Mechanism of GTP Hydrolysis by p21$^{N\text{-}ras}$ Catalyzed by GAP: Studies with a Fluorescent GTP Analogue†

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ABSTRACT: The mechanism of the hydrolysis of GTP by p21$^{N\text{-}ras}$ and its activation by the catalytic domain of p120 GTPase activating protein (GAP) have been studied using a combination of chemical and fluorescence measurements with the fluorescent GTP analogue, 2′(3′)-*O*-(*N*-methylanthraniloyl)GTP (mantGTP). Since the concentration of active p21 is important in these measurements, various assays for both total protein and active p21 were investigated. All assays gave good agreement except the filter binding assay of [^{3}H]-GDP bound to p21, which gave values of 35–40% compared to the other methods. Concentrations of p21 were thus based on the absorbance of the mant-chromophore of the p21·mant-nucleotide complexes. The rate constants of the elementary steps of the p21 intrinsic GTPase activity and the GAP activated activity were similar between GTP and mantGTP. Incubation of a stoichiometric complex of p21·mantGTP results in a biphasic decrease in fluorescence. The second phase occurs with the same rate constant as the cleavage step and is accelerated by GAP. No other steps of the mechanism are affected by GAP. Incubation of a stoichiometric complex of p21·mantGpp[NH]p also results in a biphasic decrease in fluorescence even though cleavage does not occur. This is interpreted that the cleavage step of p21·GTP is preceded by and controlled by an isomerization of the p21·GTP complex. GAP accelerates the rate constant of the second fluorescence phase occurring with p21·mantGpp[NH]p. This result shows that GAP accelerates the proposed isomerization which limits GTP cleavage rather than the cleavage step itself.

The protein products of the N-, K-, and H-*ras* genes, termed p21^{ras}, form part of a family of low molecular mass guanine-nucleotide-binding proteins that have high sequence homology [for reviews, see Barbacid (1987) and Bourne et al. (1990, 1991)]. They bind GTP and GDP with high affinity and have a low intrinsic GTPase activity (Manne et al., 1985). These proteins have been extensively studied, since overexpression or mutations at certain characteristic sites leads to oncogenic transforming properties (Der et al., 1986; Volgestein et al., 1988; Bos, 1989). In common with other GTPases, they appear to exist in a biologically active conformation when bound to GTP and hydrolysis to the GDP complex causes deactivation (McCormick, 1989). Their function is not known although it has generally been assumed that they form part of a signal-transducing pathway responsible for activating a process important for cell growth or differentiation (Bar-sagi & Feranisco, 1985; Birchmeier et al., 1985; Barbacid, 1987; Satoh et al., 1985). The GTPase-activating protein (p120-GAP) accelerates GTP hydrolysis of the normal protein but not of the oncogenic mutants (Trahey & McCormick, 1987). Crystal structures have been obtained for the GTP, Gpp-[NH]p, and GDP bound forms of p21[1] (Milburn et al., 1990; Pai et al., 1990; Schlichting et al., 1990; Tong et al., 1991) and show changes in the effector binding region between the GTP and GDP structures.

To understand the mechanisms by which the active and inactive concentrations of p21 are regulated *in vivo*, we are investigating the elementary steps of the p21 GTPase in solution. We have previously measured most rate constants in the minimal mechanism for the hydrolysis of GTP by p21 using radiolabeled nucleotides (Scheme I where R represents p21) for both the wild-type (Gly 12) and two mutant proteins (Asp 12, Neal et al., 1988; Val 12, Eccleston et al., 1991). Subsequently, we probed for conformational changes in p21 occurring during the GTP hydrolysis cycle using a fluorescence analogue of GTP, 2′(3′)-*O*-(*N*-methylanthraniloyl)GTP (mantGTP) (Neal et al., 1990). MantGTP and mantGDP are good analogues of the parent nucleotide in the p21 GTPase mechanism. The rate constants determined for the mant-nucleotides are all within a factor of 2 of those for the physiological nucleotide.

Scheme I

$$R + GTP \overset{1}{\rightleftharpoons} R{\cdot}GTP \overset{2}{\rightleftharpoons} R{\cdot}GDP{\cdot}P_i \overset{3}{\rightleftharpoons} R{\cdot}GDP \overset{4}{\rightleftharpoons} R + GDP$$

Scheme II

$$R{\cdot}GTP \overset{2a}{\rightleftharpoons} R^*{\cdot}GTP \overset{2b}{\rightleftharpoons} R{\cdot}GDP{\cdot}P_i$$

The cleavage of mantGTP by wild-type (Gly 12) p21 is accompanied by an 8–10% decrease in the fluorescence intensity of the p21·mantGTP complex that occurs with the same rate constant as the chemical cleavage of mantGTP. In addition, an exponential decrease in fluorescence of ca. 5% occurs with the mant-derivative of the nonhydrolyzable analogue of GTP, Gpp[NH]p. Since the rate of the fluorescence change with mantGpp[NH]p is similar to that observed with mantGTP, we proposed that the fluorescence change is monitoring an isomerization of the p21·GTP complex which precedes and controls the rate of GTP cleavage (Neal et al., 1990; Scheme II).

A similar conclusion was reached by Antonny et al. (1991) from fluorescence measurements of a single tryptophan-

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[1] Abbreviations: p21, the p21^{ras} protein product of the N-*ras* gene unless otherwise stated; GAP, the C terminal region (aa 684–1047) of the p120 GTPase activating protein; GTP(γS), guanosine 5′-(γ-thiotriphosphate); Gpp[NH]p, guanosine 5′-(β,γ-imidotriphosphate); mantGTP, 2′(3′)-*O*-(*N*-methylanthraniloyl)guanosine 5′-triphosphate; mantGpp[NH]p, mantGDP, and mantGTP(γS) are similarly abbreviated.

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containing mutant of p21 on its interaction with GTP and GTPγS. If the conformational change occurs on the GAP-activated pathway, this mechanism predicts that the fluorescence change occurring on the interaction of p21 with both mantGTP and mantGpp[NH]p should be accelerated by GAP, and this is investigated here. Rensland et al. (1991) investigated these reactions using complexes of $p21^{H\text{-}ras}$. They showed that there was a 10% decrease of fluorescence with the p21 mantGTP complex which could be better fitted to a double exponential than a single exponential with rate constants of 2.7×10^{-3} s^{-1} and 3.8×10^{-4} s^{-1}. In contrast they showed only a 5% decrease of fluorescence with the p21·mantGpp[NH]p complex which occurred with a half time of about 1 min. On the addition of 50 nM GAP to both these complexes, the rate of the fluorescence changes with p21·mantGTP was accelerated 40-fold but that with p21·mantGpp[NH]p remained the same. This led Rensland et al. (1991) to conclude that the mechanism shown in Scheme II may not be correct.

This paper presents our results with GAP-activated p21. First, we explored the accuracy of methods for determining the concentration of p21, since this is crucial for these measurements because the observed rates depend on the concentration of p21. We found that the usual filter binding assay seriously underestimated the concentration of p21, and we developed a method based on the absorbance of the mant-chromophore. We also used a method for quickly preparing p21·analogue complexes at high concentrations, so that observations could be made more quickly by diluting them into buffer at the appropriate temperature. In order to resolve discrepancies with the results of Rensland et al. (1991), we have repeated our experiments in the absence of GAP using this modified procedure. We found that, on incubating either p21·mantGTP or p21·mantGpp[NH]p at 30 °C, there is a biphasic decrease in fluorescence, the slower rates being similar to that of p21·mantGTP hydrolysis. The first process was not observable on the time scale of the experiments of Neal et al. (1990). The second process is accelerated in a linearly dependent manner on the concentration of GAP for both the p21·mantGTP and p21·mantGpp[NH]p complexes. This is consistent with Scheme II being correct and with the observation that GAP accelerates the rate of the R·GTP to R*·GTP isomerization step.

