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Studies on the Structure and Mechanism of H-ras p21 [and Discussion]

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Studies on the structure and mechanism of H-ras p21

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[Plate 1]

SUMMARY

Current knowledge of the structure of H-ras p21 is reviewed with particular emphasis on the interaction between guanine nucleotides and the active site of the protein. The nature of the conformational change induced by GTP hydrolysis is discussed. The major change is seen in the region known as the effector loop (loop 2), with significant but less well-defined changes occurring in loop 4, which is implicated in the GTPase reaction. Other evidence concerning the mechanism of GTP hydrolysis and its activation by GAP (GTPase-activating protein) is also discussed. Evidence regarding the rate limiting step in the p21 GTPase reaction, and the manner in which this and possibly other steps are accelerated by GAP, is inconclusive.

1. INTRODUCTION

The protein product of the H-ras proto-oncogene is a low molecular mass GTPase (21 kDa), which appears to be involved in signal transduction. Mutation at certain characteristic points in the sequence results in malignant transformation. Although normally membrane-bound via modification at the C-terminus, it has the characteristics of a soluble globular protein in the absence of this post-translational modification. Like many other guanine nucleotide binding proteins (GNBs), including the classical G-proteins, it appears to exist in an active state when GTP is bound to the protein, and an inactive state when GDP is bound. Active and inactive are terms which are used to define the state of p21 or of other GNBs in their respective signal transduction pathway, i.e. in the active state the signal is transmitted to the next species in the chain (via interaction with that species), whereas in the inactive state this interaction is interrupted. In the case of p21, this signal pathway has not yet been identified, although it is apparent that it interacts with at least two classes of molecules, these being exchange factors, which catalyse the intrinsically slow exchange of GDP and GTP, and GTPase-activating proteins. A representative of the latter class, a cytosolic protein known as GAP, has been characterized fairly extensively. Interaction of GAP with p21.GTP accelerates the extremely slow rate of GTP hydrolysis without affecting the rate of nucleotide exchange. There appear to be two fundamental molecular mechanisms of oncogenic transformation by p21 mutants. In one of these, the p21.GTP state is still able to interact with GAP, but the slow intrinsic GTPase rate (even slower for many mutants than with proto-oncogenic p21) is not accelerated by this interaction. In the other type, the spontaneous rate of GDP dissociation, and thus the rate of GTP–GDP exchange, is accelerated. Both effects lead to an increase in the concentration of the p21.GTP relative to the p21.GDP state.

We review here some of the results of structural and kinetic investigations on p21 and its interaction with nucleotides and with GAP.

2. STRUCTURE OF p21.GppNHp

Work on the structure of recombinant H-ras p21 from our laboratory has been exclusively on a C-terminally truncated form of the protein which is 23 amino acids shorter than the native length. This reduces the sensitivity of the protein to C-terminal proteolysis and improves its crystallization behaviour while retaining the most important biochemical properties apart from the ability to be modified and bound to a membrane. This form of p21 was found to crystallize much more readily when the non-hydrolysable GTP analogue GppNHp was bound at the active site than when GDP, which is bound to the protein as normally isolated after expression in *E. coli*, is bound (Scherer *et al.* 1989). After the structure was initially determined at a resolution of 2.6 Å‡ (Pai *et al.* 1989) it was possible to extend this to 1.35 Å (Pai *et al.* 1990), allowing a more detailed view of the structure and the most important interactions, as well as leading to the location of a large number of ordered water molecules. Some of the most important features concerning the

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‡ $1\text{Å} = 10^{-10}\text{m} = 10^{-1}\text{nm}$.

