide binding proteins (*9–11*). The carboxylate group of Asp-119 makes four hydrogen bonds. One oxygen interacts with the exocyclic amino group and Wat-292; the other one binds to the endocyclic nitrogen N1 and to the hydroxyl of Ser-145. The keto group at position 6 of the guanine base makes a hydrogen bond to the main chain -NH of Ala-146. Asn-116, which has been proposed to be involved in the binding of the O6 based on the three-dimensional structure of EF-Tu-GDP (*12*), is seen here to make strong hydrogen bonds to the side chain of Thr-144 and to the main chain oxygen atom of Val-14. The main function of Asn-116 is thus to tie together the three elements that are involved in nucleotide binding: the phosphate binding loop ^{10}GXXXXGKS, the ^{116}NKXD, and the ^{146}SAK motifs. It is supported by Lys-117, which links the phosphate binding loop and the NKXD motif by binding the main chain carbonyl of Gly-13. It makes only weak contacts to O1 of the ribose and N7 of the base moiety. Another element responsible for the tight binding of the guanine base is the hydrophobic interaction with the aromatic side chain of Phe-28 and the aliphatic side chain of Lys-117 on either side of the base. Phe-28 itself is also held in place by another hydrophobic interaction between its aromatic ring and the aliphatic side chain of Lys-147, lying on top of the plane of the ring in a stretched-out conformation.

The ribose ring is in the 2′-endo conformation. The angle X of the N-glycosidic bond is −112°, which is just at the border of an anti-conformation for nucleotides having the 2′-endo puckering. As reported (*4*), the 2′- and 3′-hydroxyl groups of the ribose are more or less exposed to the solvent with only weak hydrogen bonds to the side chain of Asp-30. The 3′-hydroxyl is involved in an additional hydrogen bond to the main chain carbonyl of Val-29. O1′ is weakly bound by the ε-amino group of Lys-117.

The phosphate binding site is characterized by a magnitude of interactions. Each of the eight phosphate oxygens of GppNHp has at least two hydrogen bond donors or the Mg^{2+} ion close enough for an interaction. The hydrogen bond donors include the main chain-NH groups of residues 13 to 18, 35, and 60, the hydroxyl groups of Ser-17 and Thr-35, and the phenolic hydroxyl of Tyr-32 from a neighboring p21, which contacts the γ-phosphate. The ε-amino group of Lys-16 binds to the β- and γ-phosphate oxygens, but it is closer to the latter. It should be mentioned that the main chain nitrogens