MATERIALS AND METHODS

Proteins. Wild-type (Gly 12) $p21^{N\text{-}ras}$ was isolated from overproducing strains of *Escherichia coli* (Hall & Self, 1986). All procedures were carried out at 4 °C. Cells from a 40-L culture were broken in a French Press in 20 mM Tris·HCl, pH 7.5, 10 mM NaCl, 1 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM PMSF (buffer A), and after centrifugation, the cell supernatant was loaded onto a Q-Sepharose ion-exchange column (25 × 6 cm) pre-equilibrated in buffer A. p21 was eluted with a 4-L linear salt gradient from 0 to 250 mM NaCl in buffer A at 4 mL min^{-1}. p21 elution was monitored by a [8-^{3}H]GDP filter binding assay described by Hall and Self (1986) except that the exchange of [8-^{3}H]GDP for GDP bound to p21 was accelerated by incubation in 50 mM Tris·HCl, pH 7.5, 20 mM EDTA, 200 mM ammonium sulphate, 10 mM dithiothreitol (Hoshino et al., 1987) (see below). After 5 min at 20 °C the exchange reaction (40 μL) diluted to 1 mL with 50 mM Tris·HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 10 mM dithiothreitol was cooled on ice and the amount of p21·[8-^{3}H]GDP determined by nitrocellulose filtration (Millipore, 0.45 μM, HAWP). Fractions containing p21·GDP were made 65% saturated ammonium sulfate and centrifuged, and the pellet was resuspended in buffer A plus 100 μM GTP and purified on an ACA 44 (LKB) gel filtration column (200 × 2.5 cm) in buffer A at 20 mL h^{-1}. The p21·GDP peak was concentrated by ultrafiltration, and the residue was stored at −80 °C. The purified protein was characterized by a series of assays as described below. The typical yield from 200 g of cells was 100 ± 20 mg measured by Bradford assay using bovine serum albumin as a standard, and the typical product was >95% pure on SDS polyacrylamide gel electrophoresis.

The C-terminal region of GAP (aa 684–1047) was isolated from an *E. coli* expression system (a gift of Dr. Alan Hall, ICR, London, U.K.) described by Hart and Marshall (1990). Cells from a 4-L culture were harvested, broken with a French Press in 20 mM Tris·HCl, pH 7.5, 0.1 mM dithiothreitol, and the soluble cell extract, after centrifugation, was applied to a DEAE-Sephacel column (10 × 2.5 cm) equilibrated in 20 mM Tris·HCl, 0.1 mM dithiothreitol, pH 7.5. A gradient (500 mL) of 0–250 mM NaCl in this buffer was applied. Fractions were assayed for GAP activity as described by Tsai et al. (1989) or in some preparations as described by Webb & Hunter (1992). The fractions containing GAP activity were concentrated and applied to a column of Q-Sepharose (20 × 1.6 cm), equilibrated with the same buffer as above. GAP was eluted by a gradient (800 mL) of 0–400 mM NaCl in this buffer. The pool of GAP activity was concentrated, and protein concentration was determined by the BCA assay (Pierce Chemical Company) using bovine serum albumin as a standard (Smith et al., 1985). The protein was stored at −80 °C.

Nucleotides. MantGTP, mantGDP, and mantGpp[NH]p were synthesized as previously described (Hiratsuka, 1983; Neal et al., 1990) except that a 2-fold excess of *N*-methylisatoic anhydride was used. Nucleotides were analyzed on a Whatman SAX HPLC column (250 × 4.6 mm) using a gradient system. The starting buffer (buffer C) was 0.5 M $NH_4H_2PO_4$ (adjusted to pH 4.0 with HCl), and the end buffer (buffer D) was the same solution with the addition of 25% (v/v) methanol. After the sample was injected, the column was eluted isocratically with buffer C at 1.5 mL min^{-1} for 5 min, followed by a 5-min linear gradient to 100% buffer D, followed by buffer D for 15 min. GDP, Gpp[NH]p, GTP, mantGDP, mantGpp[NH]p, and mantGTP eluted at 3.9, 9.1, 10.4, 12.3, 15.6, and 18.6 min, respectively. Each of the three mant-nucleotides synthesized were >97% pure by the elution profile at 252 nm, and no parent nucleotides were detectable.

Mant-nucleotides exist as an equilibrium mixture of the 2′ and 3′ isomers (Cremo et al., 1990), which were not separated by the above system. However, they could be resolved by reverse-phase HPLC on a C18 Novapak column (150 × 3.9 mm) eluting isocratically with 100 mM potassium phosphate, pH 6.0/acetonitrile (96:4 v/v) at 2 mL min^{-1} (Figure 7).

Protein·Nucleotide Complexes. p21·nucleotide complexes were prepared by one of two methods: (a) *via* the apoprotein by hydrophobic interaction HPLC (Feuerstein et al., 1987; Neal et al., 1988) or (b) by direct exchange of the bound nucleotide for added nucleotide utilizing the increased exchange rate of p21 in the presence of EDTA and ammonium sulfate (Hoshino et al., 1987). In this second method, the exchange reaction was performed with a 20-fold excess of mantGTP or a 50-fold excess of mantGpp[NH]p over p21 for 5 min in 50 mM Tris·HCl, pH 7.5, 20 mM EDTA, 200 mM ammonium sulfate, 10 mM dithiothreitol at 20 °C. The p21·nucleotide complex was separated from excess unbound nucleotide by gel filtration on a Pharmacia PD-10 column

pre-equilibrated in 50 mM Tris·HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 10 mM dithiothreitol at 4 °C. All fluorescence experiments were performed in this buffer (buffer B), prefiltered through Millipore 0.45-μm filters (Millipore, HAWP). HPLC analysis of the p21·mantGTP complexes using a Whatman SAX column with the system described above after this procedure indicated 5–10% hydrolysis of the bound triphosphate had occurred during this procedure.

Assays for p21. Since the concentration of active p21 is of critical importance in analyzing our data and in our hands the [^{3}H]GDP filter binding assay (Hall & Self, 1986) gave much lower values than determinations of total protein concentration (see Results), solutions of p21 were analyzed in several ways. These involved methods based on total protein concentration and methods based on the GDP-binding activity of the protein.

(*a*) *Total Protein Concentrations.* Total protein concentration was determined in one of three ways: first, by the Bradford assay (Sedmarck & Grossberg, 1977) using a standard of bovine serum albumin; second, from its absorbance at 280 nm and calculated from the predicted molar extinction coefficient as described by Gill and von Hippel (1989) but also taking into account the presence of a stoichiometric amount of GDP (This calculated value was 24.0 mM^{-1} cm^{-1}); third, by quantitative amino acid analysis of an acid hydrolysate of the protein by the method of Moore & Stein (1954).

(*b*) *GDP-Binding Assays.* The GDP-binding activity of p21 was determined in three ways: by measuring the amount of bound GDP in the p21 as isolated from *E. coli*, by the measurement of the amount of [8-^{3}H]GDP or [γ-^{32}P]GTP that can exchange into the p21, and by measuring the amount of mantGDP that can exchange into p21.

