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Time-Resolved Crystallography on H-ras p21 [and Discussion]

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Phil. Trans. R. Soc. Lond. A 1992 **340**, 263-272
doi: 10.1098/rsta.1992.0065

Time-resolved crystallography on H-ras p21

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[Plate 1]

We describe here the results obtained to date on a project aimed at characterizing the changes occurring in the protein product (p21) of the H-ras proto-oncogene during and as a result of hydrolysis of GTP at its active site. The approach used involves crystallization of p21 with a photosensitive precursor of GTP (caged GTP) at the active site followed by generation of GTP by photolysis and collection of X-ray diffraction data using the Laue method at a synchrotron source. The structure of p21 complexed with a single diastereomer of caged GTP is presented here. In contrast to crystals obtained with mixed diastereomers, the nucleotide appears to bind in a manner which is very similar to that of other guanine nucleotides (GDP, GTP, GppNHp). The current state of time resolved structural experiments using these crystals is presented.

1. Introduction

H-ras p21 is the protein product of the H-ras proto-oncogene. It appears to be involved in a signal transduction pathway which has not yet been identified but which leads to cell growth and proliferation. p21 belongs to the family of guanine nucleotide binding proteins, and in common with other members of this group exists in two states, depending on the nature of the nucleotide at the active site. When GTP is bound, the protein is in the active state, meaning that a signal is passed on to the next molecule in the transduction pathway. This signal is turned off by GTP hydrolysis, i.e. by generation of the p21-GDP state, which is the normal resting state of the system. In its isolated form, p21 is a very slow GTPase ($t_{\frac{1}{2}}$ for GTP hydrolysis at room temperature is *ca.* 30 min), but in the cell, this rate is accelerated by interaction with the cytosolic protein GAP (GTPase activating protein). In vitro experiments have shown that the rate of GTP hydrolysis in the GAP-p21-GTP complex is *ca.* 10^5 fold faster than in p21-GTP (Gideon *et al.* 1992), but the actual rate of GTP hydrolysis *in vivo* will depend on the prevailing effective concentrations of p21 and GAP. In many oncogenic mutant forms of p21, GAP is able to bind to the p21-GTP complex, but this interaction does not result in activation of the GTP cleavage rate.

Some of the most important aspects of the ras/GAP system concern the nature of

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Phil. Trans. R. Soc. Lond. A (1992), **340**, 263–272
Printed in Great Britain

the structural change occurring in p21 as a result of GTP hydrolysis, the chemical mechanism of GTP hydrolysis, the mechanism of GAP activation and the underlying mechanism (or mechanisms) of oncogenic transformation. We have adopted the approach of time-resolved protein crystallography to answer some aspects of these questions. The underlying strategy involves crystallization of p21 with a photolabile precursor, caged GTP, at the active site, which allows photolytic generation of GTP in the crystal. We describe here the results obtained to date in this project.

2. Properties of the p21-caged-GTP complex

Caged GTP exists as a mixture of diastereoisomers because of the presence of a chiral centre in the 1-(2-nitrophenyl)ethyl group. Early work with p21 and other systems was with the mixture of diastereomers (ratio *ca.* 1:1). The affinity of p21 for caged GTP was determined using stopped flow kinetics, as described by John *et al.* (1990), which allowed determination of the association rate constant (Schlichting *et al.* 1990). For the mixed isomers, this was found to be 2.5×10^6 M^{-1} s^{-1} at 25 °C, compared with 8.5×10^5 M^{-1} s^{-1} for GDP. The dissociation rate constant was determined to be 2.5×10^{-4} s^{-1}, compared with 1.2×10^{-5} s^{-1} for GDP. Thus, caged GTP binds to p21 with an affinity which is only a factor of *ca.* 10 lower than GDP, and the very high binding constant of 10^{10} M^{-1} allows preparation of 1:1 complexes of p21 and caged GTP without using a large excess of the analogue, which could create problems in the crystallization and photolysis experiments. The crystals of p21-caged-GTP that were obtained contained approximately equal amounts of the two diastereoisomers (Schlichting *et al.* 1989). They diffracted X-rays to a resolution of 2.8 Å†, and were used for the original time-resolved diffraction experiments. (Schlichting *et al.* 1990).

