Biochem. J. (1992) 287, 555–559 (Printed in Great Britain)

Interaction of GTPase-activating protein with p21^{ras}, measured using a continuous assay for inorganic phosphate release

Martin R. WEBB* and Jackie L. HUNTER
National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K.

The mechanism of GTPase-activating protein (GAP) activation of p21^{ras} GTP hydrolysis has been investigated by measuring the kinetics of release of P_i during the hydrolysis. The measurement uses a continuous spectroscopic assay for P_i, based on a guanosine analogue, 2-amino-6-mercapto-7-methylpurine ribonucleoside, as substrate for purine nucleoside phosphorylase [Webb, M. R. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 4884–4887]. This phosphorolysis gives an absorbance increase at 360 nm, so that when the reaction is coupled to GTP hydrolysis, the change in absorbance gives the total amount of P_i released from the p21^{ras}. The rate of the absorbance increase gives the GTPase activity. This provides a non-radioactive method of determining p21^{ras} concentration and GAP activity. It was used to determine the interaction of GAP with wild-type p21^{ras} and two mutants (Leu-61/Ser-186 and Asp-12), all in the GTP (or guanosine 5′-[$\beta\gamma$-imido]triphosphate) form. The Leu-61/Ser-186 mutant binds 10-fold tighter than does the wild-type protein. The Asp-12 mutant binds to GAP with the same affinity as the wild-type protein. A novel GTPase activity was characterized whereby the EDTA-induced nucleotide release and GAP-activated cleavage of bound GTP leads to steady-state turnover of GTP hydrolysis. An assay for GAP is described based on this activity.

INTRODUCTION

p21^{ras} is a member of a family of low-molecular-mass proteins that bind guanine nucleotides tightly, have low GTPase activity and show a high degree of sequence identity. The mechanism of action of p21^{ras} is of considerable interest, both because of its postulated role in cellular growth control, and because single point mutants of ras are found in a wide variety of cancers. Although some features of the protein are known in great detail, for example the crystal structure (de Vos *et al.*, 1988; Krengel *et al.*, 1990; Schlichting *et al.*, 1990), the cellular role(s) of the protein remains obscure. The general mechanism is likely to be similar to that of the heterotrimeric G proteins, for which the GTP-bound form is active for cell signalling and the GDP-bound form is inactive.

GTPase-activating protein (GAP) accelerates the formation of protein-bound GDP from protein-bound GTP for wild-type p21^{ras}, although GTP hydrolysis is not accelerated for a variety of mutants (Trahey & McCormick, 1987). GAP has been implicated either as part of the effector system of the ras signal transduction pathway or as a regulator of ras activation (Hall, 1990). We wish to understand the molecular mechanism of the interconversion of active and inactive forms of p21^{ras}, and how this interconversion is modulated by other proteins in the ras system, such as GAP.

We describe here the use of a new spectroscopic assay for inorganic phosphate (P_i) (Webb, 1992) to probe GAP-activated GTP hydrolysis. The measurements are based on coupling the GTP hydrolysis reaction to the reaction catalysed by purine nucleoside phosphorylase (EC 2.4.2.1) using 2-amino-6-mercapto-7-methylpurine ribonucleoside ('methylthioguanosine'; MESG), a guanosine analogue, as substrate (Fig. 1). This reaction is essentially irreversible and gives an absorbance increase with a change in molar absorption coefficient of 11000 $M^{-1} \cdot cm^{-1}$ at 360 nm, well away from absorbance of guanine nucleotides and proteins. Based on steady-state measurements, the K_m for P_i is 26 μM and the $k_{cat.}$ is 40 s^{-1} at 25 °C (Webb, 1992). Measurements under single turnover conditions with a slightly different GAP construct suggest that the maximum rate at 30 °C is $> 80\ s^{-1}$ (A. E. Nixon & M. R. Webb, unpublished work).

The MESG/phosphorylase system is used with the p21^{ras} GAP system to quantify p21^{ras}, to assay GAP activity and to investigate the interaction of GAP with a variety of p21^{ras}·nucleotide complexes. A novel GTPase activity of GAP and p21^{ras} is also described.