The total amount of guanine nucleotide in the sample was determined by denaturing a sample with 0.6 M $(NH_4)_2HPO_4$, pH 4.0, and after the denatured protein was removed by centrifugation, the supernatant was analyzed for guanine nucleotides by HPLC as described by Webb & Eccleston (1981). A known amount of GTP was added to the solutions as an internal standard, and corrections were made for the presence of ~15% p21·GTP in the samples of p21·GDP.

The activity of p21 in binding [^{3}H]GDP was measured by equilibration of p21·GDP with excess [^{3}H]GDP either in the presence of 10 mM $MgCl_2$ for 180 min at 37 °C (Hall & Self, 1986) or in 20 mM EDTA and 200 mM ammonium sulfate for 10 min at 20 °C. Separations of bound from free nucleotide were achieved either by nitrocellulose filtration (Millipore, 0.45 μm, HAWP) or by gel filtration (Pharmacia, PD10) (HPLC analysis of the [8-^{3}H]GDP used for these measurements showed that 90% of the radioactivity eluted as GDP).

The activity of p21 in binding [γ-^{32}P]GTP and [^{3}H]GTP was determined in the same way using the EDTA/ammonium sulfate exchange conditions to prevent hydrolysis of GTP occurring during the assay.

A spectrophotometric assay was used to measure the concentration of solutions of p21·mant-nucleotide complexes. A solution of p21·GDP was equilibrated with an approximately 20-fold molar excess of mantGDP in 20 mM EDTA, 200 mM ammonium sulphate. After 5 min at room temperature, the excess nucleotide was removed by gel filtration (PD10, Pharmacia) and the absorbance of the p21·mantGDP complex at 350 nm was measured. The concentration of p21·mantGDP was calculated from the molar extinction coefficient of mantGDP of 5700 M^{-1} cm^{-1} (Hiratsuka, 1983). Although this is the value of the free nucleotide, displacement of mantGDP from p21·mantGDP by excess GDP resulted in no change in absorbance, and so this value was also taken as the molar extinction coefficient of p21·mantGDP at 350 nm.

Finally, the concentration of active p21 was determined by forming the p21·GTP complex (as described above for p21·[γ-^{32}P]GTP) and measuring the quantitative release of P_i on GTP hydrolysis using the purine nucleoside phosphorylase assay of Webb & Hunter (1992).

The comparative values obtained by these assays are described in the Results section.

Fluorescence Measurements. Fluorescence intensity measurements were made in a thermostatically controlled SLM 8000 S spectrofluorimeter in the L format using 4-mm or 10-mm square quartz cuvettes. Excitation of the mant-moiety was at 366 nm (0.5-nm excitation slits), and emission was observed either through a monochromator at 442 nm (4-nm emission slits) or through a KV 399 cutoff filter (Schott). Fluorescence anisotropy measurements were made with the same fluorimeter but used in the T format where parallel and perpendicular polarized emitted light was measured simultaneously through two KV 399 filters.

For all time course measurements of more than 15-min duration, data were collected manually and the shutters closed between readings to prevent photobleaching. For time course measurements of less than 15 min, the data were collected continuously using an HP 86 computer. The fluorescence intensity of the sample cuvette was corrected for fluctuations in lamp intensity and/or photomultiplier tube efficiency by ratioing measurements to that of a second cuvette containing mantGDP. The noise level in control experiments with mantGDP in both cuvettes was usually <0.1% between points for rapid time course measurements (<40 min) and <1% for measurements over 6 h. Using this procedure, we are confident that fluorescence changes of 4–8% occurring over several hours are due to a change in the fluorescence of the p21·mant-nucleotide complexes and not due to instrumental artifacts.

In all the fluorescence measurements, complexes of p21 with mant-nucleotides were prepared at 4 °C and then the fluorescence intensity was monitored after diluting the complex to 15 μM and warming the solution to 30 °C. When prepared from the apoprotein, the concentration of the stock solutions of the complexes were typically 20 μM. They therefore required little dilution and so required several minutes to warm up from 0 to 30 °C when diluted with 30 °C buffer. When complexes were made by the direct exchange procedure in 20 mM EDTA, 200 mM ammonium sulphate, stock solutions of up to 100 μM were obtained. This allowed the experiments to be initiated rapidly by dilution of a small volume of p21·nucleotide complex into a large volume of buffer at 30 °C. In these cases, the time taken for diluted samples to reach 30 °C was less than 1 min. In all experiments, measurements were only taken when the temperature of the solution reached 30 ± 0.05 °C. For experiments involving GAP, the solution of p21·nucleotide complex was warmed to 30 °C before the addition of GAP preincubated at 30 °C.

Other Measurements. The rate constants of mantGTP cleavage (k_{+2}), mantGTP dissociation (k_{-1}), and mantGDP dissociation (k_{+4}) at 30 °C (Scheme I) were measured as previously described (Neal et al., 1990) except that 1:1 protein/nucleotide complexes were prepared by direct exchange described above. The rate of GTP cleavage was measured by removing aliquots from a solution of 15 μM p21·[γ-^{32}P]GTP in buffer B at 30 °C and assaying for [γ-^{32}P]GTP bound to the nitrocellulose filter as described above.

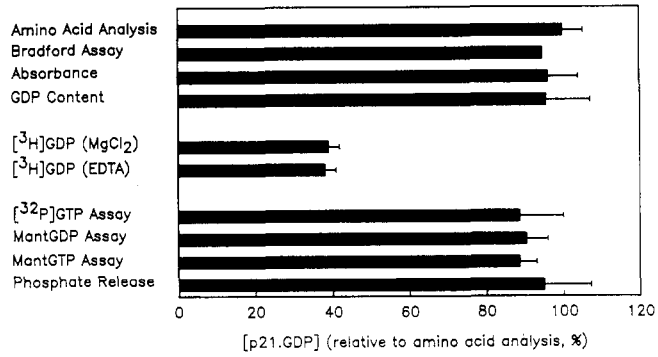

FIGURE 1: Protein and GDP binding assays of p21. Assays for total protein and GDP-binding activities of a single sample of p21 were determined as described in Materials and Methods. Error bars show standard deviations with the number of measurements from top to bottom as 14, 1, 4, 14, 2, 2, 3, 3, 3, and 2, respectively. All assays were normalized with respect to the value obtained by amino acid analysis.

RESULTS

Measurement of p21^{ras} Concentration. For the experiments described by Neal et al. (1988, 1990), all of the reactions studied were first-order or pseudo-first-order and therefore the observed rate constants were independent of the active concentration of p21. However for the conditions used, in the presence of GAP, the observed rate constants will depend critically on the active p21·GTP concentration. p21 was therefore assayed by several methods in order to obtain a reliable estimate of the active p21 concentration.

A typical set of results for the different assays of protein concentration and active p21 on one preparation of p21 is summarized in Figure 1. The assays for active p21 concentration based on the extinction coefficient of the mantGDP or mantGTP, the binding of [γ-^{32}P]GTP, the concentration of GDP, or the release of P_i all give values close to the concentration of protein based on amino acid analysis, absorption at 280 nm, or the Bradford assay. Since p21 is >95% pure by gel electrophoresis, the preparations contain little or no denatured protein. The [8-^{3}H]GDP-binding assays (Hall & Self, 1986, or modifications thereof) typically give only 35–40% binding activity relative to total protein concentration. It is unlikely that this is due to incomplete binding of the p21·[8-^{3}H]GDP complex to the nitrocellulose filters, since similar binding extents are observed when separation is achieved by gel filtration. Due to the anomalous results with the filter binding assay and also the variability of results between different batches of the same filters, all concentrations of p21 are based on the absorbance at 350 nm of the p21·mantGTP complex.