Separate diastereoisomers of caged ATP have become available recently (Corrie *et al.* 1992), and we have used the same chemistry to prepare the separate diastereoisomers of caged GTP, which were then used to generate their complexes with p21. As already suggested by the presence of equal amounts of both diastereoisomers at the active site in crystals of p21 in the presence of the mixed isomers, the affinity of the diastereoisomers to p21 is similar, as shown by kinetic experiments that demonstrate that the rate of dissociation of the two diastereoisomers differs by less than a factor of 2. As described below, crystals of p21 with pure diastereoisomers of caged GTP at the active site diffract X-rays to considerably higher resolution than those with mixed isomers. An interesting and completely unexpected observation concerns the stability of the isomers of caged GTP in crystals of p21. Whereas the *R*-isomer is more stable in crystals of p21 than free (i.e. not complexed to protein) in solution, the *S*-isomer is degraded significantly more rapidly (table 1). Similar rates of degradation were found for the caged GTP diastereoisomers bound to p21 in solution, so that the effects could be attributed solely to interaction with the active site of p21. The products of the degradation reaction were GDP and caged phosphate. Thus, it appears possible that the degradation occurs by a mechanism which may be directly related to the normal GTPase mechanism, although it is not accelerated by GAP.

An interesting question concerning the p21-caged-GTP complex is whether the protein is in the 'on' (or GTP-like) or the 'off' (GDP-like) state. This has been tested using p21-caged-GTP as an inhibitor of the GAP activation of hydrolysis of p21-

† 1 Å = 10^{-10} m = 10^{-1} nm

Table 1. *Kinetic data on the hydrolysis of caged GTP*

	$T_{\frac{1}{2}}$ days		
	R/S	*R*	*S*
in solution without protein	120	130	120
p21-caged-GTP in solution	n.d.	630	160
p21-caged-GTP crystal	1000	1100	70

GTP. The results of this experiment suggest that the structure resembles that of p21-GTP, since p21-caged-GTP is able to inhibit the GAP activation of the p21 GTPase but accurate quantitative data are not yet available. As discussed later, the region thought to be involved in the interaction of p21 with GAP adopts a configuration intermediate between that seen in p21-GTP (or p21-GppNHp) and that seen in p21-GDP.

3. Structure of p21-GppNHp

The structure of p21-GppNHp at 1.35 Å resolution has been described in detail (Pai *et al.* 1990). We will include a brief description of the structure emphasizing the interactions between the nucleotide and the active site for the purposes of comparison with other structures discussed here. All the work described is on recombinant H-ras p21 which is a C-terminally truncated form of the protein 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 post-translationally modified and bound to a membrane. This form of p21 was found to crystallize much more readily when the non-hydrolyzable GTP analogue GppNHp was at the active site than when GDP, which is bound to the protein as normally isolated after expression in *E. coli*, is present (Scherer *et al.* 1989).

Base specificity (GTP binds *ca.* 10^7 fold more tightly than ATP) is mainly determined by the interaction of the highly conserved (in guanine nucleotide binding proteins) 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 (*ca.* 10^{11} M^{-1} for GTP and GDP; Goody *et al.* 1991) 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 ^{145}SAK sequence, which is also conserved in guanine nucleotide binding proteins.

One of the most important features of the interaction of the triphosphate part of the nucleotide structure with the protein is the large number of interactions between the β-phosphate and the so-called P-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) guanine nucleotide binding proteins. These interactions are all with main chain NH groups, 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 by water, which is known to be direct (Feuerstein *et al.* 1989). There are also several 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 magnesium ion with the β and γ-phosphate groups. The magnesium ion is, as expected, octahedrally 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, Thr 35) 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 61–65. It is not possible, even in the 1.35 Å electron density map, 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. We have previously suggested an enzymatically competent configuration of loop 4 which represents the active conformation (i.e. that in which water can attack the γ-phosphate group).

One of the γ-phosphate group oxygens interacts only with water molecules or with Tyr 32 from a neighbouring p21 molecule in the crystal, but shows no hydrogen bonding interactions with its 'own' protein molecule. This is presumably the reason why the protecting group of caged GTP is well tolerated by p21. This is in marked contrast to the very weak binding of caged ATP to myosin or actomyosin.