EXPERIMENTAL

Bacterial purine nucleoside phosphorylase was from Sigma. The protein was approximately one-third phosphorylase by gel electrophoresis, and for some experiments was purified further on Q-Sepharose. p21$^{N\text{-}ras}$ (wild-type and Asp-12 mutant), p21$^{H\text{-}ras}$ (Leu-61,Ser-186 mutant and the *C*-terminal fragment of GAP (amino acids 684–1047) were expressed in *Escherichia coli* and purified as described previously (Hall & Self, 1986; Hart & Marshall, 1990). The expression systems were a gift from Dr. A. Hall, Institute of Cancer Research, London, U.K. The protein concentration of GAP was determined by the BCA Protein Assay (Pierce Chemical Company) (Smith *et al.*, 1985). The

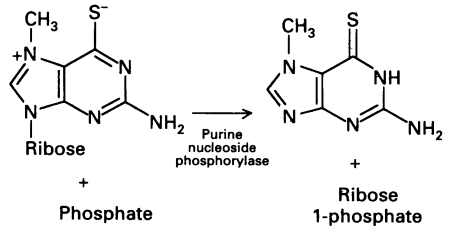

Fig. 1. Reaction catalysed by purine nucleoside phosphorylase

Abbreviations used: GAP, GTPase-activating protein; MESG, 2-amino-6-mercapto-7-methylpurine ribonucleoside; p[NH]ppG, guanosine 5′[$\beta\gamma$-imido]triphosphate; R·GTP, the complex of GTP with p21^{ras} (other nucleotide complexes are similarly abbreviated).

* To whom correspondence should be addressed.

protein was > 90% pure by gel electrophoresis. GTP complexes of $p21^{ras}$ were obtained by a 10 min incubation of a large excess of GTP with the protein in the presence of $(NH_4)_2SO_4$ and EDTA (Hoshino *et al.*, 1987). After quenching the exchange by adding excess Mg^{2+} over EDTA, the protein was desalted and excess GTP was removed on a column of Bio-Gel P6, in the buffer required for the GTP hydrolysis. Other nucleotide complexes were prepared in a similar way. Except where indicated, wild-type $p21^{N\text{-}ras}$ was used.

MESG was synthesized and purified as previously described (Broom & Milne, 1975; Webb, 1992).

The nucleotides bound to $p21^{ras}$ and the GTP remaining in solution during GTPase activity were determined by h.p.l.c. The column was a Partisil-10 SAX (0.4 cm × 25 cm) (Whatman), eluted at 2 ml·min^{-1} with 0.5 M-$(NH_4)_2HPO_4$ adjusted to pH 4.0 with HCl.

$p21^{ras}$ assay

The assay to measure wild-type $p21^{ras}$ concentration was as follows. To form the GTP complex, a mixture of 4–40 μg of $p21^{ras}$ (which should contain less than 10 nmol of free Mg^{2+}, if necessary achieved by adding extra EDTA) and 2.5 μl of 20 mM-GTP was made up to 80 μl with 20 mM-Tris/HCl/1 mM-EDTA/5 mM-dithiothreitol, pH 7.5. After 10 min of incubation at 30 °C, 2.5 μl of 100 mM-$MgCl_2$, 2.5 μl of purine nucleoside phosphorylase (500 units·ml^{-1}) and 5 μl of 4 mM-MESG were added and the absorbance was monitored at 360 nm using a Beckman DU70 spectrophotometer with a 1 cm-pathlength, 2 mm-width cuvette. The reaction was initiated by the addition of 1 μl of ~ 100 μM-GAP. The concentration of $p21^{ras}$ was determined using the relationship determined from a solution of P_i of known concentration (Webb, 1992):

$$[p21^{ras}](\mu M) = 91 \times \Delta A \cdot cm^{-1} \times \text{dilution factor}$$