Comparison of the Effect of GAP on the p21 GTPase and mantGTPase Mechanisms. The rate constants of the elementary processes of the p21*ras* GTPase and mantGTPase mechanisms in the absence of GAP have been shown to be within a factor of 2 (Neal et al., 1990). The effect of GAP on these rate constants has now been determined.

Figure 2 shows that while the rate constant for the cleavage step controlled by k_{+2} (Scheme I) in the absence of GAP is 1.8×10^{-4} s^{-1} for mantGTP and 2.7×10^{-4} s^{-1} for GTP, in the presence of GAP this rate is accelerated to a greater extent with mantGTP than with GTP. At 3 μM GAP, the rate constants are 9.8×10^{-3} s^{-1} and 3.3×10^{-3} s^{-1} respectively. Under the conditions used here (15 μM p21·mantGTP), the rate constants are linear with GAP concentration over the range used (0–3 μM for p21·mantGTP and 0–6.4 μM for

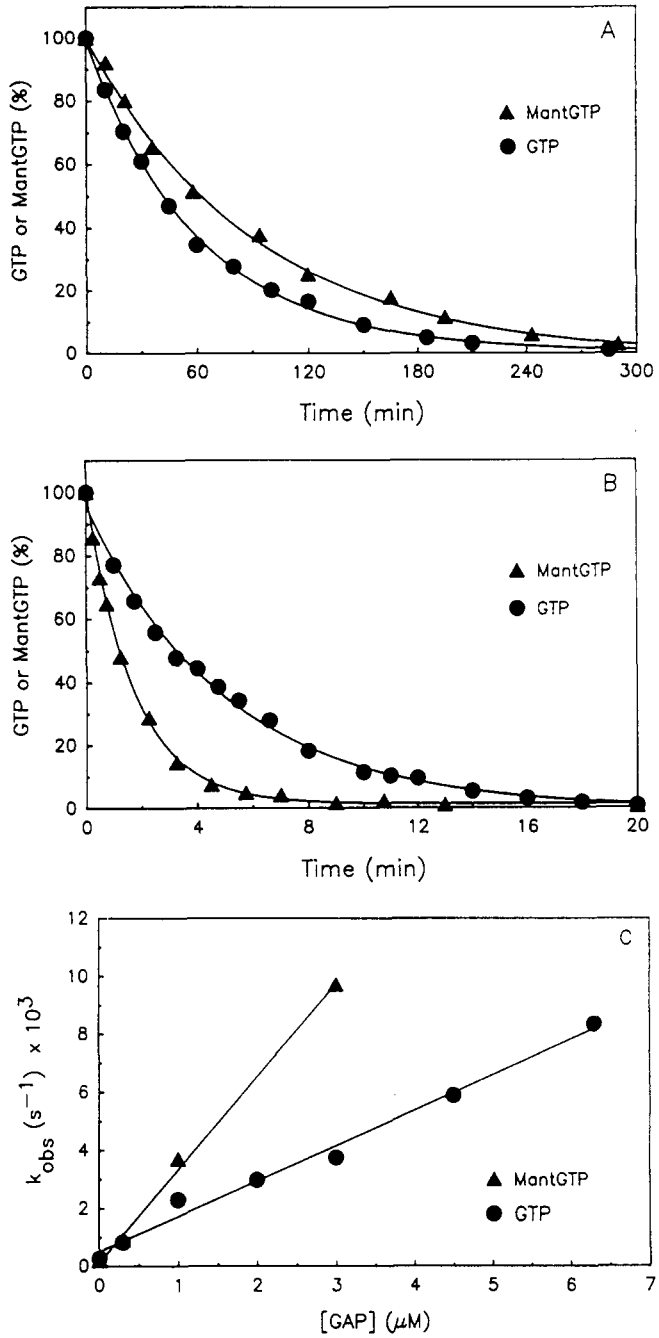

FIGURE 2: The rate of cleavage of GTP and mantGTP bound to p21. All experiments were performed in 50 mM Tris·HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 10 mM DTT (buffer B). (A) Solutions of p21·[γ-^{32}P]GTP and p21·mantGTP (15 μM) were incubated at 30 °C. At timed intervals, aliquots were removed from each sample and the extent of [γ-^{32}P]GTP and mantGTP hydrolysis was measured by nitrocellulose filtrations and HPLC, respectively. Solid lines represent the best fit to single exponentials. k_{obs}(GTP) = 2.7×10^{-4} s^{-1}, k_{obs}(mantGTP) = 1.8×10^{-4} s^{-1}. (B) As for part A except 3 μM GAP was added at t = 0 min. k_{obs}(GTP) = 3.3×10^{-3} s^{-1}, k_{obs}(mantGTP) = 9.8×10^{-3} s^{-1}. End points were <1% GTP or mantGTP. (C) Dependence on the concentration of GAP of the observed rate constants from experiments as above (A and B). Solid lines are the best fit to straight lines with gradients of 1.22×10^{-3} s^{-1} μM^{-1} GAP (GTP) and 3.21×10^{-3} s^{-1} μM^{-1} GAP (mantGTP).

p21·GTP). The high ionic strength conditions used previously (Neal et al., 1990) and in this study were chosen so that the reactions could be followed over as wide a range of GAP concentrations as possible, and consequently the activations are relatively low compared to those of low ionic strength conditions (Moore et al., 1992). However, the ionic strength

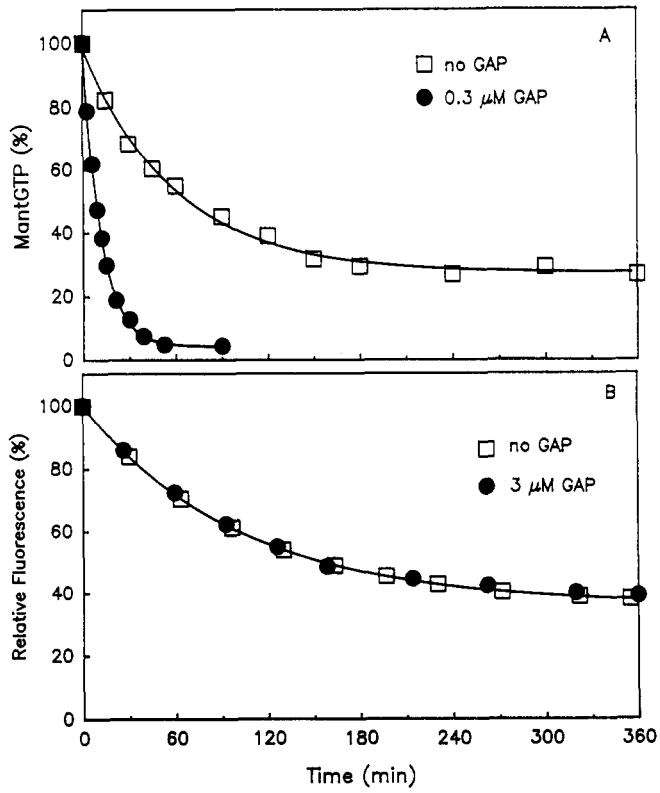

FIGURE 3: Effect of GAP on the rate of mantGTP and mantGDP dissociation (k_{-1} and k_{+4}, respectively) from p21. (A) 15 μM p21·mantGTP was incubated at 30 °C in buffer B with 1 mM GDP in the presence or absence of 0.3 μM GAP. Aliquots were removed at timed intervals and analyzed for the extent of mantGTP hydrolysis by HPLC. Solid lines are the best fits to single exponentials: □ no GAP, $k_{obs} = 2.8 \times 10^{-4}$ s^{-1}, end point = 27.2% mantGTP; ● 0.3 μM GAP, $k_{obs} = 1.4 \times 10^{-3}$ s^{-1}, end point = 4.3% mantGTP. (B) 15 μM p21·mantGDP in buffer B was incubated at 30 °C in a fluorimeter, either in the presence (circles) or absence (squares) of 3 μM GAP. At $t = 0$ min, GDP was added to 1 mM final concentration and the fluorescence intensity monitored at timed intervals. The solid line is the best fit to a single exponential. In both experiments, $k_{obs} = 1.6 \times 10^{-4}$ s^{-1} and the end point was at 36% relative fluorescence.