4. Structure of p21-caged-GTP

Crystals containing the mixture of diastereomers of caged GTP diffract X-rays to a resolution of *ca.* 2.8 Å (Schlichting *et al.* 1989, 1990). Determination of the structure of this complex by classical (monochromatic) crystallography led to the conclusion that caged GTP was bound to the active site in a completely different mode to GTP or GDP, although NMR measurements indicated that the binding to p21 in solution was normal (i.e. GDP- or GppNHp-like). We suggested that this was due to steric effects caused by interference of the cage group of the nucleotide with the neighbouring protein molecule in the crystal (Schlichting *et al.* 1990). We have now determined the structure of p21 complexed with the pure *R*-diastereomer of caged GTP, and describe the structure here (see table 2 for details of the structure determination). The improvement in resolution (to 1.8 Å) on replacing the mixed isomers by a pure diastereoisomer is striking. This leads to a better determined structure and allows a more meaningful comparison with the high resolution structure of p21-GppNHp. Surprisingly, the nucleotide is seen to bind in a GTP-like manner in this complex (see figure 1*a*, plate 1) in contrast to the structure of p21 with mixed diastereoisomers of caged GTP (Schlichting *et al.* 1990). The GTP part of caged GTP appears to bind almost identically to GppNHp. Despite the fact that there is one negative charge less on the γ-phosphate than in GTP, the magnesium ion is seen to be coordinated to caged GTP via the β- and γ-phosphates, as in the case

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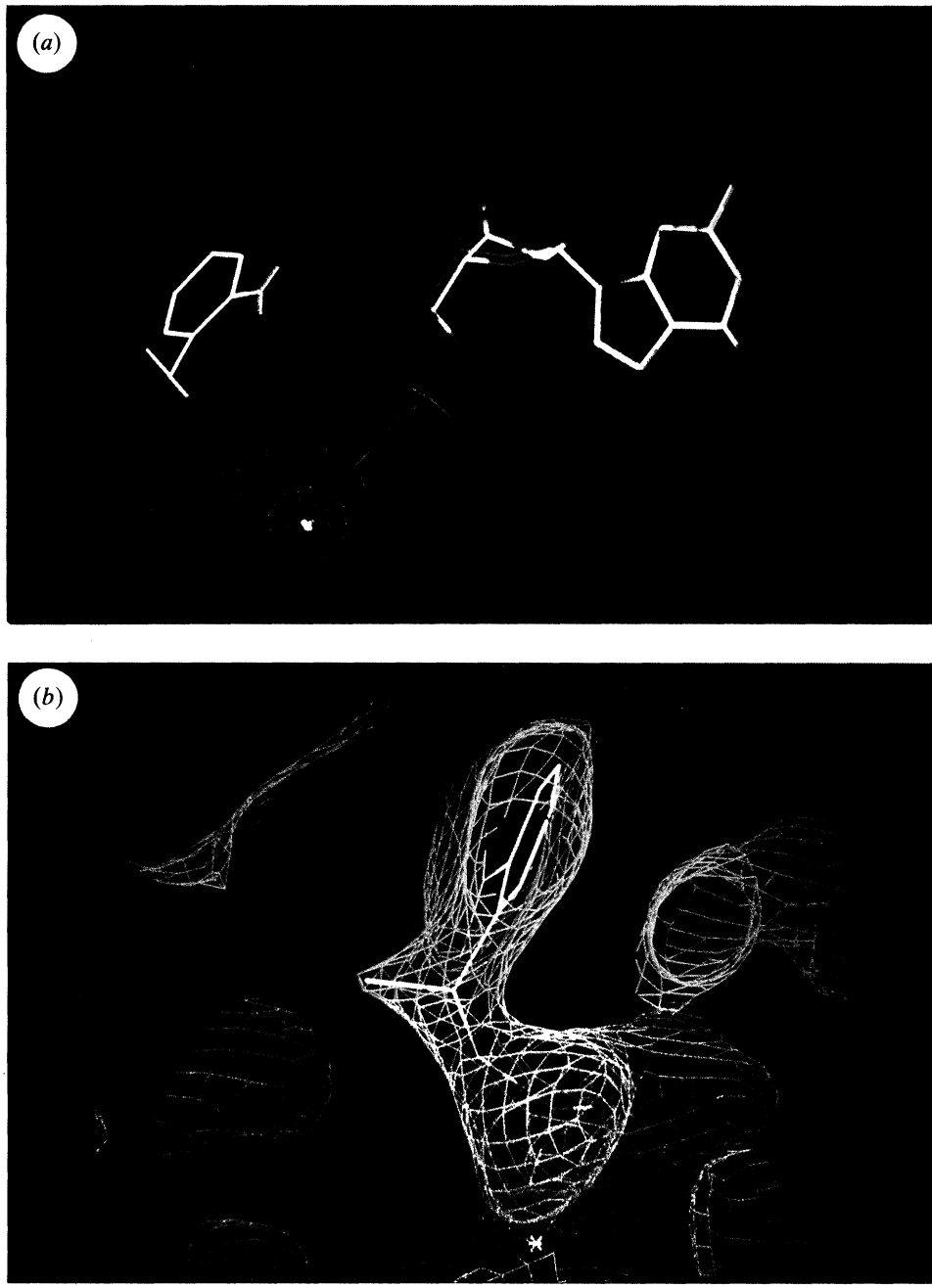