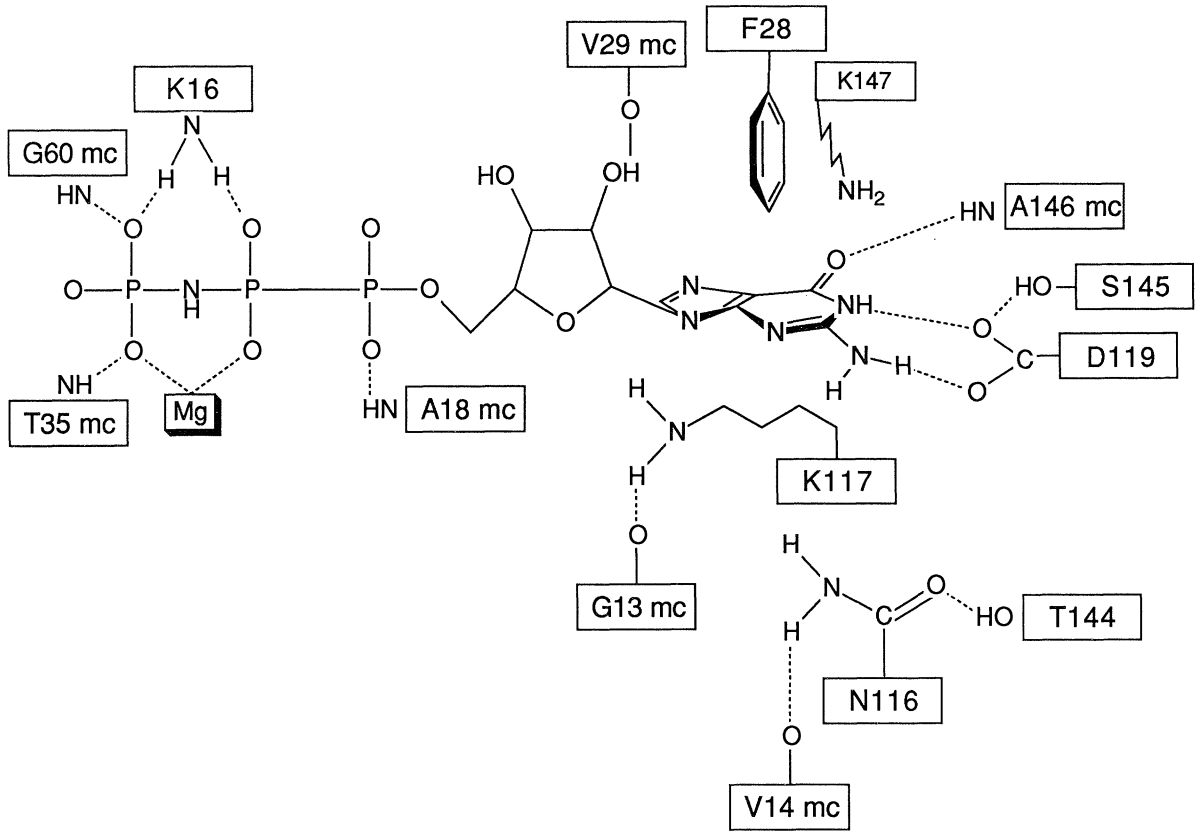

Figure 1. Schematic diagram of GppNHp at the active site of p21, showing some important interactions between the nucleotide and the protein. Hydrogen bonds have only been included if their length is less than 3.1 Å; mc, main chain.

interaction of nucleotides with the active site arising from these studies are shown in figure 1.

Base specificity (GTP binds *ca.* 10^7-fold more tightly than ATP) is mainly determined by the interaction of the highly conserved Asp-119 with both the exocyclic amino group and N1 of the guanine base, together with an interaction of the amide group of Ala-146 (also highly conserved) with the exocyclic oxygen of the guanine base. Important contributions to the high affinity for guanosine nucleotides (association constant *ca.* 10^{11} M^{-1} for GTP and GDP) are made by the aromatic interaction of the guanine base with Phe-28 and the hydrophobic interaction with the aliphatic side chain of Lys-117. Phe-28 is stabilized in its position by a hydrophobic interaction with Lys-147, which is part of the conserved ^{145}SAK sequence.

Interactions with the triphosphate moiety of the nucleotide are probably of more general significance for the larger class of phosphotransferases which use nucleoside triphosphates as phosphate donors. One of the most important features is the large number of interactions between the β-phosphate and the so-called phosphate binding loop, i.e. the highly conserved GXXXXGKS(T) motif found in a large number of ATP- and GTP-utilizing proteins including myosin, adenylate kinase and both classical (trimeric) and non-classical (small) G-proteins. These interactions are all with the main chain, and appear to serve the purpose of localizing the phosphate groups and withdrawing electrons to make GDP a better leaving group when the γ-phosphate group is subjected to nucleophilic attack. There are also a number of interactions with the oxygens of the γ-phosphate group, including the hydroxyl group and main chain NH of Thr-35 and the main chain NH of Gly-60. The electron-withdrawing effect of these interactions is supplemented by important bidentate interactions of the amino group of Lys-16 and of the essential

Table 1. *Amplitude and sign of the slow fluorescent transients seen after the initial rapid enhancement of fluorescence on interaction of p21 and some of its mutants with 2′(3′)-mantGTP and 3′-mantdGTP, respectively; the rates of these transients agree with the rates of GTP hydrolysis which can be measured by radioactive or high performance liquid chromatography (HPLC) methods*

p21 species	fluorescence change with 2′(3′)-mantGTP (%)	fluorescence change with 3′-mantdGTP (%)	GTPase rate (1/s)
wild type	−10	none	6.2×10^{-4}
G12D	none	+12	2.2×10^{-4}
G12V	−10	+12	0.6×10^{-4}
G12P	−10	+10	7.4×10^{-4}

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magnesium ion with the β- and γ-phosphate groups. The magnesium ion is, as expected, octahednally coordinated. In addition to the two phosphate ligands, there are interactions with Ser-17 and Thr-35, and with two water molecules, which in turn interact with Asp-33 and Asp-57. A key role of Mg^{2+} in the mechanism of action of GTP-binding proteins is suggested by the fact that these ligands are either highly conserved (Ser-17, Asp-57) or are part of the loop known as the effector loop (residues 30–40), which has been implicated in the interaction of p21 with GAP. First indications of the nature of this role have been obtained, as discussed below.

Several water molecules have been found in the region of the phosphate groups. The one which is most likely to be involved in GTP hydrolysis (Wat-175) forms a hydrogen bond to the main chain carbonyl group of Thr-35. It is also near to the region of the protein whose structure is least well defined. This region is termed loop 4 and comprises the residues 58–65. It is not possible, even in the electron density map at 1.35 Å, to place the amino acid residues in unambiguous positions in this region of the structure. Instead, it appears that two or more configurations of each residue could be assumed and accounted for by the density. As discussed below, it is possible that one particular configuration of loop 4 represents the active conformation (i.e. that in which water can attack the γ-phosphate group).

3. STRUCTURE OF p21.GTP

Although the resolution and quality of the electron density map obtained for p21.GppNHp are outstanding, we cannot be certain that the resulting atomic model is a perfect reflection of the p21.GTP state, i.e. the active state of p21. Some differences in the interaction of p21 with GTP and GppNHp are suggested by the fact that its affinity for the analogue is about one order of magnitude lower than that of GTP or GDP. For this reason, and because we were interested in obtaining as direct a comparison as possible of the p21.GTP and p21.GDP states, we sought methods for generating the p21.GTP complex in crystalline form, and for following the transition associated with GTP hydrolysis, i.e. with the active to inactive transition, in the crystal. A possible way of doing this was provided by crystallization of the complex between p21 and caged GTP, a photosensitive precursor of GTP (Schlichting *et al.* 1989). Photolysis of caged GTP at the active site of p21 in the crystals resulted in formation of the p21.GTP complex, which then underwent the transition to p21.GDP at approximately the same rate as in solution. Because the half-life for this reaction is about 40 min at room temperature, determination of the structure within the first few min after photolysis would lead to the structure of p21.GTP. This was possible using the recently rediscovered Laue technique (for review see Hajdu & Johnson 1990), which uses a range of wavelengths to obtain X-ray diffraction patterns, instead of the standard monochromatic technique. Collection of a data set *ca.* 4 min after removal of the protecting group led to determination of the structure of the p21.GTP complex (Schlichting *et al.* 1990). Although the spatial resolution of the electron density map was inferior to that of the map obtained from monochromatic data for the p21.GppNHp state, it is apparent that the structures are very similar, if not identical. As with p21.GppNHp, the density in loop 4 does not allow unambiguous fitting of the residues.