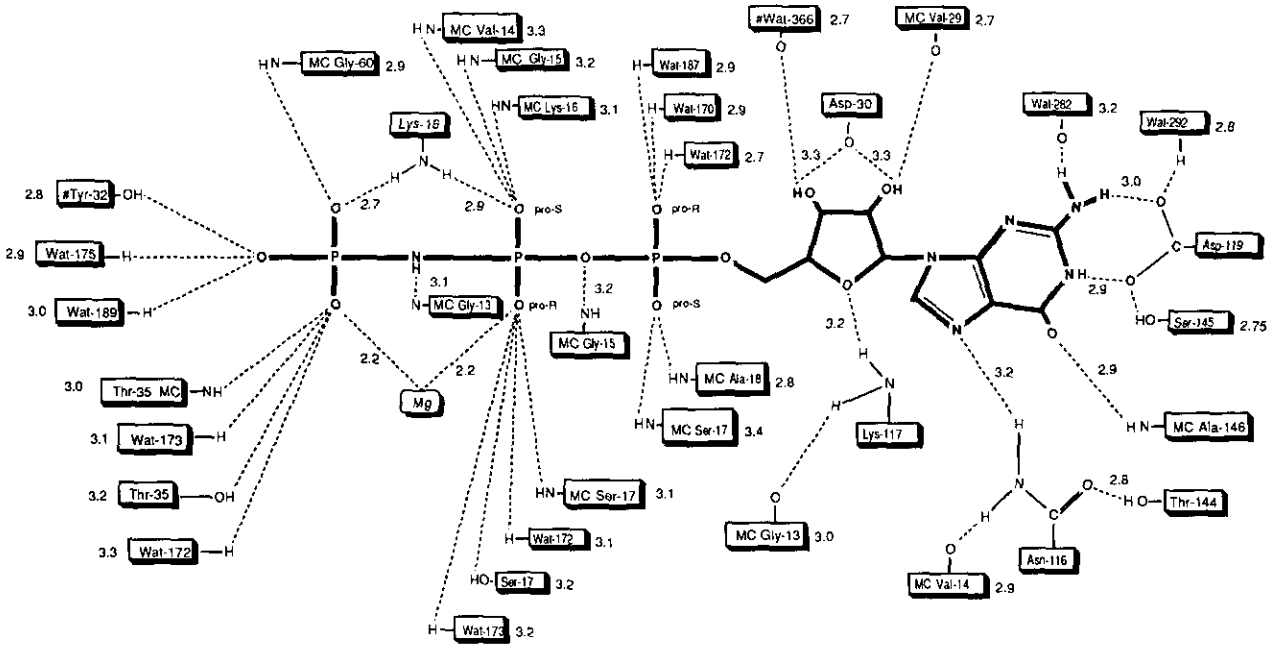

FIGURE 2. Scheme for the interactions between GppNHp and p21 or water molecules. All dashed lines correspond to hydrogen bonding interactions (below 3.4 Å) between appropriate partners. The corresponding distances of the hydrogen bonds are given behind the residue, except where one residue makes several bonds, in which case the distances are given on the dashed line. The distances are between the corresponding heteroatoms.

of residues 13 to 18 point toward the phosphate groups, thereby creating a strong electrostatic field. There is a hydrogen bond between the -NH of Gly-13 and the atom briding the β- and γ-phosphate, in this case GppNHp. Since this hydrogen bond should be stronger with a bridging oxygen atom, it is likely that this interaction is responsible for the lower affinity of GppNHp as compared to GTP (Schlichting et al., unpublished data).

Mg^{2+} Binding Site

It is generally believed that phosphoryl transferases require at least one divalent cation for catalytic activity, complexed directly to phosphoryl group oxygens. One can distinguish various possible catalytic functions of the Mg^{2+} ion such as shielding the negative charge on the attacked γ-phosphate, increasing the acid strength of the leaving group (β-phosphate), or activation of the nucleophile (*13*). Mg^{2+} could also be involved in the stabilization of the transition state of the reaction. The precise role of the metal ion for the quanine nucleotide binding proteins has not been proven and may indeed be different for each enzyme, as can be seen from the fact that there is no preferred ligation pattern of the metal ion to the enzyme or the phosphate atoms for related enzymes (*14*). As reported before (*4*), Mg^{2+} in the three-dimensional structure of the p21-GppNHp complex is coordinated to one oxygen of both the β- and γ-phosphate and to the side chain hydroxyl groups of Ser-17 and Thr-35, both of which are highly conserved

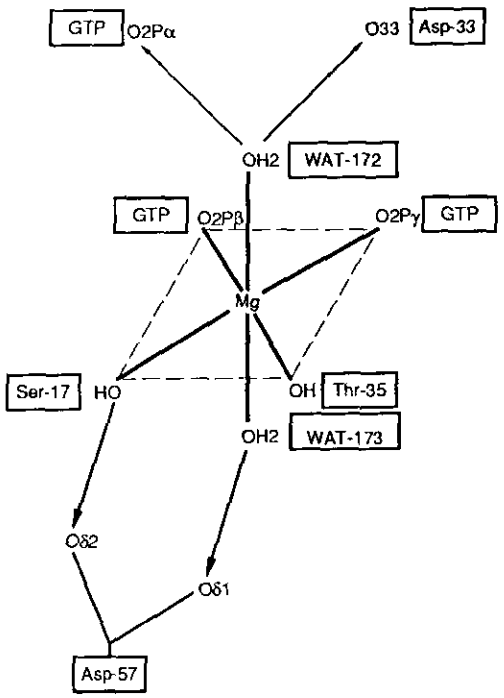

FIGURE 3. Schematic drawing of the Mg binding site showing the ligands of the first coordination sphere of the metal ion and some of the interactions of these ligands.

in all nucleotide binding proteins. In addition, the high-resolution structure proves that Asp-57, which is totally conserved as part of the DXXG motif in guanine nucleotide binding proteins, is not in the first coordination sphere of Mg^{2+} in the triphosphate structure. Instead,

it is hydrogen bonded to a water molecule, Wat-173, which is directly liganded, as shown schematically in Figure 3. Asp-57 further binds to the side chain of Ser-17. The sixth ligand of the metal ion is Wat-172, which is held in place by the interaction with the main chain oxygen of Asp-33 and the pro-R oxygen of the α-phosphate.

Mutated p21

Table 2 lists the nucleotide complexes of various mutant p21 proteins that we have crystallized and for which complete data sets have been collected. Thus we have data for four transforming mutants and mutants that do not interact with GAP, such as p21 (D38E) (*15–17*). By using molecular replacement methods to solve these structures we hope to be able to give structural explanations for the different biological behavior of mutant proteins. This should enable us and other people to possibly start with the rational design of drugs that could interfere with the effect of transforming p21 mutants without harmful effects on the normal protein.

The most frequent *ras* mutations found in human tumors have a mutation of Gly-12, which is part of the phosphate binding loop that wraps around the β- and γ-phosphates. It has also been shown by *in vitro* mutagenesis studies that the mutation of Gly-12 to any amino acid except proline renders the protein oncogenic (*18*). It has been postulated that Gly-12 has such unusual phi and psi angles that it cannot tolerate any side chain

Table 2. Crystals and data sets of ras-proteins.