Figure 1. (*a*) Superposition of caged GTP (*R*-diastereoisomer; varied colours) and GppNHp (red; from the p21.GppNHp structure reported by Pai *et al.* (1990)) at the active site of p21. The red crosses represent the positions of water molecules, the green sphere the Mg^{2+} ion. (*b*) The 1-(2-nitrophenyl)ethyl group of caged GTP at the active site of p21, showing that the position of the methyl group, and thus the absolute stereochemistry at the chiral centre, is well defined. The model is shown in varied colours, the electron density in blue.

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Table 2. *Crystallographic data from Ha-ras p21 (wild-type, truncated) crystallized with the R-isomer of* P^3*-1-(2-nitrophenyl)ethyl guanosine triphosphate ('caged-GTP')*

(SA stands for simulated annealing.)

spacegroup	$P3_221$
cell axes/Å	$a = b = 40.4$, $c = 158.8$
resolution range/Å	8.0–1.8
observed reflections	13998
R_{sym}[a] (%)	7.4
cycles of SA refinement	7
amino acids	166
non-hydrogen atoms	1457
protein	1322
nucleotide	43
metal ion	Mg^{II}
water molecules	91
R-factor[b] (%) with water	21.3
without water	24.1
RMS-deviation from ideality of	
bond lengths/Å	0.018
bond angles/deg	3.561

[a] R_{sym} is defined as $\Sigma_{i,hkl}(|I(i, hkl)| - \langle I(hkl)\rangle)/\Sigma_{i,hkl}\langle I(hkl)\rangle$, where i runs through symmetrically related reflections.

[b] R factor $= R_{cryst} = (\Sigma|F_{obs} - F_{calc}|)/(\Sigma|F_{obs}|)$.

of GppNHp or GTP. The coordination of the magnesium ion to the protein is the same as in the p21-GppNHp structure, i.e. to the side chain hydroxyl groups of Ser 17 and Thr 35, the remaining two positions in the octahedral coordination being occupied by water molecules, which in turn interact with Asp 33 and Asp 57 respectively. The protecting group can be seen to interact via its nitro group with the main chain in the region of the amide bond of Asp 33, which is in the effector loop, and with water 172, a water molecule found in p21-caged-GTP and p21-GppNHp. The quality of the electron density map is good enough to distinguish the stereochemistry at the chiral centre in the protecting group. As shown in figure 1*b*, plate 1, the positions of the proton and the methyl group attached to the asymmetric carbon can be unambiguously assigned, and the configuration is seen to be *R* using the Ingold–Cahn–Prelog nomenclature. This confirms the expected stereochemistry of the caged GTP isomers based on the known stereochemistry of the caged phosphate isomers (Corrie *et al.* 1992) used for the synthesis of caged GTP.

The effector loop in these crystals appears to differ from both p21-GppNHp (or p21-GTP) or p21-GDP, adopting a conformation which is intermediate between the two. This is particularly clear in the case of Tyr 32. This residue undergoes the largest change in the GTP/GDP transition seen in crystals, but it is not clear whether this large change may be partly due to interactions between neighbouring molecules in the crystal. In the p21-GppNHp and p21-GTP structures, the hydroxyl group of Tyr 32 is seen to interact with the γ-phosphate of the nucleotide at the active site of a neighbouring p21 molecule. In p21-*R*-caged-GTP, the hydroxyl group of Tyr 32 interacts with the corresponding group of Tyr 40, at the C-terminal end of the effector binding loop. In the p21-GppCH$_2$p structure, which crystallizes with a different mode of packing, the Tyr 32 hydroxyl group interacts with the γ-phosphate of the nucleotide at the active site of the same p21 molecule (Milburn *et al.* 1990).