4. STRUCTURE OF p21.GDP ARISING FROM HYDROLYSIS OF GTP IN p21 CRYSTALS

After removal of the protecting group from caged GTP in crystals of p21, the intrinsic GTPase results in the formation of p21.GDP. Because this is a stable state, its structure could be determined by classical (i.e. monochromatic) X-ray methods. This has allowed a comparison of the structure with that of p21.GTP (Schlichting *et al.* 1990). The most obvious changes occur in the effector loop, where it can be seen that Tyr-32, Asp-33, Pro-34, Tyr-35 and IIe-36, in particular, change their positions drastically. These changes are triggered by the loss of the γ-phosphate group. Thr-35 appears to be intimately involved with these changes. In the p21.GTP and p21.GppNHp states, there is an interaction between the main chain NH group of this residue and an oxygen of the γ-phosphate, which is not present in the p21.GDP state. In addition, the interaction of the side chain hydroxyl of Thr-35 to Mg^{2+} is also lost after GTP hydrolysis. The loss of these interactions allows the effector loop to take up a new configuration. This, presumably, is not recognized by GAP, which binds much more weakly to p21.GDP than to p21.GTP.

It is of interest to speculate on the causal relations involved in this structural change. As there are no obvious interactions between GDP or Mg^{2+} and the effector loop, the conformation adopted in the p21.GDP state must be assumed to be the intrinsically stable conformation of this region. Although accurate quantitative data are not available, it seems that the affinity of the p21.GDP complex for GAP is at least a factor of 100 lower than that of the p21.GTP complex. If we assume that the change in affinity is due solely to the change in structure of the effector loop, and also assume that the 'high affinity' conformation is attainable, even if not stable, in the p21.GDP complex, it must exist at a relative concentration which is at least a concentration of 100 below that of the predominant conformation to explain the change in affinity. According to this interpretation, the presence of GTP at the active site would stabilize an intrinsically unstable conformation of the effector loop, this conformation having a higher affinity for GAP. The interactions responsible for holding the effector loop in this conformation appear to be the backbone NH interaction of Thr-35 with an oxygen of the γ-phosphate group already mentioned and an interaction of the same phosphate oxygen with the hydroxyl group of the Thr-35 side chain, which is additionally coordi-

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nated with Mg^{2+}. It is the loss of these interactions in the p21.GDP state which appears to trigger the change in conformation of the effector loop.

Significant changes on GTP hydrolysis are also seen in another region of the protein structure, namely in loop 4 (residues 58–65). These changes are, however, difficult to define because, as mentioned above, clearly interpretable electron density is not seen in any of the structures solved in our work, nor in that of others. A change in attitude of the helix following this loop (helix 2), seen on comparison of the structures of p21.GppCH$_2$p and p21.GDP (Milburn *et al.* 1990), was not seen in our work. Another point of disagreement concerns the interaction of Asp-57 with Mg^{2+} in the p21.GDP complex. In the p21.GppNHp (Pai *et al.* 1989, 1990), p21.GppCH$_2$P (Milburn *et al.* 1990) and p21.GTP (Schlichting *et al.* 1990) complexes, Asp-57 is seen to interact via a water molecule with Mg^{2+}. In the p21.GDP complex arising from photolysis of p21.caged GTP, the interaction appears to be direct. It is possible that this is partly responsible for loss of the interaction of Mg^{2+} with the side chain of Thr-35. However, the model of the structure of p21.GDP obtained by direct crystallization of the complex does not appear to show the Mg^{2+}–Asp-57 interaction (Milburn *et al.* 1990), i.e. it is still indirect, presumably via a water molecule.

The studies outlined here have done much to define the structural differences between the active and inactive states of p21. However, more work is needed to be certain of the nature and significance of the differences, particularly in the loop 4 and helix 2 region, and to understand the causal relations linking the state of the nucleotide and the conformation of the protein. A significant improvement would arise from determination of the structures of p21.GTP and p21.GDP at higher resolution. Progress in this direction has been made recently with an improvement in the quality of the p21.caged GTP crystals. Caged GTP exists as a mixture of diastereomers because of an assymmetric centre in the protecting group. The crystals of p21.caged GTP used in previously published work contained a mixture of these diastereomers present at approximately equal concentrations. As these isomers are separate chemical entities, it is possible that the relatively poor quality of the p21.caged GTP crystals (diffraction to *ca.* 2.8 Å) is caused by the presence of this mixture at the active site. Pure diastereomers of caged GTP have now been synthesized (J. Corrie, G. P. Reid & D. R. Trentham, submitted), and crystals of p21 with the individual isomers have been obtained. These diffract X-rays to much higher spatial resolution than crystals with the mixed isomers (for the *R* diastereomer to at least 1.8 Å, for *S* to 2.2 Å), and the structure of p21 with the *R* isomer has been solved at 1.8 Å resolution. A major difference has emerged when the results are compared with the structure of p21 with mixed isomers of caged GTP at the active site. In this case (Schlichting *et al.* 1990), caged GTP was seen to bind in a different manner to GTP or GDP, and this appeared to be a result of steric hindrance between the nitrophenylethyl group and a neighbouring protein molecule in the crystal. None of the interactions identified between p21 and GppNHp could be identified in the p21.caged GTP structure, although nuclear magnetic resonance (NMR) evidence suggested that the interaction in solution was normal. In the p21.*R*-caged GTP structure the situation is different. The caged GTP molecule appears to be bound in an identical manner to GppNHp, with the exception that the additional group on the γ-phosphate is present. The Mg^{2+} ion can also be located, and is bound in the same manner as in p21.GppNHp. Interestingly, the effector loop in these crystals can be seen to be similar to that in p21.GppNHp. In accordance with this, preliminary solution experiments suggest that p21.caged GTP interacts with GAP in a similar manner to p21.GppNHp or p21.GTP. This suggests an interesting use of caged GTP in systems involving GTP binding proteins. If a particular GTP binding protein when loaded with caged GTP adopts the 'on' state, so that the system which involves the G-protein is also turned on, removal of the protecting group by photolysis followed by GTP hydrolysis will lead to the system being switched off in a single turnover type of experiment.