GAP activity assay

The following (in 100 μl) were mixed in a 2 mm-width, 10 mm-pathlength cuvette: 20 mM-Tris/HCl buffer, pH 7.5, 1 mM-EDTA, 10 mM-ammonium sulphate, 0.2 mM-dithiothreitol, 0.2 mM-MESG, purine nucleoside phosphorylase (12.5 units·ml^{-1}), 4 μM-$p21^{ras}$ and 0.5 mM-GTP. The absorbance was monitored at 360 nm. After ~ 2 min, the reaction was initiated by addition of 5 μl of GAP. The slope of the absorbance increase is proportional to the GAP activity:

$$p21^{ras}\cdot GTP \text{ hydrolysed } (\mu M \cdot s^{-1}) = 91 \times \Delta A \cdot cm^{-1} \cdot s^{-1}$$

Control experiments were performed at higher GAP concentrations to ensure that the GAP-catalysed reaction, rather than the release of bound GDP, is the rate-limiting process (see the Discussion section).

RESULTS

The MESG/phosphorylase assay was developed to measure the amounts of P_i in solution and the kinetics of P_i release from biological systems, such as GTPases and ATPases (Webb, 1992). The method was applied to the ras–GAP system, first showing that it could be used to measure the concentration of active $p21^{ras}$ in solution and the activity of GAP. The work in this paper uses the *C*-terminal domain fragment of GAP (amino acids 688–1047) expressed in *E. coli*, that interacts with $p21^{ras}$ (Marshall *et al.*, 1989; Hart & Marshall, 1990).

The reaction catalysed by the phosphorylase was coupled to GAP-catalysed hydrolysis of GTP bound to $p21^{ras}$, to measure rapidly and simply the total concentration of $p21^{ras}$·GTP complex (R·GTP) and the rate of P_i release from the catalytic site of the $p21^{ras}$. Fig. 2 shows the absorbance traces for three different reactions. The amount of R·GTP is the same and the reaction is initiated by addition of different amounts of GAP. The total absorbance change can be used to calculate [R·GTP] as described in the Experimental section.

The sloping baselines in Fig. 2 are caused by the slow breakdown of MESG to its purine base in the presence of an impurity in the $p21^{ras}$ solutions. This impurity has not been found in other proteins tested with this assay system, and it varies from one $p21^{ras}$ preparation to another. It is partly due to oxidation products of dithiothreitol, so that in most subsequent experiments the level of added thiols was decreased.

The measurement of phosphate release was developed into an assay to quantify wild-type $p21^{ras}$, and a set of assay conditions is given in the Experimental section. As prepared from *E. coli*, the nucleotide bound to $p21^{ras}$ is >80% GDP and 2′-deoxy-GDP. In the presence of EDTA (i.e. very low free $[Mg^{2+}]$), nucleotide release is fast (Hall & Self, 1986; Hoshino *et al.*, 1987), so that a short incubation of $p21^{ras}$ with excess GTP gives the R·GTP complex. Addition of excess $MgCl_2$ quenches this exchange reaction. The R·GTP can then be hydrolysed by GAP, with coupled measurement of P_i release as described above. The response is linear up to 17 μM-$p21^{ras}$ (Fig. 3); concentrations above this were not tested. The non-zero intercept is due to a

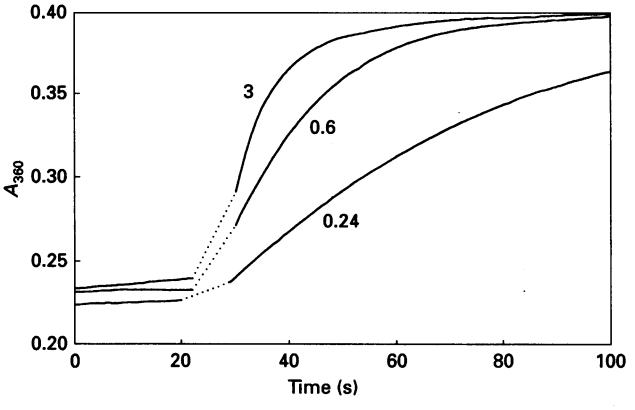

Fig. 2. P_i release from the GTP complex with ras, initiated by GAP, as measured by absorbance at 360 nm with the MESG assay system

The reaction mixture (0.5 ml) at 30 °C contained 20 mM-Tris/HCl buffer, pH 7.6, 1 mM-$MgCl_2$, 5 mM-dithiothreitol, 14.5 μM-R·GTP, purine nucleoside phosphorylase (2.5 units·ml^{-1}), and 85 μM-MESG. The reaction was initiated by adding GAP at the micromolar concentrations indicated. The dotted lines represent the period of addition and mixing.

