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Crystallization and Preliminary X-ray Structure Analysis of Thermally Unstable p21$^{H\text{-}ras}$ Guanosine Complexes

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Abstract

p21 is a small guanine nucleotide binding protein that is involved in intracellular signal transduction. Biochemical data suggest that the presence of the β-phosphate is essential for strong binding of guanine nucleotides to the protein. Guanosine or GMP bind six orders of magnitude more weakly to p21 than GDP or GTP. Moreover, the thermal stability of the protein is dramatically reduced when bound to GMP or guanosine. We have crystallized C-terminally truncated forms of p21$^{H\text{-}ras}$, with guanosine or GMP bound, in the space groups $P4_32_12$, $P2_12_12$ and $P2_1$. The crystals diffract in the range 2.8–2.2 Å. Details of the crystallization procedures, the characterization of the crystals and preliminary results of structure determination are described. An unexpected electron-density peak was found close to the position of the β-phosphate in the phosphate-binding loop.

Abbreviations

[p21c(1–166)], C-terminally truncated form of the human c-Ha-*ras* proto-oncogene product, consisting of amino-acid residues 1–166. Gua, guanosine. GMPS, guanosine 5′-phosphorothioate. GPPCP, guanosine 5′-(β,γ-methylenetriphosphate). GPPNP, guanosine 5′-(β,γ-imidotriphosphate). DTE, dithioerythritol. PEG, polyethylene glycol. P_i, inorganic phosphate.

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Introduction

p21$^{H\text{-}ras}$ is a now well studied protein (M_r = 21 000) which is believed to play a crucial role in intracellular signal transduction (McCormick, 1989) and in mammalian carcinogenesis as well, since mutant forms of the protein have been found in a great number of human tumours (Reddy, Reynold, Santos & Barbacid, 1982; Bos, 1989).

p21 binds GTP/GDP with high affinity and has a very low intrinsic GTPase activity that is accelerated by GTPase-activating proteins such as GAP (Trahey & McCormick, 1987; Vogel *et al.*, 1988; McCormick, 1989) or the neurofibromin gene product NF1 (Xu *et al.*, 1990). Biochemical and biological properties of p21 were reviewed by Barbacid (1987) and Grand & Owen (1991).

Crystal structures of the catalytic domain of p21$^{H\text{-}ras}$ complexed with GDP, non-hydrolyzable analogs of GTP and even with GTP itself are now available at medium to high resolution (Pai *et al.*, 1989, 1990; Brünger *et al.*, 1990; Schlichting *et al.*, 1990).

p21 also binds guanosine and GMP (John *et al.*, 1990) but no detailed structural information is available for the protein complexed with nucleotides lacking the β-phosphate. In this work we report the crystallization and preliminary results of X-ray structure analysis of p21$^{H\text{-}ras}$ in the presence of guanosine-like nucleotides. In the following text p21$^{H\text{-}ras}$ is denoted as 'p21'.

The motivation for our crystallization studies was threefold: firstly, p21 binds GMP/Gua six orders of

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magnitude more weakly than it binds its natural substrates GDP/GTP (John *et al.*, 1990); the affinity is $10^5\ M^{-1}$ for GMP as compared to $10^{11}\ M^{-1}$ for GDP. The binding strength of GMP/Gua is comparable to that of ATP (Rensland, 1992); this could be interpreted to mean that interactions of the β-phosphate with the protein create a high-affinity binding site for the guanine base.

Comparing the corresponding crystal structures might provide a structural explanation for the large differences in affinity between GDP/GTP and GMP/Gua and give some insight into the nucleotide-binding mechanism.

Secondly, although guanosine has stabilizing effects on the protein, p21:Gua is still thermally unstable (John *et al.*, 1990). At room temperature it precipitates within a few hours, whereas p21:GDP can be stored for several weeks without losing significant amounts of GDP-binding activity. X-ray structure analysis could reveal elements important for protein stabilization.