5. MECHANISM OF GTP HYDROLYSIS

Although knowledge of the structures of individual states of p21 do not automatically lead to clarification and understanding of the chemical mechanism of GTP hydrolysis, certain insights into the nature of the catalysis can be gleaned and some specific suggestions made. Some of the interactions leading to activation of the γ-phosphate group, to nucleophilic attack, and to improvement of the leaving group properties of GDP have already been mentioned. In general, it will be necessary to correlate structural and other data to arrive at this mechanism. An important piece of information in this respect concerns the question of the possible involvement of a phosphorylated enzyme intermediate. It was shown several years ago, by using GTP(γ-*S*) in which the thiophosphate group was made chiral by labelling with oxygen isotopes, that the configuration at the phosphorus atom was inverted on transfer of the group of water (Feuerstein *et al.* 1989). This suggests that attack of water on the terminal phosphate group is a direct in-line displacement, as two inversions, resulting in net retention of configuration, would be expected if a phosphorylated intermediate was involved. In accordance with this, a water molecule has been identified in the structure of p21.GppNHp which appears to be well placed for attack on the γ-phosphate group. This water molecule (Wat-175) makes a hydrogen-bonding interaction with the backbone carbonyl group of Thr35. In addition, if one particular possible configuration of the carbamoyl group of Gln-61 in the less well-defined electron density of loop 4 is assumed, there could also be an interaction of this side group with Wat-175, and Glu-63 could interact with the side group of Gln-61. This situation, with the appropriate orientations of the Gln-61 and Glu-63 side chains, is shown in figure 2. A formally reasonable mechanism for the activation of

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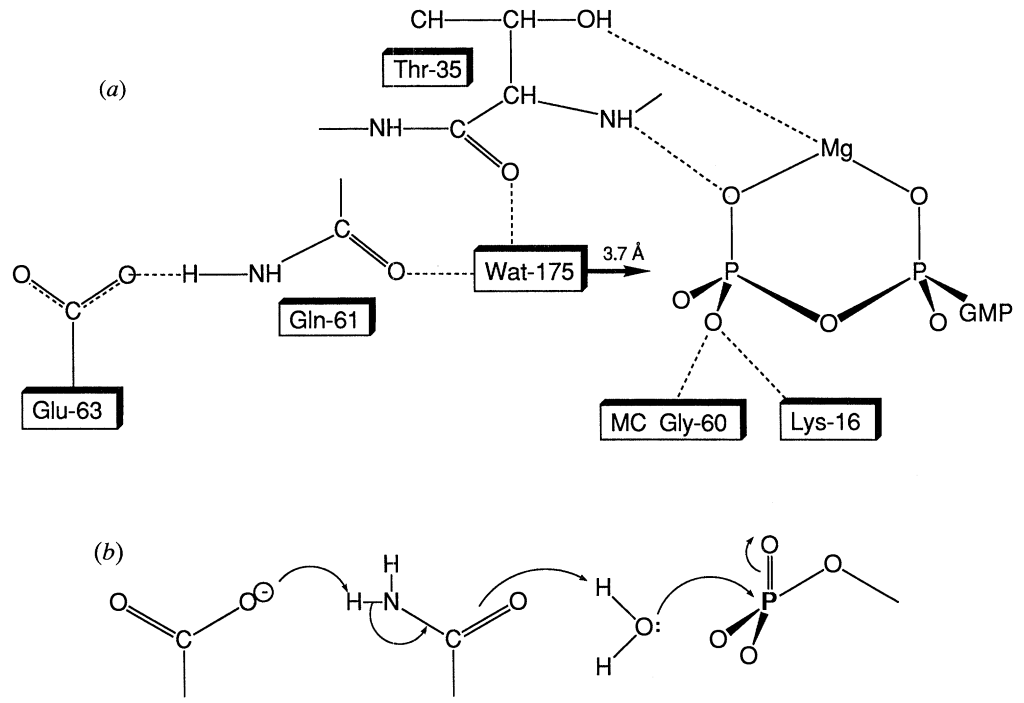

Figure 2. (*a*) Representation of the proposed catalytically active configuration of residues in loop 4. (*b*) Formal representation of a possible reaction mechanism.

the water molecule and attack on the γ-phosphate group of GTP is also shown in figure 2. More evidence, perhaps from spectroscopic methods, including NMR, electron paramagnetic resonance (EPR) and Fourier transform infrared measurements, will be needed to prove, disprove or add substance to this proposal. Such studies are currently in progress in our and other laboratories.

6. INTERACTION OF p21 WITH GAP

Both the molecule known as GAP and a region of the neurofibromatosis type 1 (NF 1) gene product, which is highly homologous to GAP, are able to activate the rate of GTP cleavage by p21. In the case of GAP, this acceleration is by a factor of 10^5 (i.e. in the complex between GAP and p21.GTP, the rate of GTP cleavage is *ca.* 10^5-fold faster than in the p21.GTP complex; as the very slow rate of GDP released is not accelerated by GAP, p21 is still, in classical terms, a very slow GTPase). An understanding of the mechanism of this activation, and of the reason for its absence with many oncogenic mutants of p21, is obviously of major importance for understanding the system in general and oncogenic transformation in particular. There have been no reports to date of structural work on GAP or on the NF 1 gene product so the available information comes from solution studies on the interaction of p21 with these proteins.