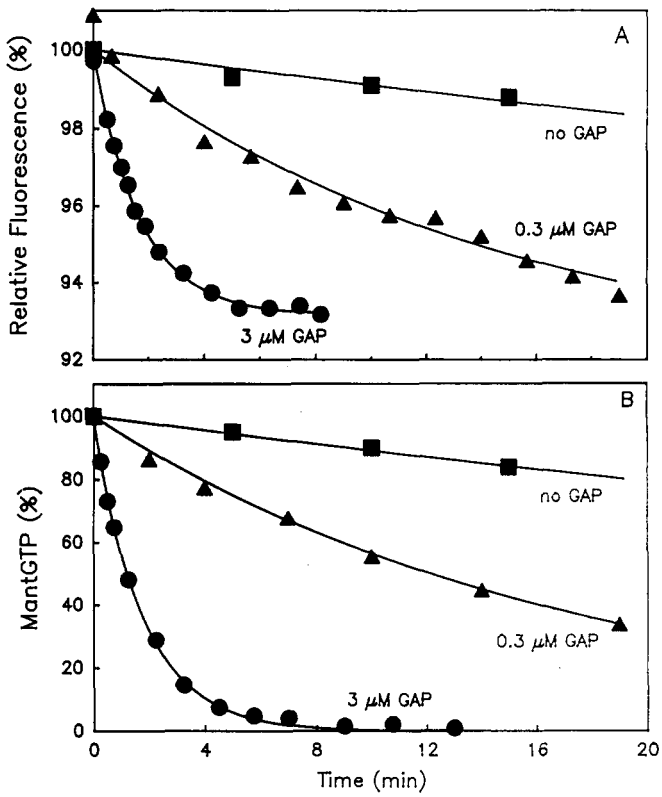

FIGURE 4: Effect of GAP on the fluorescence intensity of the p21·mantGTP complex and on the chemical cleavage rate of p21·mantGTP. 15 μM p21·mantGTP in buffer A at 4 °C was warmed to 30 °C in a fluorimeter cell (typically within 6–7 min). Recording was not initiated until the temperature of the solution had reached 30(±0.05) °C. Chemical cleavage of mantGTP was monitored by HPLC. Each data set is normalized to the zero time measurement. (A) Effect of GAP on the fluorescence intensity of p21·mantGTP. GAP was added to 0.3 or 3 μM final concentration at $t = 0$ min as indicated. Solid lines are the best fits to single exponentials: ■ no GAP, $k_{obs} = 1.9 \times 10^{-4}$ s^{-1}, $A = 7.6\%$; ▲ 0.3 μM GAP, $k_{obs} = 0.90 \times 10^{-3}$ s^{-1}, $A = 8.3\%$; ● 3 μM GAP, $k_{obs} = 9.5 \times 10^{-3}$ s^{-1}, $A = 6.3\%$. (B) Time course for the chemical cleavage of mantGTP bound to p21 measured by HPLC under conditions identical to those in part A. Solid lines are best fits to single exponentials with end points at <1% mantGTP in all cases. ■ no GAP, $k_{obs} = 1.8 \times 10^{-4}$ s^{-1}; ▲ 0.3 μM GAP, $k_{obs} = 0.95 \times 10^{-3}$ s^{-1}; ● 3 μM GAP, $k_{obs} = 9.8 \times 10^{-3}$ s^{-1}.

used here is closer to physiological values than the conditions required for maximal activation.

Using the phosphate release assay of Webb and Hunter (1992), the larger activations of p21·mantGTP hydrolysis by GAP compared to that of p21·GTP hydrolysis have been shown to be due to a decreased K_m of GAP for p21·mantGTP and the fact that k_{cat} remains unchanged (M. R. Webb and A. E. Nixon, unpublished results).

The rate constant of the step controlling mantGTP dissociation from p21 (k_{-1}, Scheme I) was measured in the absence and presence of GAP. The rationale for these experiments has been described by Neal et al. (1988). On addition of GDP to p21*ras*·mantGTP, the nucleotide can either dissociate (k_{-1}) or undergo the cleavage step (k_2). The observed rate constant of the formation of mantGDP is the sum of k_{-1} and k_2, and the ratio of mantGTP and mantGDP at the end of the reaction is k_{-1}/k_2. Thus, both k_{-1} and k_2 can be calculated. Figure 3A shows that, on incubating p21·mantGTP with 1 mM GDP in the absence of GAP, mantGDP is formed with a rate constant of 2.8×10^{-4} s^{-1} and an end point of 27.2% mantGTP. In the presence of 0.3 μM GAP, the rate constant was 1.4×10^{-3} s^{-1} and the end point was 4.3% mantGTP. From these measurements, calculation of k_{-1} gave values of 0.7×10^{-4} s^{-1} in both the absence and presence of 0.3 μM GAP. At higher concentrations of GAP, the cleavage rate predominates and only an upper limit of k_{-1} is obtained.

The rate constant of mantGDP dissociation from p21 was identical in the absence and presence of 3 μM GAP (Figure 3B).

Fluorescence Changes of p21·mantGTP in the Presence of GAP. In the absence of GAP, the fluorescence of p21·mantGTP decreases by ~8% with the same rate as that of the cleavage step (Neal et al., 1990). The effect of GAP on the cleavage rate and on the fluorescence change is shown in Figure 4. GAP accelerates both processes to the same extent over a 10-fold range of GAP concentration (Figure 5). In a control experiment there was no fluorescence change observed with p21·mantGDP over the same time course in the presence of 0.3 and 3 μM GAP (data not shown). Gel filtration analysis of the p21·mantGTP solutions at the end of the reactions showed that, in either the absence or presence of GAP, there was no detectable free nucleotide present; greater than 99% of the fluorescence eluted with the protein.