Since it seems unlikely that all the differences in the effector loop seen between

p21-GppNHp and p21-caged-GTP are due solely to flexibility, and since the structure of this region, according to currently accepted theories, depends on the state of the nucleotide at the active site, it is of interest to examine the interactions between the phosphate residues of caged GTP and the active site of p21 in detail in order to identify a causal relationship. This examination reveals that, apart from differences in the presence and position of certain water molecules, all of the interactions seen between the protein and the phosphate groups in the p21-GppNHp structure are also seen in the p21-caged-GTP structure, with one exception. This concerns the interaction of Thr 35 with the γ-phosphate group. In the p21-GppNHp structure, three interactions connect this residue, which is in the effector loop, to the active centre of the protein. These are a direct coordination of the side chain hydroxyl group with the Mg^{2+} ion and interactions of this same hydroxyl group (via the proton) and the main chain NH with one oxygen of the γ-phosphate group. While the magnesium coordination is preserved in the p21-*R*-caged-GTP complex, the interaction of the proton of the hydroxyl group with the γ-phosphate appears to be weaker, and the main chain NH is now too far away from the γ-phosphate to interact. One result of this is that the sidechain of Thr 35 in the caged (*R*)-GTP structure has a significantly higher flexibility. The *B* factor of the sidechain is about 60 Å^2 whereas in the GppNHp structure the Thr 35 sidechain is very well defined (*B*-factor between 10 and 20 Å^2). We have speculated (Schlichting *et al.* 1990 and below) that the loss of all three of these interactions is decisive in the transition between the GTP and GDP forms of the effector loop, so that it is reasonable to assume that the loss or weakening of two of the interactions is responsible for the conformation of the effector loop seen in the p21-*R*-caged-GTP, which is neither completely GTP- nor GDP-like. However, since p21-caged-GTP (mixed isomers) can interact with GAP, it seems likely that the correct conformation of this interaction is easily (i.e. energetically) available.

Crystals of p21 with the *S*-isomer of caged GTP diffract X-rays to lower resolution (*ca.* 2.2 Å) than crystals containing the *R*-isomer. Due to their instability, it is more difficult to collect complete data-sets. Using synchrotron radiation (SRS, Daresbury, beamline 9.6), a data-set has now been obtained, but not yet evaluated. Determination of this structure may help to understand the differences seen in the manner in which nucleotide binds to the active site in crystals of p21 with mixed and with single diastereomers of caged GTP, and give some clues to the reason for the difference in stability of the two diastereoisomers in p21 crystals.

5. Laue diffraction experiments

The first work on the p21 system using the Laue diffraction method at a synchrotron source was done at the EMBL facility on the storage ring DORIS at DESY (Deutsches Elektronensynchrotron) in Hamburg. These experiments used crystals of p21 with mixed diastereoisomers of caged GTP as the starting point and led to determination of the structure of the p21-GTP complex *ca.* 4 min after photolytic removal of the protecting group (Schlichting *et al.* 1990). The three phosphate groups of GTP were easily recognized in the electron density map, and it became clear that they were positioned exactly as in the p21-GppNHp complex. A data set collected 14 min after removal of the protecting group led to an electron density map which had lost most, but not all of the density associated with the γ-phosphate group. From the rate of the GTP cleavage reaction at 25° (the ambient

temperature in these experiments), we would expect that much less intensity should be lost at this time. A likely explanation for this discrepancy is that heating of the crystal in the white X-ray beam leads to a faster rate of hydrolysis.

The structure of the p21-GTP complex from data obtained 4 min after photolysis was seen to be very similar to that of p21-GppNHp. Indeed, within the limits imposed by the relatively poor resolution of the p21-GTP electron density map, they appear to be identical, so that it appears to be reasonable to use the p21-GppNHp structure as a model of the p21-GTP state. Thus, although caged GTP appeared to be bound in these crystals in an anomalous fashion, proper interaction with the active site is seen shortly after removing the protecting group. This suggests that structural changes in the protein occurring as a result of correct binding of GTP at the active site are not dramatic.

When crystals of p21 with mixed diastereoisomers of caged GTP were photolysed and then allowed to stand overnight, crystals of p21-GDP were obtained (Schlichting *et al.* 1990). Their structure was solved from data obtained using monochromatic radiation to a resolution of 2.6 Å. Complete loss of density at the position of the γ-phosphate group was apparent, and the Mg^{2+} ion was now bound only to the β-phosphate group of the nucleotide. In addition to this, Thr 35 was no longer coordinated to the Mg^{2+} ion, and together with the loss of the interactions of the side chain and the main chain NH of this residue with the γ-phosphate group, this appears to be of importance in a major change of structure of the effector loop, Encompassing in particular Tyr 32, Asp 33, Pro 34, Thr 35 and Ile 36. We interpret the results to indicate that the effector loop in the GTP state is held in an intrinsically unstable configuration, mainly by the interactions of Thr 35 with the γ-phosphate and the Mg^{2+} ion. After these interactions are lost on loss of the γ-phosphate group, the loop adopts its most stable configuration, and this is not able to interact with GAP.