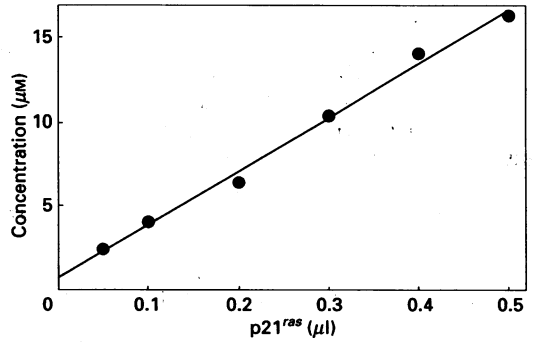

Fig. 3. Dependence of $p21^{ras}$ concentration, as measured by GAP-catalysed phosphate release, on the amount of p21 added to the assay solution

Assay conditions are described in the Experimental section.

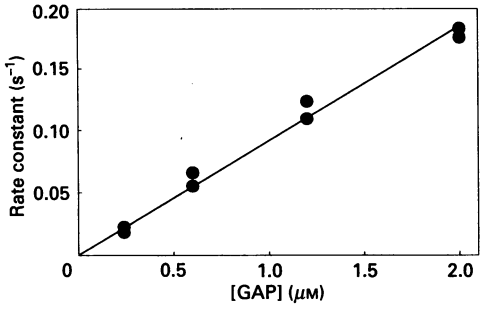

Fig. 4. Rate of P_i release from the GTP complex with p21^{ras} as a function of GAP concentration

The conditions were as in Fig. 3.

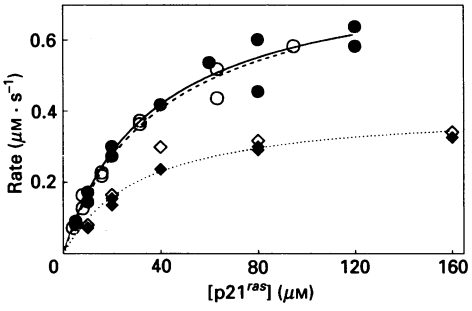

Fig. 5. Rate of GTP hydrolysis as a function of p21^{ras} concentration

The conditions were as follows: ●, 80 μl of solution at 30 °C, containing 20 mM-Tris/HCl buffer, pH 7.5, 5 mM-dithiothreitol, 1 mM-$MgCl_2$, phosphorylase (12.5 units · ml^{-1}), 0.2 mM-MESG and R·GTP. The reaction was initiated by addition of 0.4 μM-GAP and the rate of P_i release was measured. The data shown by ○ were from similar solutions, except the $MgCl_2$ was replaced by EDTA and GTP was present at 400 μM for [p21^{ras}] < 40 μM, and at 800 μM for [p21^{ras}] > 40 μM. The initial rates were measured at 360 nm. The lines are the best fit for Michaelis–Menten kinetics. The solid line is $+Mg^{2+}$ (K_m = 36.5 μM; $V_{max.}$ = 0.80 μM · s^{-1}); the broken line is +EDTA (K_m = 39.5 μM; $V_{max.}$ = 0.81 μM · s^{-1}). The steady-state rates of phosphate release were measured for similar solutions, except that the p21^{ras} was added as the GDP complex. The rate of hydrolysis was measured by h.p.l.c. of nucleotides (◇) or by P_i release (◆). The dotted line is the best fit for Michaelis–Menten kinetics (K_m = 32 μM; $V_{max.}$ = 0.40 μM · s^{-1}).

small amount of P_i in the GAP preparation (approximately equimolar with GAP), which can be removed by gel filtration. Advantages and limitations of this assay are described in the Discussion section.