The results above were obtained using complexes which required several minutes to reach 30 °C before measurements were initiated (see Materials and Methods). However, by using the direct exchange procedure for the preparation of

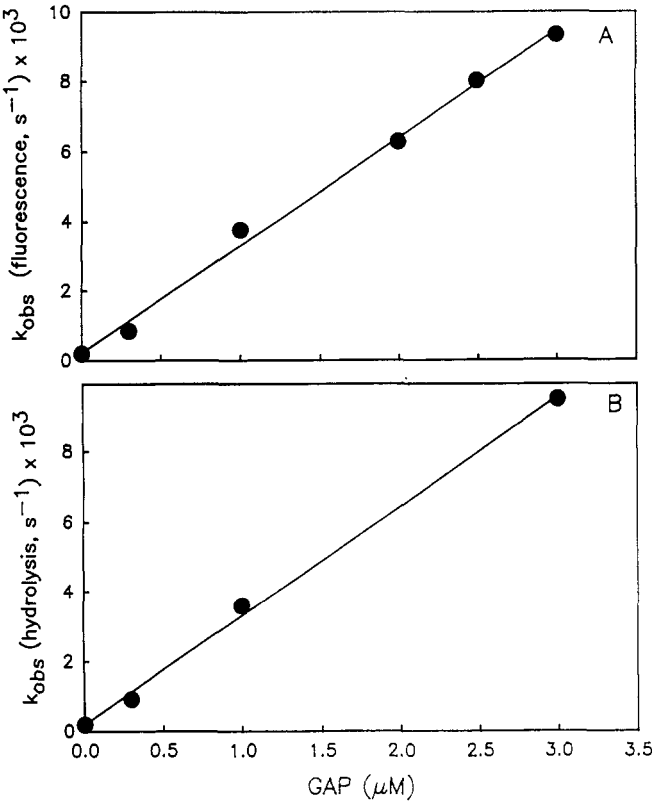

FIGURE 5: Dependence of the observed first-order rate of the fluorescence change or the chemical cleavage of mantGTP on GAP concentration. GAP was added to a solution of 15 μM p21·mantGTP at 30 °C in buffer B to a final concentration of 0–3 μM, as described in Figure 4. The observed rate of the fluorescence change (A) and the chemical cleavage of mantGTP (B) is shown as a function of the final GAP concentration. The solid lines are best fits to straight lines with gradients of (A) 3.18 × 10^{-3} s^{-1} μM^{-1} and (B) 3.21 × 10^{-3} s^{-1} μM^{-1}.

p21·mantGTP, the fluorescence experiments could be initiated more rapidly by dilution of a small volume of stock p21·mantGTP into buffer at 30 °C. When the fluorescence change was re-examined using this modified procedure, it was biphasic (Figure 6A). An initial decrease of 4.8% occurred with a rate constant of 3.3 × 10^{-3} s^{-1} followed by a further decrease of 7.9% with a rate constant of 2.1 × 10^{-4} s^{-1}. The second phase is that identified by Neal et al. (1990) which occurs with the same rate constant as that of mantGTP cleavage and is the same as that shown in Figure 4A. The first phase was almost complete before measurement began using the previous procedure. Again, gel filtration showed that no dissociation of nucleotide had occurred during the time course of the measurement.

To obtain more information about the nature of the fluorescence changes, the anisotropy and the intensity were followed simultaneously during the time course of the mantGTP hydrolysis reaction in the absence of GAP (Figure 6). The measurements of fluorescence anisotropy show a rapid decrease occurring with the same rate constant as that of the fast phase of the intensity data. However, there is no further change in anisotropy associated with the slow phase of the intensity. These data provide further evidence that there was no significant dissociation of nucleotides from p21 during the time course of these experiments, since there is a large difference between the anisotropy of p21·mantGDP and mantGDP (0.21 and 0.02, respectively) (data not shown). GAP has no significant effect on the rate or amplitude of the fast phase of the fluorescence-intensity change.

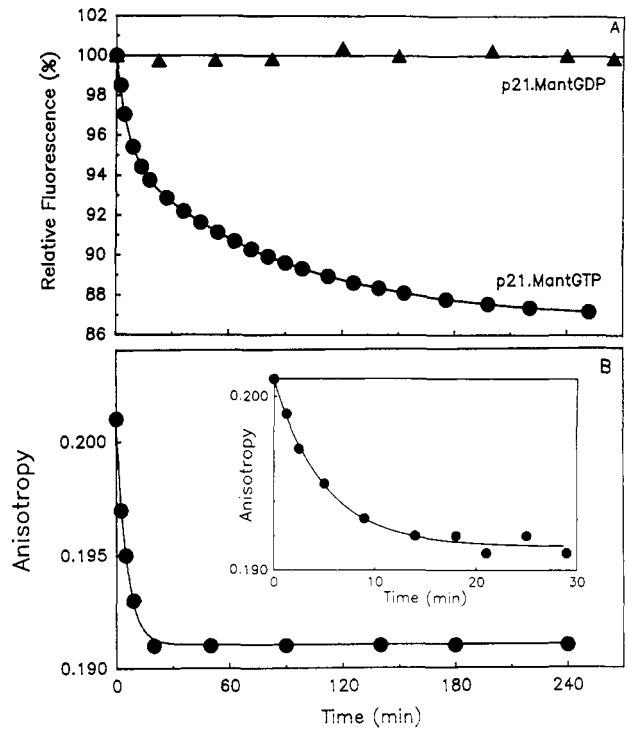

FIGURE 6: Fluorescence and anisotropy changes associated with mantGTP hydrolysis by p21. (A) p21·mantGTP (56.3 μM) at 4 °C was diluted into buffer B at 30 °C to a final concentration of 15 μM. The temperature of the solution reached 30 °C in ca. 45 s. The fluorescence intensity was followed with time (circles). The fluorescence intensity of a solution of p21·mantGDP is also shown (triangles). The solid line through the circles is a double exponential best fit to the p21·mantGTP data with k_{obs1} = 3.3 × 10^{-3} s^{-1}, A_1 = 4.8%, k_{obs2} = 2.1 × 10^{-4} s^{-1}, and A_2 = 7.9%. The solid line through the triangles is a linear regression best fit to the p21·mantGDP data. (B) as in part A except that fluorescence anisotropy was measured on the same time scale. Inset: data plotted on a shorter time scale. The data is fitted to a single exponential. k_{obs} = 2.8 × 10^{-3} s^{-1} and A = 0.010.

Since the fluorescence intensity of the 3′ isomer of mantGTP and mantGDP is approximately double that of the 2′ isomer (Eccleston et al., 1991), interconversion of the 3′ isomer to the 2′ isomer might provide one possible explanation for the reduction of the fluorescence intensity during the mantGTP hydrolysis reaction although the relative fluorescent intensities are not known for the bound species. Quenching p21·mantGTP complexes into perchloric acid (to stop acyl migration and denature the protein) followed by reverse-phase HPLC of the supernatant demonstrated that, at the start of the hydrolysis reaction, only 8% of the fluorescence of mantGTP eluted in the position of the 2′ isomer (Figure 7). Correcting for the intensity changes between the 2′ and 3′ isomers, this represents 15% of the bound mantGTP being the 2′ isomer. The same ratio between the two isomers was maintained during the hydrolysis reaction. Thus, acyl transfer does not explain either the fast or slow phases of the fluorescence changes associated with the mantGTP hydrolysis.