We are presently attempting to extend the spatial and temporal resolution of the Laue experiments by using crystals with pure diastereoisomers of caged GTP and using a higher intensity Laue station. The first Laue experiments at DESY/HASYLAB (Hamburg, Germany) (1989 and 1990) showed that very few crystals of the 1:1 complex between p21 and caged GTP are good enough for Laue diffraction. Most of the crystals had a very high mosaic spread or showed split reflections. As a result, data were collected only at two time points after initiation of reaction. In more recent work, we have used crystals of p21 containing pure diastereoisomers of caged GTP. During Laue experiments on beamline 9.5 of the SRS at Daresbury, U.K., 28 crystals were tested. Only one crystal with wild-type p21 but nine of the mutant p21 in which glycine at position 12 has been replaced by proline (G12P) were of high enough quality to give good Laue diffraction patterns. This mutant is a good substitute for wild-type p21 in this type of experiment, since neither the overall structure nor the intrinsic GTPase reaction are affected and G12P is the only mutation of Gly 12 which does not lead to (oncogenic) transforming properties. The statistics of the crystals tested are shown in table 3.

After initiations of the GTPase reaction in the crystals, diffraction patterns at 10 different time intervals were collected. The data-set at each time step consists of four exposures at four different orientations of the crystal. Each exposure was 0.5 to 1 s long. Only two data-sets per crystal could be collected due to radiation damage, which became severe after *ca.* 10 s exposure time.

Experiments with different crystal sizes, temperature changes and increasing DTE

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Table 3. *Crystal statistics*

p21	number of good crystals (number of tested crystals) *R*/*S*-mixture	*R*-isomer	*S*-isomer
wild-type	2 (> 100)	1 (11)	0 (5)
G12P-mutant	not used	5 (5)	4 (8)

Table 4. *Data statistics*

time/min	*R*-merge[a]	reflections total	reflections unique	*d*/Å	completeness of possible (%)
2–4	7.2/7.6/7.8	3817	2193	2.8	50[b]
14–16	5.6/7.2/7.4	4338	2322	2.8	50[b]
2–4	still in refinement	—	—	—	—
2–4	still in refinement	—	—	—	—
11–13	6.7/6.7/7.9/9.5	8642	2413	2.8	52[c]
30–32	8.4/9.0/9.9/10.3	8254	2700	2.8	55[c]
55–57	7.5/10.2/10.7	5777	2024	2.8	48[c]
90–92	still in refinement	—	—	—	—

[a] *R*-merge defined as $R = \Sigma\{\mathrm{abs}[I(i) - I(j)]\}/\Sigma[I(i) + I(j)]$.
[b] DESY/HASYLAB (Hamburg, F.R.G.).
[c] SRS (Daresbury, U.K.).

(dithioerythritol) concentration in a flow cell were done to determine optimal conditions for the experiments. Several points emerged as being of importance for the design of the time-resolved Laue experiments. For short exposure times and the possibility of translating the crystal before each exposure, it is convenient to use large crystals. However, we observed that with increasing crystal size, the probability of obtaining Laue diffraction patterns with split reflections increased. The best results were obtained with a crystal size of 400–500 μm.

To protect the crystals against warming during photolysis, we tried cooling the crystals and keeping them at 4 °C. Cooling the crystals caused an increase in mosaic spread. Interestingly, a relaxation of the lattice seems to occur after incubation of the crystal at the lower temperature for periods of *ca.* 30 min, so that after this period the original quality of diffraction pattern returned. However, since no damage was seen after photolysis at room temperature, cooling was not used.

High DTE concentrations in the crystal are necessary to protect the protein against damaging effects caused by the nitrosoamine-group of the leaving group of caged GTP. In a flow-cell experiment it could be shown that a change of the DTE concentration from 5 mM to 100 mM over 2 min does not affect the crystal quality.

For the data processing and refinement only data sets were used which were obtained from crystals having a residual content of non-photolysed caged GTP of less than 20% (determined by HPLC). The results of the Laue experiments are shown in table 4. Data processing is not yet complete.

6. Conclusions

Time resolved crystallography on H-ras p21 has led to determination of the structure of the p21-GTP complex, i.e. of the enzyme–substrate complex in enzymatically active crystals without resorting to artificial (and possibly artefact-

inducing) methods of slowing down the normal enzymatic reaction (e.g. use of low temperatures, of substrate analogues or of slow mutants of the protein). This has allowed characterization of the structural change occurring on GTP hydrolysis in this protein. While several features of the protein and the way in which it interacts with and cleaves GTP were favourable factors leading to the achievement of this aim, it seems that the principles developed may also be applicable to other systems. Extension of the methods to the observation of processes which are faster than the GTPase rate of p21 is limited at present more by the problems of reaction initiation than by X-ray intensity, especially with the forthcoming availability of the next generation of dedicated synchrotron light sources.