As GAP is a protein with a very high molecular mass (*ca.* 120 kDa), attempts have been made to produce recombinant versions which are very much reduced in length but which retain the basic property of accelerating the p21 GTPase reaction. Thus some of the work discussed here was done with a preparation designated GAP_{334}, which corresponds to residues 714–1047 of the native molecule. We will not distinguish between this and the full length form in the discussion here.

A basic question about the mechanism of GAP activation of p21 GTPase is whether the mechanism of GTP cleavage is fundamentally the same in p21 and in the p21.GAP complex. One of the first steps in defining the chemical mechanism of an enzyme is to define the 'kinetic mechanism' of catalysis, which usually involves determining the number of steps which occur, measuring their rate and equilibrium constants, which allows identification of the rate limiting step (or steps), and chemical definition of the individual steps. We have used fluorescent analogues of guanosine nucleotides to obtain some of this information. It was shown that binding of GTP or GDP occurs in at least two steps, the first step being an easily reversible and rapid equilibrium reaction, the second an essentially irreversible isomerization (John *et al.* 1990). Studies using the fluorescent GTP analogue 2′(3′)-methylanthraniloyl GTP (mant GTP) have been interpreted to suggest that the rate limiting step in the GTPase reaction is a second isomerization reaction which occurs immediately before the cleavage step (Neal *et al.* 1990). A possible candidate for this isomerization which arises from the crystallographic work is the conformational change required to reach the catalytically active constellation of residues defined as described above. The role of GAP would be to accelerate this transition, after which attack of water on the γ-phosphate would then be rapid (and perhaps not influenced in its rate by GAP). This mechanism would belong to the class in which GAP exerts its effect purely by influencing the rate of steps that can take place even in its absence.

Regardless of whether the suggestion for the actual

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structural change preceding GTP cleavage is correct or not, the kinetic mehanism suggested by Neal *et al.* (1990) can be tested by observing the effect of GAP on the fluorescence change which was suggested to be a signal of the slow isomerization. It should be accelerated by GAP, and this is indeed the case using mantGTP (Rensland *et al.* 1991). In addition, a similar but smaller change seen with the corresponding fluorescent derivative of GppNHp (Neal *et al.* 1989) should also be accelerated by GAP. In our experiments this does not occur, i.e. the slow fluorescence change seen in the p21.mant GppNHp complex is insensitive to GAP (Rensland *et al.* 1991). It thus seems more likely that the GAP-sensitive fluorescence change seen with mant GTP is associated directly with GTP cleavage (or occurs immediately after it); this theory is further strengthened by the observation that mant GTP(γ-*S*), which is cleaved much more slowly than mant GTP, also exhibits a much slower fluorescence transient than mant GTP, both of these rates being accelerated by GAP (Rensland *et al.* 1991). A slightly different conclusion was reached by Antonny *et al.* (1991) by using the intrinsic fluorescence of tryptophan-containing p21 mutants as a signal. Their results are interpreted to suggest that a slow conformational change is rate limiting in the hydrolysis of GTP by p21 alone, but that this step is bypassed in the GAP-activated reaction.

7. STRUCTURE OF MANT dGppNHp

Although the fluorescent signals observed with the mant GTP and GDP derivatives have been useful in helping to establish mechanistic features of the p21 GTPase, their use is limited, as is usually the case with fluorescence studies, by the inability to relate the spectroscopic changes observed directly to structural or mechanistic properties of the system. To attempt to remove this shortcoming, we have begun to investigate the structures of p21 complexes with mant nucleotides. The first complex which was crystallized is that between p21 and 3′-methylanthraniloyl-2′-deoxy GppNHp (mant dGppNHp). The deoxyribonucleotide derivative was chosen because it can be obtained in pure form, in contrast to the corresponding ribonucleotide, which is a mixture between the 2′- and 3′-isomers which are in equilibrium with each other. Needle-shaped crystals of the p21.mant dGppNH diffract X-rays to a resolution of 2.5 Å. The structure, which is isomorphous to that of p21.GppNHp, has been refined to 2.7 Å, and an electron density map has been calculated. In general, the structure of the protein looks identical to that of p21.GppNHp, including the effector binding loop, which explains the observation that p21 complexes with mant nucleotides appear to interact with GAP in the normal fashion. The features most relevant to the present discussion concern the mode of binding of the nucleotide at the active site of the protein. Figure 3*a* shows that the nucleotide is well defined by the electron density, and a comparison with the model of p21.GppNHp shows that most of the nucleotide, in particular the base and the triphosphate moieties, appear to be in identical positions, within the limits of resolution, to GppNHp in the p21.GppNHp structure. The ribose moiety is somewhat displaced from its position in p21.GppNHp. This is in part a result of the missing interaction of the 2′-hydroxyl group with the carbonyl group of the backbone amide function of Val-29. As can be seen in figure 3*a*, the 3′-methylanthraniloyl group projects from the ribose ring at *ca.* 180° to the base in the direction of the affector loop. The only major interaction of the fluorescent group appears to be between its aromatic ring and the aromatic ring of Tyr-32. A comparison with the p21.GppNHp structure shows that this occurs with minimal displacement of this residue (Tyr-32) from its normal position in the active state of p21. This interaction is of particular interest, because Tyr-32 is the residue which undergoes the most significant change on GTP hydrolysis. In figure 3*b*, the model of the effector loop region of the structure is shown and compared with the model for the same region in the p21.GDP complex. The large change in the position of Tyr-32 is probably the origin of the change seen in fluorescence quenching on GTP hydrolysis. We are at present attempting to crystallize a methylanthraniloyl derivative of GDP to gain more insight into the changes occurring on GTP hydrolysis.