Fluorescence Changes of p21·mantGpp[NH]p in the Presence of GAP. A solution of p21·mantGpp[NH]p also shows a fluorescence decrease with a rate constant similar to that with p21·mantGTP even though no hydrolysis occurs (Neal et al., 1990). On this basis, we proposed that the cleavage step was controlled by a preceding isomerization during the hydrolysis of the p21·mantGTP complex. On re-examination of the p21·mantGpp[NH]p reaction in the absence of GAP making measurements at earlier time points as described above, we found that this reaction was also biphasic (Figure 8A). A

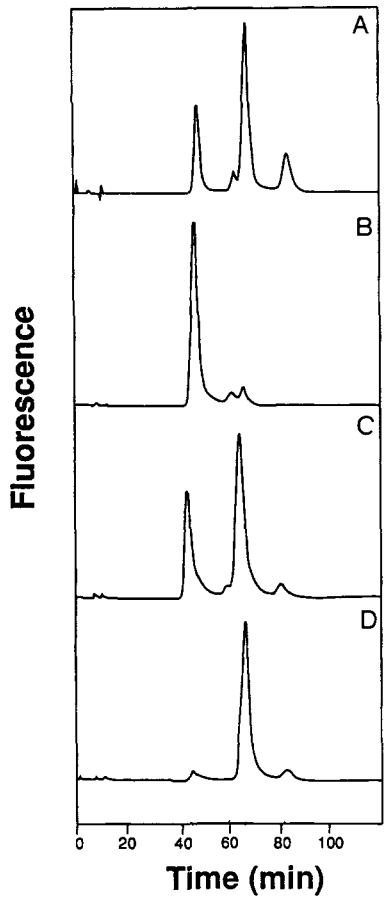

FIGURE 7: Separation of the 2′ and 3′ isomers of mant-nucleotides bound to p21. At timed intervals, aliquots were removed from a solution of 15 μM p21·mantGTP in buffer B at 30 °C. The mant-nucleotides were displaced from the protein/nucleotide complex by acid treatment and analyzed by reverse-phase HPLC for the presence of the 2′ and 3′ isomers, as described under Materials and Methods. (A) Standard mixture of mantGDP and mantGTP (>98% pure by ion-exchange HPLC). The retention times for the 3′ and 2′ isomers of mantGTP and mantGDP under these conditions are 3′ mantGTP = 48 min, 2′ mantGTP = 63 min, 3′ mantGDP = 68 min, and 2′ mantGDP = 85 min. (B) Reaction mixture after 2 min. (C) After 60 min. (D) After 240 min.

4.3% decrease of fluorescence intensity occurred with a rate constant of 2.9×10^{-3} s^{-1} followed by a 3.9% decrease with a rate constant of 1.9×10^{-4} s^{-1}. These rates are similar for both mantGTP and mantGpp[NH]p although the amplitude is smaller in the latter case. HPLC analysis at intervals during the time course of the process indicated <1% hydrolysis of mantGpp[NH]p. As with p21·mantGTP the second phase was that identified by Neal et al. (1990).

When GAP was added to a solution of p21·mantGpp[NH]p after the fast phase was essentially complete (15 min), the rate of the slow phase was accelerated although the amplitude remained the same (Figure 8B). The rate constant of this process was linear with GAP concentration up to 3 μM (Figure 8C). The observed rate constants in the presence of GAP are approximately 3-fold lower than those for mantGTP. HPLC analysis of the p21·mantGpp[NH]p complex over the time course of the reaction with 3 μM GAP (40 min) indicated <1% hydrolysis of mantGpp[NH]p, and gel filtration demonstrated <1% free nucleotide.

DISCUSSION

Since kinetic studies of the mechanism of the hydrolysis of p21·GTP catalyzed by substoichiometric concentrations of

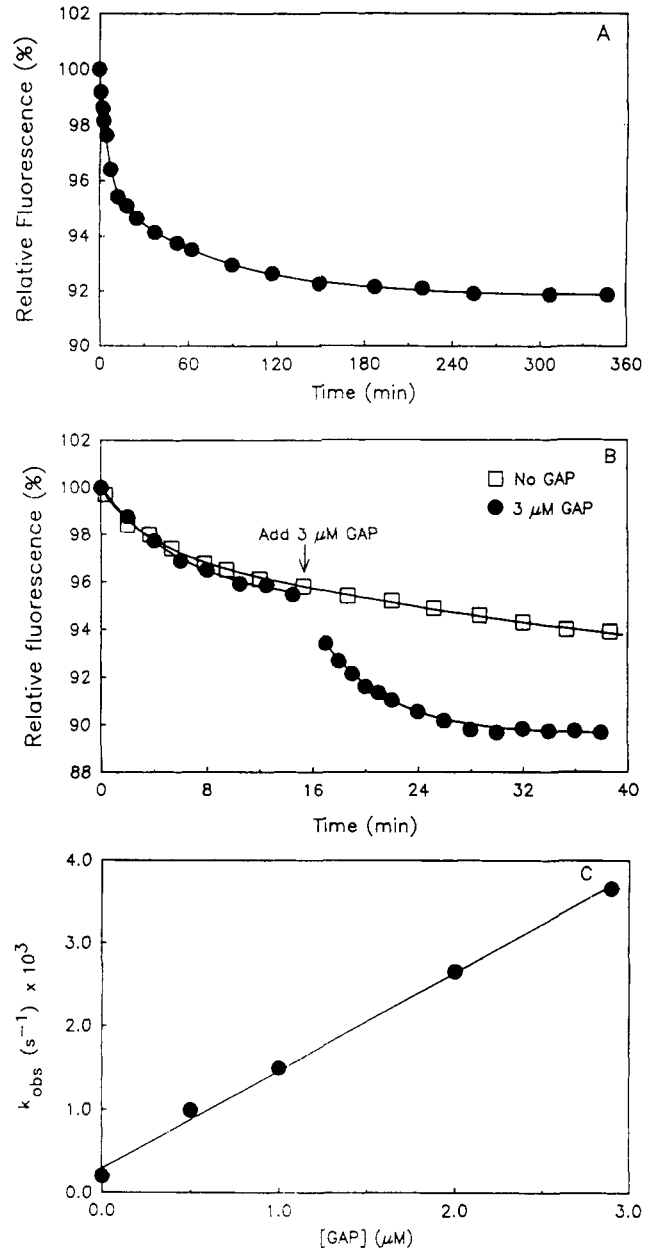

FIGURE 8: Change in fluorescence intensity on incubation of the p21·mantGpp[NH]p complex in the absence and presence of GAP at 30 °C. (A) 15 μM p21·mantGpp[NH]p in buffer B was warmed to 30 °C in a fluorimeter cell in ca. 1 min and the fluorescence intensity monitored at timed intervals. The solid line is the best fit to a double exponential, $k_{obs1} = 2.9 \times 10^{-3}$ s^{-1}, $A_1 = 4.3\%$, $k_{obs2} = 1.9 \times 10^{-4}$ s^{-1}, $A_2 = 3.9\%$. (B) 15 μM p21·mantGpp[NH]p in buffer B was warmed to 30 °C and the fluorescence intensity monitored at timed intervals over 40 min: open squares, the fluorescence intensity initially decreased in a biphasic manner with the initial phase occurring at 3.5×10^{-3} s^{-1} ($A = 3.2\%$), and this was followed by a further slow decrease in intensity; filled circles, as above except that GAP is added to 2.9 μM final concentration at 16 min. The data after the addition of GAP can be fitted to a single exponential ($k_{obs} = 3.6 \times 10^{-3}$ s^{-1}, $A = 4.2\%$). (Addition of GAP resulted in an immediate 2% reduction of fluorescence intensity due to dilution.) (C) Dependence of k_{obs} on GAP concentration. The solid line is the best fit to a straight line with a gradient of 1.22×10^{-3} s^{-1} μM^{-1} GAP.