References

Corrie, J., Reid, G. R., Trentham, D. R., Hursthouse, M. B. & Mazid, M. 1992 Synthesis and absolute stereochemistry of the two diastereomers. of P^3-1-(2-nitrophenyl)ethyl adenosine triphosphate ('caged' ATP). *J. chem. Soc. Perkin Trans.* I **8**, 1015–1018.

Feuerstein, J. F., Goody, R. S. & Webb, M. R. 1989 The mechanism of guanosine nucleotide hydrolysis by p21 c-Ha-ras: the stereochemical course of the GTPase reaction. *J. biol. Chem.* **264**, 6188–6190.

Frech, M., Wittinghofer, A. & Goody, R. S. 1991 The affinity of guanosine binding proteins for GDP and GTP: Facts and artefacts. *TIBS* **16**, 327–328.

Goody, R. S., Schlichting, I. & Pai, E. F. 1990 Eine neue Dimension in der Proteinkristallographie. *Nachr. Chem. Tech. Lab.* **38**, 842–850.

Gideon, P., John, J., Frech, M., Lautwein, A., Clark, R., Scheffler, J. E. & Wittinghofer, A. 1992 Mutational and kinetic analysis of the GTPase-activating protein (GAP)-p21 interaction: The C-terminal domain of GAP is not sufficient for full activity. *Molec. Cell Biol.* **12**, 2050–2056.

John, J., Sohmen, R., Feuerstein, J., Wittinghofer, A. & Goody, R. S. 1990 Kinetics of interaction of GDP and GTP with H-ras p21 proteins. *Biochem.* **29**, 6058–6065.

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

Pai, E. F., Krengel, U., Petsko, G. A., Goody, R. S., Kabsch, W. & Wittinghofer, A. 1990 Refined crystals 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.

Reinstein, J., Schlichting, I., Frech, M., Goody, R. S. & Wittinghofer, A. 1991 p21 with a Phe28-Leu mutation has transforming properties although its GTPase can be activated normally by GAP. *J. biol. Chem.* **266**, 17 700–17 706.

Scherer, A., John, J., Linke, R., Goody, R. S., Wittinghofer, A., Pai, E. F. & Holmes, K. C. 1989 Crystallisation and preliminary X-ray analysis of the c-H-*ras*-oncogene product p21 complexed with GTP-analogues. *J. molec. Biol.* **206**, 257–259.

Schlichting, I., Almo, S. C., Rapp, G., Wilson, K., Petratos, K., Lentfer, A., Wittinghofer, A., Kabsch, W., Pai, E. F., Petsko, G. A. & Goody, R. S. 1990 Time resolved X-ray structural studies on H-ras p21: Identification of the conformational change induced by GTP hydrolysis. *Nature, Lond.* **345**, 309–315.

Schlichting, I., John, J., Rapp, C., Wittinghofer, A., Pai, E. F. & Goody, R. S. 1989 Biochemical and crystallographic characterisation of a complex of p21 and caged GTP using flash photolysis. *Proc. natn. Acad. Sci. U.S.A.* **86**, 7687–7690.

Discussion

A. Liljas (*University of Lund, Sweden*). You have described two of the conformations of p21, the GTP and GDP conformations. Do you have any information about the conformation that is induced by GAP, the GTPase activating enzyme?

E. F. Pai. GAP specifically recognizes the GTP conformation of p21. As soon as the guanine nucleotide has been converted to the diphosphate form, the p21-GAP complex dissociates. At present, we do not have any structural information on the GAP induced conformation. Our time-resolved work dealt only with the intrinsic GTPase activity.