A third aspect is the investigation of the intrinsic GTPase mechanism. p21 is one of the rare examples where time-resolved Laue studies have been successful (Schlichting *et al.*, 1990). The catalytic domain of $p21^{H\text{-}ras}$ [p21c(1–166)] was crystallized as a 'caged GTP' complex (Schlichting *et al.*, 1989). After removal of the photolabile cage group by flash photolysis, GTP is hydrolyzed in the crystal. The corresponding idea with p21:Gua was to flow substrate (GTP) into crystals, which should displace guanosine from the active site since it binds to it much more strongly. Thus, diffusion could be used as another trigger for time-resolved crystallographic studies on the intrinsic GTPase mechanism of p21.

The basic questions of our studies were: What happens to the crystal structure if p21 has a bound nucleotide without a β-phosphate? Do the crystals tolerate the addition of GTP?

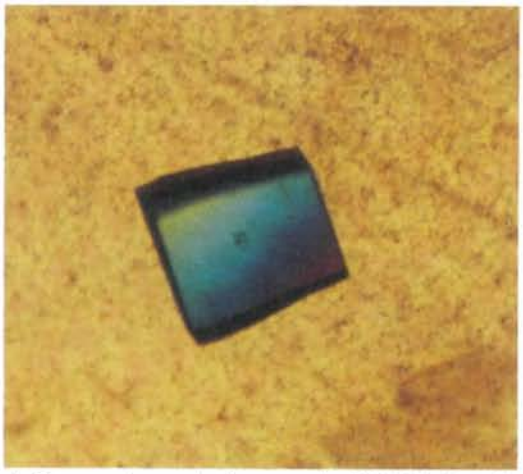

Fig. 1. Tetragonal crystal of truncated p21(Q61L):Gua obtained by microseeding with spherulites crushed with a needle.

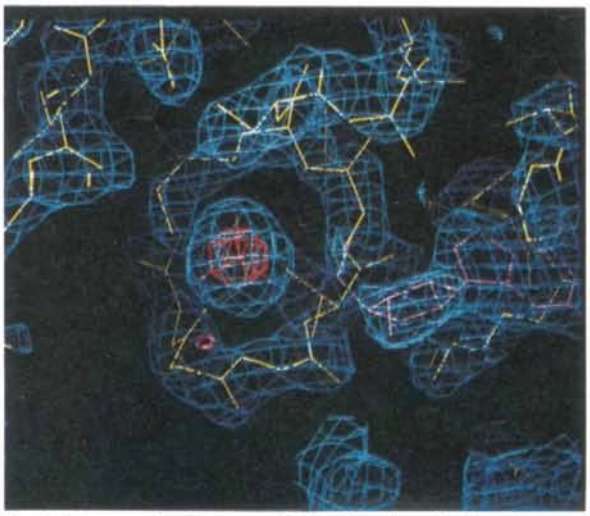

Fig. 2. Electron-density map of the phosphate-binding loop and bound nucleoside (30% of the maximum value, shown in light blue) in the p21(Q61L):Gua structure (after two cycles of refinement) with an intense spherical density peak (70% of the maximum value of the electron density, shown in red) close to the position of the β-phosphate (see Fig. 3). The map and structure representation were produced with the graphics program *FRODO* (Jones, 1978) modified for an IRIS 4GT (Silicon Graphics Inc., Mountain View, California) by C. M. Cambillau.

Protein purification, crystallization and crystal characterization

All crystallization experiments were performed with C-terminally truncated forms of p21 (residues 1 to 166, $M_r \simeq 19\,000$) (John, Schlichting, Schiltz, Rösch