GAP (i.e., multiple turnovers of p21·GTP) depend critically on the concentration of active p21·GTP, we have made a detailed comparison of various methods for determining this value. There was good agreement between all of the methods for determining total protein concentration and most of the assays for active p21 (Figure 1). The only method which significantly deviated from the other assays was that involving

the binding of [^{3}H]GDP. This consistently gave values of approximately 30% of those obtained by the other methods. This occurred whether the excess nucleotide was removed by cellulose nitrate filtration or by gel filtration. The reason for this discrepancy is not known despite extensive studies. Similar low values of concentrations determined by the binding of ^{3}H nucleotides to p21*ras* have been obtained by others (Santos et al., 1988 and references therein). Concentrations of p21·nucleotide complexes in this study were obtained by measuring the absorbance of the bound mant-nucleotide at 354 nm. Re-examination of the fluorescence changes of the mant-fluorophore on incubating complexes of p21·mantGTP or p21·mantGpp[NH]p at 30 °C in the absence of GAP but using a method of preparation which allowed observations to be made at a time point earlier than that previously used (Neal et al., 1990) showed biphasic decreases of fluorescence intensity (Figures 6 and 8). With p21·mantGTP, the more rapid phase was a decrease of 4.8% with a rate constant of 3.3×10^{-3} s^{-1} and the second phase was a decrease of 7.9% with a rate constant of 2.1×10^{-4} s^{-1}. With p21·mantGpp-[NH]p the equivalent values are 4.3%, 2.9×10^{-3} s^{-1}, and 3.9%, 1.9×10^{-4} s^{-1}, respectively. The second phase in each case was equivalent to that described by Neal et al. (1990) and with p21·mantGTP occurs with the same rate constant as that for p21·mantGTP cleavage. Although the observed amplitudes of the fluorescence changes are small, the careful controls performed (see Materials and Methods) ensure that the changes do reflect changes of the fluorescence of the p21·mant-nucleotide complexes.

The effects of catalytic amounts of GAP on the fluorescence changes occurring with both p21·mantGTP and p21·mantGpp-[NH]p were investigated (Figures 4, 5, and 8). In both cases, the rate constant of the second phase of the fluorescence change increased with a linear dependence on GAP concentration up to 3 μM.

Although there is extensive information about the structure of p21 available from X-ray diffraction studies (Schlichting et al., 1990; Pai et al., 1990; Milburn et al., 1990; Tong et al., 1991) including that of the p21·mantGpp[NH]p complex (Goody et al., 1992), it is difficult to correlate the fluorescence changes observed here with specific structures of the complexes. The decrease in anisotropy which occurs concomitant with the fast phase of intensity decrease with p21·mantGTP in the absence of GAP (Figure 6) is interpreted as showing that, during this process, the mant-fluorophore has increased motion. This could be the result of increased local motion of the fluorophore resulting from a conformational change of the complex or of the global rotation of the complex. The latter possibility could occur if p21·mantGTP existed as a dimeric structure and the process represented dissociation of p21 dimer to monomer. There is some evidence that p21·nucleotide complexes can form oligomers from studies using gel filtration (John et al., 1989), radiation inactivation (Santos et al., 1988), and time-resolved fluorescence measurements (Hazlett et al., 1990).

We have previously suggested that the slow phase of the fluorescence decrease of p21·mantGTP and p21·mantGpp-[NH]p represents a conformational change of the complex and, in the case of p21·mantGTP, that this process precedes and controls the rate of the cleavage step. The results in Figures 4, 5, and 8 show that this fluorescence decrease is accelerated in a linearly dependent manner with constant amplitude on GAP concentration at catalytic amounts of GAP and that the amplitude of the process remains constant. This provides strong support for the model. On the basis of the X-ray crystallographic structure of the p21·Gpp[NH]p complex, Pai et al. (1990) have proposed a mechanism for the hydrolysis of GTP. The region around Glu 61 shows ill-resolved density but appears to consist of at least two conformations, only one of which has a structure in which the Gln 61 can activate a water molecule, which has been proposed to be the nucleophile in the GTP hydrolysis reaction. The slow fluorescence change may be due to the formation of this active complex, and it is accelerated by GAP.

Possible alternative explanations for the fluorescence processes associated with mantGTP hydrolysis that can be excluded are nucleotide dissociation (on the basis of gel filtration and fluorescence anisotropy measurements) or acyl migration of the mant group from the 3′-*O*- to the 2′-*O*-position (Figure 7).

Antonny et al. (1991) used a different spectroscopic approach to study the mechanism of the GAP-activated p21 GTPase. They introduced a tryptophan residue at position 56 of p21$^{H\text{-}ras}$ and showed that with p21·GTP(γS) there was a 5% decrease in tryptophan fluorescence occurring with a rate constant similar to that of GTP cleavage. Since GTP(γS) would be expected to be cleaved more slowly than GTP, they concluded that, in the absence of GAP, p21·GTP cleavage was controlled by a preceding isomerization step. However, in the presence of GAP, the fluorescence decrease was not obviously accelerated although the amplitude was increased. Without direct chemical determination of the state of the nucleotide, it is difficult to define this process. Rensland et al. (1991) showed that the rate of p21$^{H\text{-}ras}$·mantGTP(γS) cleavage is similar to that of a fluorescence change although as they point out this is an extremely slow reaction and difficult to measure accurately. It would be expected that the rate of the conformational change would be similar with both p21·mantGTP and mantGpp[NH]p but that the cleavage reaction would be slower in the latter complex.

Rensland et al. (1991) also measured the fluorescence changes occurring with the mantGTP and mantGpp-[NH]p complexes with p21$^{H\text{-}ras}$. They showed that, with p21$^{H\text{-}ras}$·mantGTP at 37 °C, a biphasic decrease in fluorescence occurred with rate constants of 3.8×10^{-4} s^{-1} and 2.7×10^{-3} s^{-1} although the relative amplitudes are not given. With p21$^{H\text{-}ras}$·mantGpp[NH]p, a 5% decrease in fluorescence occurred. The rate constant of this protein was not given although it appears to have a half time of approximately 100 s, which corresponds to a rate constant of 7×10^{-3} s^{-1}. Since their experiments were at 37 °C, this probably corresponds to the fast process we are observing at 30 °C (2.9×10^{-3} s^{-1}). They did not publish measurements at times greater than 17 min, which would have been the relevant time domain to reveal the slow phase described in this work.

Rensland et al. (1991) also showed that, in the presence of ~50 nM GAP, the fluorescence change occurring with p21$^{H\text{-}ras}$·mantGTP is accelerated to give a reaction with a half time of about 50 s. By contrast, with p21$^{H\text{-}ras}$·mantGpp[NH]p the fluorescence decrease in the presence of GAP remains with a half time of 100 s. With p21$^{H\text{-}ras}$·mantGTP(γS), the zero change of fluorescence in the absence of GAP changes to a 15% decrease in fluorescence with a half time of about 10 min. Rensland et al. (1991) conclude from these results that a conformational change does not precede and control the cleavage step by p21$^{H\text{-}ras}$·mantGTP hydrolysis.

Apart from the different time domains of the p21·mantGpp-[NH]p data of the two studies, there are several other possible reasons for the discrepancies. They could be due to differences between p21$^{N\text{-}ras}$ and p21$^{H\text{-}ras}$ or differences between buffer

conditions. No details of the latter are given by Rensland et al. (1991). They may also be due to differences in the preparation of the complexes, since as they point out, the exact method of preparation results in complexes containing different proportions of the 2′ and 3′ mant isomers.

The most likely reason for the discrepancy, however, is that the two studies of the p21·mantGpp[NH]p complex were made over two diferent time scales, as noted above. Rensland et al. only measured the fluorescence process over 17 min and would not have observed the slower process described in this work. Their finding that GAP does not affect the fast phase is not in disagreement with our results.

Despite the possible differences in the results presented here and those of Rensland et al. (1991), we believe that the careful control of instrumental artifacts over long time periods in the present study, combined with characterization of the chemical state of the nucleotides in the complex with respect to the state of its phosphorylation and the position of the mant isomer, and proof of no dissociation of nucleotide occurring from the complexes provide strong evidence that an isomerization controls the rate of the p21·mantGTP cleavage step and that this is accelerated by GAP.

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