8. STUDIES ON STRUCTURE AND MECHANISM OF p21 MUTANTS

Structural work on oncogenic mutants of p21 with reduced GTPase activity has shown that only very minor changes in structure are seen in comparison with wild type protein, apart from the vicinity of the position of substitution (Krengel *et al.* 1990). A common feature of both position 12 and postion 61 mutants is that the suggested catalytically active constellation of residues in loop 4 (figure 2) cannot be adopted. In addition, the different nature of the side chain of position 61 mutants is likely to exclude the activation of Wat-175 suggested in figure 2. More work is needed to enable a definitive model for the effects of these and other mutations. In addition to X-ray crystallography, spectroscopic and kinetic methods are likely to add detail to the explanation outlined here.

Fluorescent nucleotides have already been used fairly extensively with p21 mutants, and we describe some of the results briefly here. The oncogenic mutant of p21 G12D was reported by Neal *et al.* (1980) not to show the slow fluorescent transient seen in the complex between wild type p21 and 2′(3′)-mantGTP, although it is hydrolysed by this mutant. This was interpreted to mean that the complex between this mutant and GTP was locked into the 'active' form throughout the GTPase cycle. Our results suggest that this fluorescence change is not a good parameter on which to base such a conclusion. We have used the 3′-mant derivative of 2′-deoxyGTP (3′-mantdGTP) because its use avoids the potential problems associated with using the mixture of 2′- and 3′-isomers of 2′(3′)-mantGTP. In contrast to 2′(3′)-mantGTP, when 3′-mantdGTP is bound to p21 there is no slow

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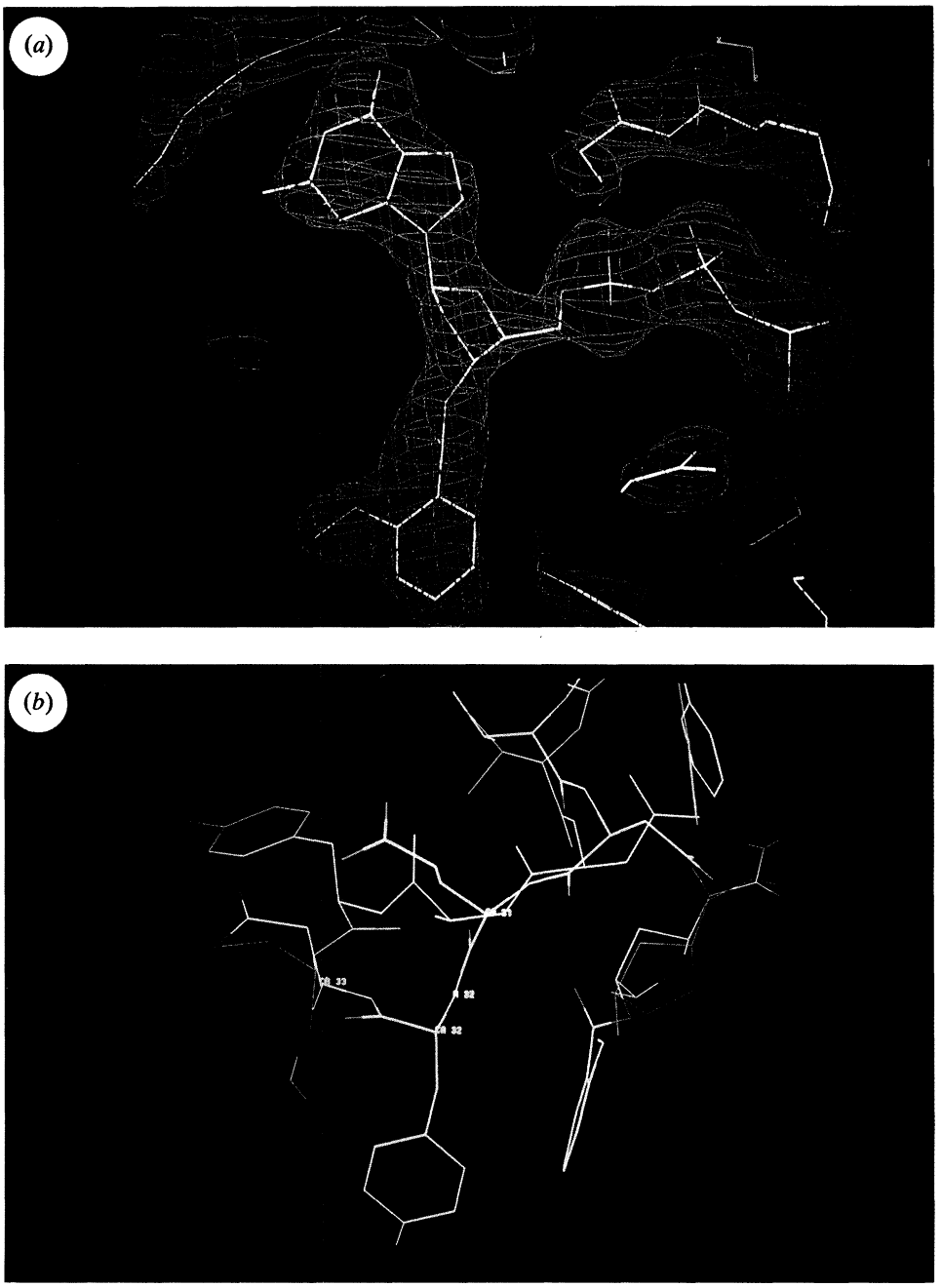

Figure 3. (*a*) Model of 3′-mantdGppNHp at the active site of p21 together with the 2.7 Å electron density map. (*b*) Region around Tyr-32 in the model of the p21.mantdGppNHp structure (multicoloured) and of the p21.GDP state derived from p21.cagedGTP crystals after photolysis and enzymic cleavage of GTP (pink). The mant nucleotide is on the right side, the aromatic ring of the methylanthraniloyl group being perpendicular to Tyr-32 pointing out of the picture plane.