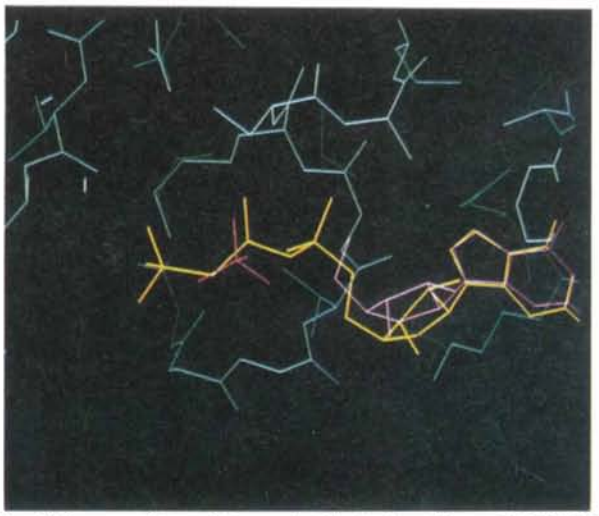

Fig. 3. Superposition of the nucleotide (yellow) from p21:GPPNP on the p21(Q61L):Gua structure (protein in light blue, guanosine in light red) showing the position of the putative phosphate or sulfate ion (light red).

& Wittinghofer, 1989). It must be emphasized that the C-terminal truncation by 23 amino acids which are highly variable among proteins of the *ras* family was a breakthrough critical to all our X-ray crystallographic studies on p21 up until now.

Purification of C-terminally truncated p21 that was overexpressed in *E. coli* followed the scheme described by Tucker *et al.* (1986). The purified protein has GDP bound.

The p21:Gua complexes were prepared essentially as described by John *et al.* (1990). Briefly, GDP was removed by alkaline phosphatase from calf intestine (Boehringer) in the presence of ammonium sulfate and GPPCP, which both accelerate the release of GDP from the protein. After degradation of GDP to guanosine (monitored by HPLC) GPPCP was removed by adding phosphodiesterase from snake venom (Boehringer) to the reaction mixture. The buffer was changed to 64 m*M* Tris–HCl pH 7.6, 10 m*M* $MgCl_2$, 5 m*M* DTE, 1 m*M* guanosine using a G25 PD10-column (Pharmacia). For a preparation of p21 with GMPS a similar procedure was used except that the protein was eluted in a buffer containing 3 m*M* GMPS. After concentration to about 20 mg ml^{-1} by Centricon 10 microconcentrators (Amicon), the protein was used for crystallization trials.

Since initial attempts with truncated wild-type protein were not promising, several mutant forms of p21 that had already been crystallized in the triphosphate form were tested; all mutations were in regions interacting with the nucleotide or in the effector loop (residues 32–40). The overall similarity of their structures (Krengel *et al.*, 1990) suggested that any of them could be suitable for studying the binding of guanosine in a crystal structure.

Of the 12 mutant forms of p21 tested, the substitutions G12P (position 12 glycine replaced by proline) and Q61L (position 61 glutamine replaced by leucine) yielded the best crystallization results.

Three different crystal forms were obtained depending on the mutant protein and nucleotide present in the crystallization setups.

The overall crystallization conditions were similar to those described for the p21:GPPNP complex (Scherer *et al.*, 1989) [12–25% PEG 400 (4000), Tris–HCl pH 7.6, 10 m*M* $MgCl_2$, 5 m*M* DTE].

Crystals useful for X-ray diffraction studies could only be obtained using the batch technique in chambers of 14 mm in diameter and 4 mm in depth sealed with transparent tape; the drop size was 10 μl (5 μl protein + 5 μl precipitant). Hanging-drop and sitting-drop methods were also tried but did not give satisfactory results.

Crystals were washed with artificial mother liquor, dissolved in buffer, and analyzed by SDS–PAGE and HPLC as described by Scherer *et al.* (1989) to prove that the crystals contained p21 and nucleotide or nucleoside.

To characterize the unit-cell contents further, the density of the crystals was determined in a 10 ml CCl_4/Xylol gradient calibrated with CsCl solutions of known density.

Crystals were mounted in thin-walled glass or quartz capillaries. Data sets were collected using an oscillation camera with a Siemens/Nicolet area detector. The X-ray source (Cu $K\alpha$) was a GX-18 rotating anode (Elliott/Enraf–Nonius, Delft) with Franks double-mirror optics for focusing. Space-group determination (confirmed by precession photography) and data processing were carried out with an extended version of the program *XDS* (Kabsch, 1993).