The curves in Fig. 2 were fitted to single exponentials to obtain first-order rate constants for P_i release at different GAP concentrations. For the assay conditions used, the rate constants were linear with [GAP] up to ~ 0.2 s^{-1} (Fig. 4).

In order to investigate the interaction of GAP with p21^{ras}, the initial rate of P_i release was measured as a function of [R·GTP] at a fixed GAP concentration (Fig. 5). The reaction can be treated as following Michaelis–Menten kinetics, with GAP acting as the enzyme catalyst, R·GTP as the substrate and R·GDP and P_i as the products. The data were fitted to give a K_m for R·GTP of 36.5 μM and a $k_{cat.}$ for P_i release of 2 s^{-1}. The values of $k_{cat.}$ in this paper assume that the GAP is fully active, although there is currently no recognized way to determine this. The measured K_m values are independent of the assumed percentage activity of GAP.

The interaction of several mutant p21^{ras} nucleotide complexes

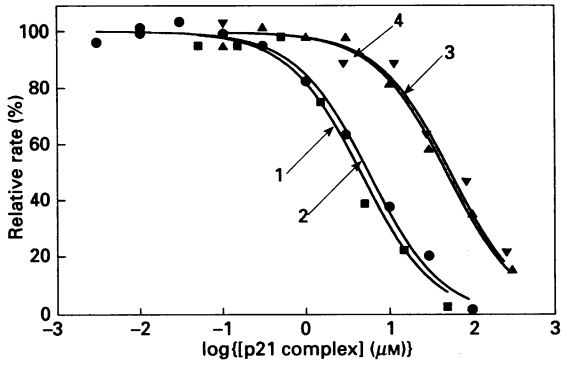

Fig. 6. Interaction of non-hydrolysable p21^{ras}·nucleotide complexes with GAP, as measured by inhibition of GAP-stimulated wild-type p21$^{N\text{-}ras}$·GTP hydrolysis

The conditions were as in Fig. 5 ($+Mg^{2+}$). Wild-type R·GTP was at 15 μM. p21$^{H\text{-}ras}$ (Leu-61/Ser-186 mutant) as the GTP complex (●) and p[NH]ppG complex (■) are based on a filter-binding assay, assuming that this gives 30% of the true concentration. Forming a 1:1 complex of 2′(3′)-methylanthraniloyl-GTP with this mutant and measuring the fluorescence intensity (Neal *et al.*, 1990) indicates that the estimated concentration is correct. The lines are the best fit for K_I = 5.7 μM ([Leu^{61}]p21^{ras}·GTP complex; curve 1), 4.6 μM ([Leu^{61}]p21^{ras}·p[NH]ppG complex; curve 2), 56 μM ([Asp^{12}]p21^{ras}·GTP complex; curve 3), and 50 μM (p21^{ras}·p[NH]ppG complex; curve 4).

with GAP was also determined. p21$^{H\text{-}ras}$ (Leu-61, Ser-186 mutant) in the GTP-bound form binds tightly to GAP, but the GTP hydrolysis is only very weakly accelerated by GAP. This complex inhibits the hydrolysis of wild-type R·GTP by sequestration of GAP. By choosing a concentration of wild-type R·GTP below the K_m value, where the P_i release fits an exponential, the rate of hydrolysis is approximately proportional to the concentrations of both wild-type R·GTP and free GAP. As GAP is complexed by increasing concentrations of the mutant, the rate of wild-type R·GTP hydrolysis decreases. This is shown in Fig. 6, and the best-fit line gives a K_i of 5.7 μM. Similar data are also shown for the guanosine 5′-[$\beta\gamma$-imido]triphosphate (p[NH]ppG) complex with this Leu-61 mutant. Replacing the GTP with the non-hydrolysable analogue had no measurable affect on the steady-state interaction with GAP. Results with the p[NH]ppG complex of wild-type p21^{ras} and the GTP complex of an Asp-12 mutant show that these bind with affinities similar to each other, but 10-fold weaker than the Leu-61 mutant. The inhibition constants (Fig. 6) are approximately the same as the K_m for wild-type R·GTP measured under similar conditions.