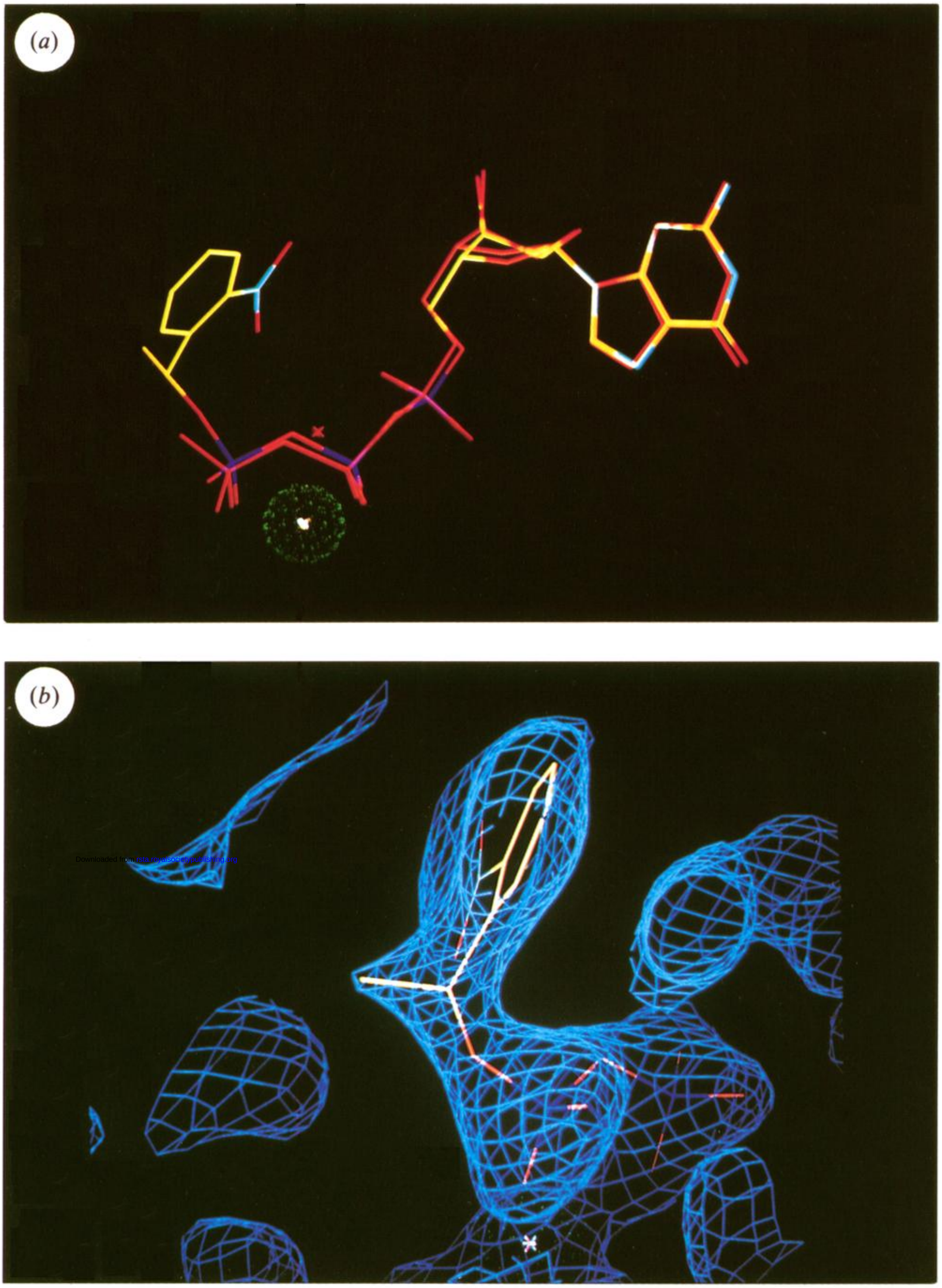
K. Moffat (*University of Chicago, U.S.A.*). Several of your difference Fouriers used Laue data. Their quality was good, substantially better than those shown by Professor Johnson. What factors might account for this?

E. F. Pai. The electron densities we showed were not difference Fouriers. One of the reasons why we did not use this method was the change in length of the *c* axis of the crystal. Instead we used the GppNHp as well as the GDP-structure as starting models for X-PLOR runs, with both runs converging on the same structure. The pictures you have just seen were then obtained from 'omit' maps based on this result.

Further, the molecular weight of the truncated forms of p21 is 18 kD, that of phosphorylase is 97 kD. One would therefore expect the differences in the intensities of the reflections to be considerably larger when the changes in the GTP-p21 complex are monitored. We were also able to obtain at least 50% of a complete data-set for each time-point.

Colour plate printed by G. Over Ltd, Rugby and London.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES



igure 1. (*a*) Superposition of caged GTP (*R*-diastereoisomer; varied colours) and GppNHp ed; from the p21.GppNHp structure reported by Pai *et al.* (1990)) at the active site of p21. The d crosses represent the positions of water molecules, the green sphere the Mg^{2+} ion. (*b*) The (2-nitrophenyl)ethyl group of caged GTP at the active site of p21, showing that the position of the ethyl group, and thus the absolute stereochemistry at the chiral centre, is well defined. The model shown in varied colours, the electron density in blue.