Crystallization results

Tetragonal crystals of p21(Q61L):Gua

Tetragonal crystals grew from 15% PEG 3350 (Sigma) in Tris–HCl buffer as described above. 5–10% glycerol was added. The setups had to be stored at 281–283 K. At room temperature few crystals appeared in a bulk mass of precipitated protein. Storage at 274–277 K resulted in a few small spherulite-like structures which could not be used for improvement procedures such as seeding. At 281–283 K mainly spherulites were obtained, which were used for microseeding procedures (Stura & Wilson, 1990) yielding good single crystals that grew to final sizes of 500 × 800 × 500 μm within a few days to several weeks (Fig. 1).

The crystals belong to space group $P4_32_12$ (the enantiomorph was determined by molecular replacement, see below) with cell dimensions $a = b = 37.05$, $c = 244.78$ Å. Assuming one molecule of p21 in the asymmetric unit, the Matthews parameter is $V_M = 2.21$ Å^3 Da^{-1}, consistent with density determination of $\rho = 1.25$ g cm^{-3}. The crystals diffract to 2.7 Å.

Orthorhombic crystals of p21(G12P):Gua

Orthorombic crystals grew from 15% PEG 6000 in the usual Tris–HCl buffer. As for p21(Q61L), 5–10% glycerol were added. Crystals suitable for X-ray diffraction could be obtained only if the setups were started at room temperature and then moved to 277–283 K. If the crystallization trials were carried out entirely at low temperature, only hundreds of very small crystals appeared in the drops. Thus, a temperature gradient seems to play an important part in the improvement of crystallization conditions. The crystals appeared after about 12 h and grew to a final size of 600 × 200 × 200 μm within 3 d.

Table 1. *Summary of crystallographic characterization*

[p21(1–166)]	Q61L:Gua	G12P:Gua	G12P:GMPS*
Size (μm)	500 × 500 × 500	600 × 200 × 200	500 × 200 × 100
Space group	$P4_32_12$	$P2_12_12$	$P2_1$
Cell parameters (Å, °)			
a	37.05	86.6	43.6
b	37.05	154.7	96.9
c	244.78	38.4	59.7
α	90	90	90
β	90	90	111.4
γ	90	90	90
ρ (g cm^{-3})	1.25	1.23	—
V_M (Å^3 Da^{-1})	2.3	2.3	2.1
Z (p21 au^{-1})†	1	3	3
Resolution (Å)	2.3	2.2	2.7

* Nucleotide content not confirmed yet, see text.
† Asymmetric unit.

The space group is $P2_12_12$ with cell dimensions $a = 86.6$, $b = 154.7$, $c = 38.4$ Å. According to self-rotation studies the asymmetric unit contains three molecules of p21 ($V_M = 2.3$ Å^3 Da^{-1}) which is consistent with calculations from density measurements ($\rho = 1.23$ g cm^{-3}). The crystals diffract to 2.2 Å.

Monoclinic crystals of p21(G12P) grown in the presence of GMPS

At the beginning of our experiments we started with GMP-like nucleotides complexed to p21 because of their higher solubility. GMP itself could not be used since it is immediately degraded to guanosine by the alkaline phosphatase in the preparation. In the presence of phosphodiesterase GMPS reacts to give a substance of the same retention time on HPLC as GMP. After some days to several weeks most crystallization drops contained only guanosine. In most cases only multi-crystalline crystals were obtained with this preparation of p21.

However, one series of crystallization trials yielded crystals good enough for X-ray structure analysis (600 × 200 × 100 μm). These crystals could not be reproduced. They were obtained from 15% PEG 8000 in the usual buffer. The crystallization conditions differ significantly from those for the crystal forms described above in that neither glycerol was added nor were the setups stored at low temperature.

The crystals with unit-cell parameters $a = 43.6$, $b = 96.9$, $c = 59.7$ Å, $\beta = 111.4°$ belong to space group $P2_1$; V_M is 2.1 Å^3 Da^{-1} assuming three molecules of p21 in the asymmetric unit. The maximum resolution is 2.6 Å.