Protein	Crystals	Data sets collected
Ha-*ras*		
c′	GDP	(+)
	GMPPNP	+
	GMPPCP	+
	GTPγS	−
	Caged GTP	+
G12V′	GDP	−
	GMPPNP	+
	GMPPCP	−
	GTPγS	−
	Caged GTP	+
D38E′	GDP	+
	GMPPNP	+
	GMPPCP	+
	GTPγS	−
	Caged GTP	−
D38A′	GDP	−
	GMPPNP	−
Q61H′	GDP	+
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	GMPPCP	+
Q61L	GMPPNP	+
	GDP	+
Ki-ras		
c′	GDP	+
G12V	GDP	−

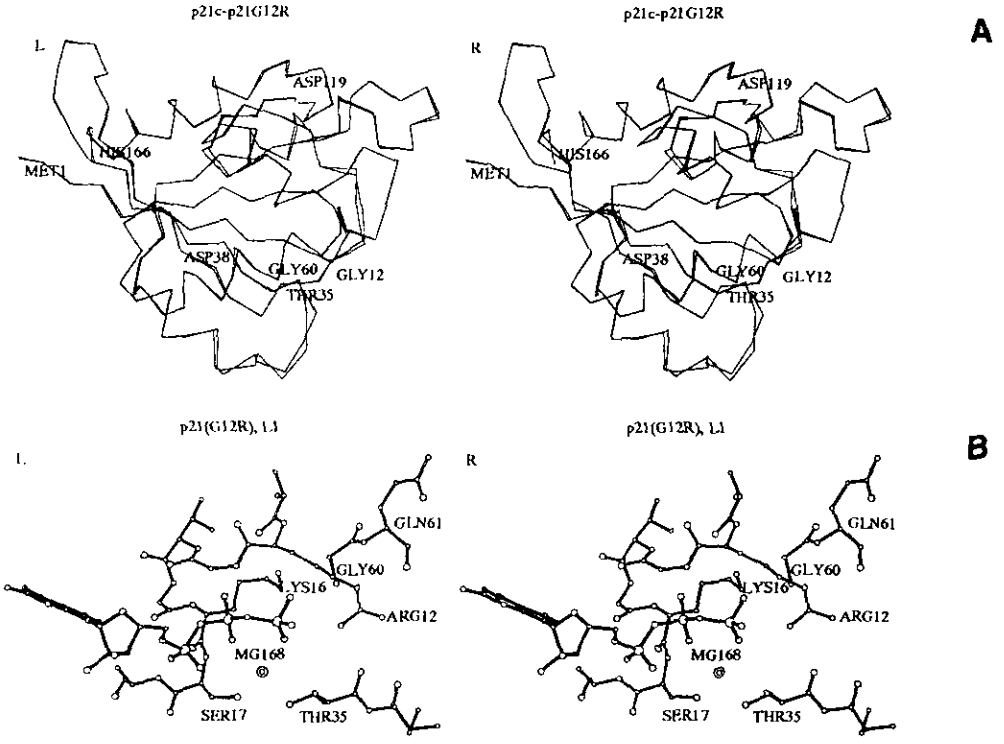

FIGURE 4. Stereodrawings of the structure of the oncogenic mutant p21 (G12R) complexed to GppNHp. (*A*) C_α plot of the mutant (thick line) in comparison to the cellular protein (thin line). (*B*) The phosphate binding region of the mutant protein, showing the Arg-12 side chain interacting with γ-phosphate.

without destroying the geometry of the phosphate binding loop. We have revealed the structure of the oncogenic mutant p21 (G12R) and find that the three-dimensional structure of the protein is very similar to the wild-type structure, as shown in the plot of C_α coordinates in Figure 4A. The only major difference between wild-type and the G12R mutant is the large side chain of Arg-12, which is coming close to the γ-phosphate and makes a hydrogen bond to one phosphoryl oxygen. Thus it is reasonable to assume that this interaction is responsible for the slow dissociation rate of GTP and for its slow GTP hydrolysis.

The interaction of p21 with GAP is governed by the conformational transition between the GDP and GTP bound states and this, obviously, is regulated by the nucleotide binding site. It is clear from our nuclear magnetic resonance and X-ray structural studies that there is no major conformational change involving large parts of the p21 molecule when the GDP and GTP (or GTP analogue) structures are compared. By comparing the structure of the nucleotide binding site in the triphosphate complex with that of the diphosphate complex, which we are currently analyzing, we hope to be able to give a detailed structural interpretation of the conformational transition at this site that is transmitted to the site of the effector interaction. This conformational change is the critical event for the functioning of all biological processes involving G-binding proteins. Preliminary experiments show that the difference between the GDP-bound and the GTP-bound form is confined to two subregions, loop L2 and loop L4.

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