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fluorescent transient, although cleavage occurs and can be activated by GAP. However, a significant slow change (12%) in fluorescence occurs with the G12D mutant. This has the same rate as GTP hydrolysis (two- to threefold slower than wild type) but is now in the opposite direction (i.e. the fluorescence increases) to that seen with 2′(3′)-mantGTP and wild type p21. Another position 12 mutant, G12V, exhibits a fluorescence change with 2′(3′)-mantGTP in the same direction and of similar magnitude to that observed with wild type p21. In keeping with the slower GTPase rate, the rate of this transient is a factor of *ca.* 10 slower than with wild type. In contrast to wild type p21, this mutant also shows a slow fluorescence change (increase) with 3′-mantdGTP. Similar behaviour is also found with the only non-transforming position 12 mutant, G12P, i.e. there is a slow decrease in fluorescence in its complex with 2′(3′)-mant GTP, and a slow increase in the fluorescence with 3′-mantdGTP, the rates in this case being similar to wild type. It is thus difficult to draw mechanistic conclusions from these studies alone. The changes in fluorescence seen are apparently very sensitive to changes in both the protein and nucleotide structure, in a manner which can probably only be interpreted when more structural information of the type shown in figure 3 is available for some of the other complexes. An interesting additional observation with wild type H-ras p21 is that 2′-mant-3′-deoxyGTP shows a very large change in fluorescence (35% decrease) at the rate of cleavage. One of the reasons for the relatively large differences in behaviour of the different derivatives probably arises from the fact that the 2′-hydroxyl group of GTP normally interacts with the backbone carbonyl group of Va-29. This is presumably also the reason for the preferred binding of the 3′-mant isomer over the 2′-isomer (Rensland *et al.* 1991).

9. CONCLUSION

A very large amount of detail of the p21 structure and the way the protein interacts with nucleotides has been gained from X-ray diffraction studies. These have been supplemented by kinetic and other studies which have led to information on the rates and nature of steps in the GTPase reaction of both p21 and GAP-activated p21. In the light of this evidence, it is sobering to realize that the mechanism of the GTPase reaction, even in the absence of GAP, is not yet fully unravelled. It is apparent that additional data from independent methods are needed to complete this picture, as well as structural information on GAP and GAP-p21 complexes. In the absence of a complete understanding of these fundamental mechanisms it will not be possible to define in detail the mechanism of oncogenic transformation at this level of resolution, even though the work done so far on p21 mutants has already offered valuable insights and allows a phenomenological description of the mechanisms involved. It is unlikely that the principles of rational drug design, which might allow an exploitation of the differences between oncogenic and proto-oncogenic forms, will be applicable until we have a more profound understanding of the mechanism of GTPase reaction, especially in the presence of GAP, and of the way this interaction is modified in oncogenic mutants.

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Discussion

T. J. Mitchison (*Department of Pharmacology, University of California, San Francisco, U.S.A.*). I am interested in a different GTP analogue ($\alpha\beta$-CH_2)GTP. Some of the older literature says it is not hydrolysed by GTP binding proteins and we found that also to be true of tubulin where it is a very useful analogue. On the basis of structure, is it possible to understand the lack of hydrolysis of that analogue, which has normal $\beta\gamma$ P-O-P bonds presumably?

R. S. Goody. On the basis of the structure, I think not. We have not actually tried that analogue with p21ras. We are at the moment, for completely different reasons, looking at the interaction of p21ras with ($\alpha\beta$-CH_2)GDP. I have always thought that ($\alpha\beta$-CH_2)ATP is a bad analogue with myosin, in the sense that it is cleaved 100–1000 times more slowly by myosin ATPase than ATP is, and it does appear to interact much more weakly with the active site. So it may be that it cannot take up exactly the correct configuration.

T. J. Mitchison. Certainly for tubulin, and some of the older literature on GTP binding proteins says it is a very good analogue in terms of affinity.

R. S. Goody. It is important to be careful, as people tend to compare K_is with K_ms, and this may not be valid, particularly in systems that have very high binding constants for the natural nucleoside triphosphates. It is a similar situation to comparing ATP and AMPPNP for myosin. It looks as if AMPPNP is an extremely good analogue of ATP for myosin, if its K_i value is compared with the K_m of ATP, but if the binding constant of ATP is measured, it is five orders of magnitude better than that of AMPPNP.

K. A. Johnson (*Department of Biochemistry, The Pennsylvania State University, U.S.A.*). I have two related questions. First, from the structure, what makes ras such a poor catalyst? Secondly, pursuing this thought of a conformational change as you proceed into the transition state, what sort of groups might be nearby that could come into play in a transient conformational change?

R. S. Goody. I mentioned that in the loop 4 region (residues 60–65) in all the structures that have been examined, both GTP and GDP analogue structures, each of those residues could have the side chain and the backbone in at least two different places (so there are at least 2^5 possible conformations). It is likely that a particular configuration of those residues is needed to activate the water molecule, and that this would be a slow, energetically unfavourable event. This is only a hypothesis and obviously more information is needed.

K. A. Johnson. The use of glutamine as an active site base is curious. Is that known in any other cases?

R. S. Goody. I do not think there is a precedent for it, and we were worried about it as well, but formally in terms of the chemistry, curly arrows do the right things and it might be possible.

K. A. Johnson. Related to that, if glutamine 61 were replaced with a glutamic acid, and glutamate 63 with histidine, it might have a better active site.

R. S. Goody. Some of these things have been done. Nothing has yet led to anything which is dramatically better. Many things have led to mutants which are much worse in terms of their GTPase activity.

R. Skinner (*Wellcome Research Laboratories, Beckenham, U.K.*). With regard to the hypothesis that GAP may contribute a catalytic residue such as arginine, our group has mutated a motif FLR (901–903) that is absolutely conserved among GAP, IRA1, IRA2, NF-1 and GAP1 (Skinner *et al.* 1991; Xu *et al.* 1990; Imai *et al.* 1991). R903 is the only arginine residue that is clearly invariant in the catalytic domains of these proteins, and the R903K mutant is some 30-fold less efficient than normal GAP in the stimulation of ras GTPase.