A summary of the characterization of the crystals is given in Table 1.

Soaking of crystals with GTP/GDP

Preliminary soaking experiments were carried out with the orthorombic and tetragonal crystal forms by adding artificial mother liquor containing 0.1–1 m*M* GTP or GDP to the drops. The substrate solution was added in small amounts to the rim of the drops to achieve a smooth concentration gradient over the crystal. However, the crystals cracked in the presence of substrate and most of them dissolved within one day. Many crystals were very sensitive to the addition of artificial mother liquor in general; the presence of substrate enhanced this effect dramatically.

At the beginning of our studies there was evidence for a rate-limiting step preceeding GTP hydrolysis with a half-life accessible to Laue crystallography and other fast data-collection techniques (Neal, Eccleston & Webb, 1990). We had planned to characterize this state structurally. However, according to recent studies there is no indication for a stable intermediate during the processes involved in GTP hydrolysis (Rensland, Lautwein, Wittinghofer & Goody, 1991). Therefore, we are presently making no further efforts to stabilize the crystals in the presence of substrate.

Discussion of crystallization results

Our crystallization experiments confirm that p21 complexes with guanosine-like nucleotides are labile proteins which must be treated with great care to obtain crystals suitable for X-ray diffraction studies. Usually stabilization by glycerol was necessary. Also, temperature (gradients) were found to be critical parameters for growing good crystals reproducibly. These two features are not observed for the crystallization conditions of the catalytic domain of p21 described previously (Jancarik *et al.*, 1988; Scherer *et al.*, 1989; Schlichting *et al.*, 1989). At room temperature and without glycerol only the monoclinic crystal form of the p21(G12P) grew to reasonable shape and size. In this case the crystallization setups contained – at least initially – GMPS. X-ray structure analysis should reveal if GMPS is indeed bound in the crystal. If this is not the case there may be further stabilizing factors in the sense of an additional crystallization agent.

Catalytic domains of p21 have been crystallized in several laboratories in different forms with space groups $P2_1$ ($a = 42$, $b = 79.9$, $c \simeq 130$ Å, $\beta = 117.5°$), $P6_522$ ($a = b \simeq 83$, $c \simeq 105$ Å), $I4$ ($a = b = 97.8$, $c = 130.5$ Å) (Brünger *et al.*, 1990; Jancarik *et al.*, 1988; Tong *et al.*, 1989; Privé *et al.*, 1992) for the domain comprising residues 1 to 171 and $I4$ in the case of the full sequence. The crystals of the domain with residues 1 to 166 belong to space groups $P3_221$ ($a = b \simeq 40$, $c \simeq 160$ Å; $a = b \simeq 83$, $c \simeq 55$ Å), $P2_1$ ($a = 36.8$, $b = 35.5$, $c = 58.1$ Å, $\beta = 107°$), $C2$ ($a = 69.9$, $b = 39.8$, $c = 56.1$ Å, $\beta = 107.4°$) (Scherer *et al.*, 1989; Krengel *et al.*, 1990; Franken *et al.*, 1993; A. Sanchez,

unpublished work; A. Scheidig, unpublished work). With this work three more crystal forms with space-group symmetries $P2_1$, $P2_12_12$ and $P4_32_12$ are added to this repertoire. With this diversity in the crystal forms there should be a reasonable foundation to account for the influence of packing interactions – especially if there is more than one molecule in the asymmetric unit – on the crystal structures and thus obtain a model of p21 closer to that in solution.

Preliminary results of structure determination

The stucture of p21(Q61L):Gua in the tetragonal crystals was solved by molecular replacement using the highly refined coordinates of p21:GPPNP (Pai *et al.*, 1990) without nucleotide. A rotation search along with PC refinement (Brünger, 1990) was carried out with the program *X-PLOR* (Brünger, Kuriyan & Karplus, 1987). For translation searches the programs *X-PLOR* and *BRUTE* (Fujinaga & Read, 1987) were used.

As expected, the overall structure is similar to the starting model. The refinement of the structure is underway. The current *R* factor is 24%. Details of structure determination and analysis will be presented elsewhere.

In the crystal structure guanosine is bound similarly to the corresponding part of GPPNP.

The first electron-density map calculated with the unrefined model after molecular replacement showed a strong spherical peak close to the position of the β-phosphate. This peak was confirmed in all density maps calculated during the subsequent refinement (Figs. 2 and 3).

At the moment we cannot assign this density peak unambigously. It could be inorganic phosphate, which was not added to the preparation buffer but is a product of the enzymatic degradation of GDP and GPPCP and was present in at least threefold excess over p21 in the first stages of complex preparation; it could also be a sulfate ion from ammonium sulfate, which was added to increase the release rate of GDP from p21. Experiments are now underway to determine the affinity of p21 to P_i and SO_4^{2-}. The results should help us in interpreting the density peak in the phosphate-binding loop.

The situation is reminiscent of the crystal structures of adenylate kinase (Dreusicke & Schulz, 1986), glycogen phosphorylase (Barford, Hu & Johnson, 1991) or myosin S1 (Rayment *et al.*, 1993). In adenylate kinase the phosphate-binding loop forms a 'giant anion hole' in which a sulfate ion from the crystallization precipitant is bound in the absence of nucleotide (Dreusicke & Schulz, 1986). In the recently published myosin subfragment-1 structure, solved from crystals grown from ammonium sulfate, a sulfate ion also binds to the phosphate-binding loop. Sulfate is a weak allosteric activator of glycogen phosphorylase. In the crystals of glycogen phosphorylase b grown from ammonium sulfate it is found in positions where phosphate is usually bound.

The situation with the p21:Gua complexes is different insofar as neither phosphate nor sulfate was added to the crystallization buffer; thus if indeed P_i or SO_4^{2-} is present in the position normally occupied by the β-phosphate it must have been bound tightly enough to be carried through the gel-filtration step in the preparation of p21:guanosine.

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References

BARBACID, M. (1987). *Annu. Rev. Biochem.* **56**, 779–827.
BARFORD, D., HU, S.-H. & JOHNSON, L. N. (1991). *J. Mol. Biol.* **218**, 233–260.
BOS, J. L. (1989). *Cancer Res.* **49**, 4682–4689.
BRÜNGER, A. T. (1990). *Acta Cryst.* A**46**, 46–57.
BRÜNGER, A. T., KURIYAN, J. & KARPLUS, M. (1987). *Science*, **235**, 458–460.
BRÜNGER, A. T., MILBURN, M. V., TONG, L., DE VOS, A. M., JANCARIK, J., YAMIZUMI, Z., NISHIMURA, S., OHTSUKA, E. & KIM, S.-H. (1990). *Proc. Natl Acad. Sci. USA*, **87**, 4849–4853.
DREUSICKE, D. & SCHULZ, G. E. (1986). *FEBS Lett.* **208**, 301–304.
FRANKEN, S., SCHEIDIG, A. J., KREUGEL, U., RENSLAND, H., LAUTWEIN, A., GEYER, M., SCHEFFZEK, K., GOODY, R. S., KALBITZER, H.-R. & WITTINGHOFER, A. (1993). *Biochemistry* **32**, 8411–8420.
FUJINAGA, M. & READ, R. (1987). *J. Appl. Cryst.* **20**, 517–521.
GRAND, R. J. A. & OWEN, D. (1991). *Biochem. J.* **279**, 609–631.
JANCARIK, J., DE VOS, A. M., KIM, S.-H., MIURA, K., OHTSUKA, E., NOGUCHI, S. & NISHIMURA, S. (1988). *Mol. Cell. Biol.* **200**, 205–207.
JOHN, J., SCHLICHTING, I., SCHILTZ, E., RÖSCH, P. & WITTINGHOFER, A. (1989). *J. Biol. Chem.* **264**(22), 13086–13092.
JOHN, J., SOHMEN, R., FEUERSTEIN, J., LINKE, R., WITTINGHOFER, A. & GOODY, R. S. (1990). *Biochemistry*, **29**, 6058–6065.
JONES, T. A. (1978). *J. Appl. Cryst.* **11**, 268–272.
KABSCH, W. (1993). *J. Appl. Cryst.* **26**, 795–800.
KRENGEL, U., SCHLICHTING, I., SCHERER, A., SCHUMANN, R., FRECH, M., JOHN, J., KABSCH, W., PAI, E. F. & WITTINGHOFER, A. (1990). *Cell*, **62**, 539–548.
MCCORMICK, F. (1989). *Cell*, **56**, 5–8.
NEAL, S. E., ECCLESTON, J. F. & WEBB, M. (1990). *Proc. Natl Acad. Sci. USA*, **87**, 3562–3565.
PAI, E. F., KABSCH, W., KRENGEL, U., HOLMES, K. C., JOHN, J. & WITTINGHOFER, A. (1989). *Nature (London)*, **341**, 209–214.
PAI, E. F., KRENGEL, U., PETSKO, G. A., GOODY, R. S., KABSCH, W. & WITTINGHOFER, A. (1990). *EMBO J.* **9**, 2351–2359.
PRIVÉ, G. G., MILBURN, M. V., TONG, L., DE VOS, A. M., YAMAIZUMI, Z., NISHIMURA, S. & KIM, S.-H. (1992). *Proc. Natl Acad. Sci. USA*, **89**, 3649–3652.
RAYMENT, I., RYPNIEWSKI, W. R., SCHMIDT-BÄSE, K., SMITH, R., TOMCHICK, D. R., BENNING, M. M., WINKELMANN, D. A., WESENBERG, G. & HOLDEN, H. M. (1993). *Science*, **261**, 50–58.