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R. S. Goody. That is interesting. There are a number of highly conserved arginines in the GAP sequence, I believe. Is that right?

R. Skinner. R903 is the one that is most conserved if the difference between, say GAP and NF1 and IRA1 and IRA2, is compared.

D. M. Blow (*The Blackett Laboratory, Imperial College, London, U.K.*). I would like to ask about the Laue work which is so beautiful. Is there a chance that the enantiomeric caged compounds will enable the authors to improve the resolution of that Laue work?

R. S. Goody. Yes, we hope so. Certainly, with monochromatic radiation we have now 1.8 Å resolution with one of the pure enantiomers, whereas we had 2.8 Å with a mixture. There are Laue data sets which have been obtained with one enantiomer but have not yet been processed, and we hope that their resolution will also be higher than the mixture.

C. Cremo (*Department of Biochemistry, Washington State University, Pullman, U.S.A.*). Can the authors comment on why G12 is so important in the transforming activity?

R. S. Goody. G12 does not appear to be involved directly in catalysis except that the backbone NH interacts with the β-phosphate group. The reason appears to be that whatever other group is put in there, the size of the group stops loop 4, which is very near to it, from taking up what we regard as the catalytically active conformation. It appears to be a purely steric effect. There is no change in the structure of the phosphate binding loop in contrast to what was maintained by other groups earlier.

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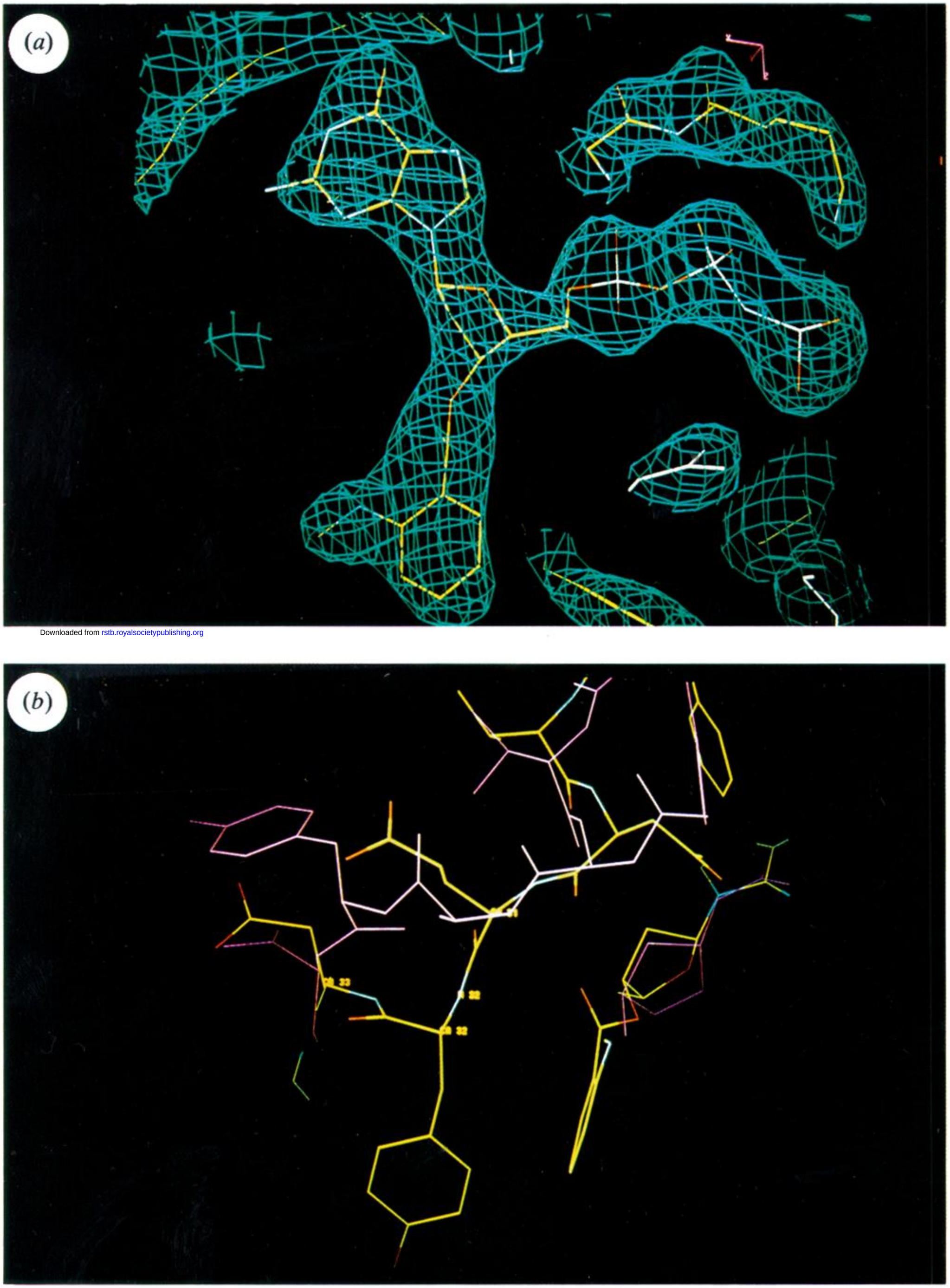

gure 3. (*a*) Model of 3′-mantdGppNHp at the active site of p21 together with the 2.7 Å electron density map.) Region around Tyr-32 in the model of the p21.mantdGppNHp structure (multicoloured) and of the p21.GDP ate derived from p21.cagedGTP crystals after photolysis and enzymic cleavage of GTP (pink). The mant nucleotide on the right side, the aromatic ring of the methylanthraniloyl group being perpendicular to Tyr-32 pointing out of the picture plane.