REDDY, E. P., REYNOLD, R. K., SANTOS, E. & BARBACID, M. (1982). *Nature* (*London*), **300**, 149–152.

RENSLAND, H. (1992). PhD thesis, Univ. of Heidelberg, Germany.

RENSLAND, H., LAUTWEIN, A., WITTINGHOFER, A. & GOODY, R. S. (1991). *Biochemistry*, **30**, 11181–11185.

SCHERER, A., JOHN, J., LINKE, R., GOODY, R. S., WITTINGHOFER, A., PAI, E. F. & HOLMES, K. C. (1989). *J. Mol. 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). *Nature* (*London*), **345**, 309–315.

SCHLICHTING, I., RAPP, G., JOHN, J., WITTINGHOFER, A., PAI, E. F. & GOODY, R. S. (1989). *Proc. Natl Acad. Sci. USA*, **86**, 7687–7690.

STURA, E. A. & WILSON, I. A. (1990). *Methods: a Companion to Methods in Enzymology*, Vol. 1, pp. 38–39. New York: Academic Press.

TONG, L., DE VOS, A., MILBURN, M. V., JANCARIK, J., NOGUCHI, S., NISHIMURA, S., MIURA, K., OHTSUKA, E. & KIM, S.-H. (1989). *Nature* (*London*), **337**, 90–93.

TRAHEY, M. & MCCORMICK, F. (1987). *Science*, **238**, 542–545.

TUCKER, J., SCZAKIEL, G., FEUERSTEIN, J., JOHN, J., GOODY, R. S. & WITTINGHOFER, A. (1986). *EMBO J.* **5**(6), 1351–1358.

VOGEL, U. S., DIXON, R. A. F., SCHABER, M. D., DIEHL, E. E., MARSHALL, M. S., SCOLNICK, E. M., SIGAL, I. S. & GIBBS, J. B. (1988). *Nature* (*London*), **335**, 90–93.

XU, G., LIN, B., TANAKA, K., DUNN, D., WOOD, D., GESTELAND, R., WHITE, R., WEISS, R. & TAMANO, F. (1990). *Cell*, **63**, 835–841.