A novel GTPase activity of GAP + p21^{ras} in the presence of EDTA was investigated by measurement of P_i release and GDP formation. In the presence of excess EDTA, GAP + p21^{ras} catalyse the multiple turnover of GTP to GDP + P_i. The reaction was linear up to ~ 50% hydrolysis at 500 μM-GTP. After that the slope decreased, presumably due to product GDP inhibition. This turnover arises because of the acceleration of nucleotide release by EDTA.

A detailed study of the early part of the reaction using P_i release measurements showed two phases. If the nucleotide initially bound to p21^{ras} was GTP, there was a rapid phase followed by a slower (of approximately half the initial rate) steady-state reaction. If the initially bound nucleotide was GDP, there was a lag phase, followed by the steady-state rate, which was similar to the steady-state rate with initially bound GTP. The steady-state rates observed by measurement of P_i release were similar to the rates of GTP loss measured by h.p.l.c.

These results suggest that there is a balance of velocities for

hydrolysis and GDP release. When R·GTP is present initially it is hydrolysed to a lower steady-state level. This model is supported by measuring the steady-state rates of P_i release (and hence GTP turnover) at a fixed initial R·GDP concentration with excess free GTP and increasing the GAP concentration. At 10 μM-R·GDP and up to ~ 1 μM-GAP the steady-state rate is proportional to [GAP]. Above this the rate becomes independent of GAP concentration, presumably limited by GDP release.

In the presence of 10 mM-ammonium sulphate in addition to the EDTA, the rate is linear over a much wider range of GAP concentrations (up to 10 μM) at 1 μM-R·GDP or R·GTP; there is no difference between traces depending on which nucleotide is initially bound. This is presumably due to the much faster rate of nucleotide release induced by EDTA + sulphate, compared with each alone (Hoshino *et al.*, 1987). This enables a large range of GAP activities to be assayed, and this was developed into an assay for GAP activity as described in the Experimental section.

The dependence of the steady-state rates of P_i release on the initial R·GTP concentration is shown in Fig. 5. These data fit Michaelis–Menten kinetics with a K_m of 32 μM for $p21^{ras}$ and a $k_{cat.}$ of 1.0 s^{-1} relative to GAP. The K_m is similar to that obtained from the initial-rate data, but the $k_{cat.}$ is decreased by approx. 50%. This decrease may be due to modified kinetics of the cleavage step in the presence of EDTA.

DISCUSSION

The MESG/phosphorylase assay to quantify $p21^{ras}$ is described in the Experimental section. Current methods of measuring GAP-activated hydrolysis of GTP bound to $p21^{ras}$ depend on radioactive nucleotides and filter-binding, t.l.c. or h.p.l.c. for analysis of the extent of hydrolysis at single time points (e.g. Gibbs *et al.*, 1988). Concentrations of $p21^{ras}$ are normally determined by a filter-binding assay using radioactively labelled nucleotide (e.g. Manne *et al.*, 1984). The use of the phosphate release assay described here for $p21^{ras}$ has a number of advantages. It is non-radioactive and specific for $p21^{ras}$. Other guanine nucleotide-binding proteins, such as elongation factor Tu, will not give false positive readings, unlike the filter-binding assay. Most importantly, it is quantitative. In our hands the filter-binding assay is not quantitative, possibly due to low protein binding to the filter. In particular, different batches of filters from the same manufacturer give up to 5-fold differences in values of bound radioactivity. We find that filter-binding assays of $p21^{N\text{-}ras}$ optimally give only one-third to one-quarter of the concentration found by the P_i release assay. Other assays based on total protein, amino acid analysis or binding of fluorescent guanine nucleotide analogues support the accuracy of the phosphate release assay (K. Moore & J. F. Eccleston, personal communication).

The sensitivity of our assay is about 10-fold less than for the filter-binding assay, and so it is not suitable for dilute protein solutions. The assay requires that the GTP hydrolysis is GAP-activated, and therefore it can be applied to the wild-type and some mutant ras proteins. A large amount of $MgCl_2$ would complicate the assay, as excess EDTA over Mg^{2+} is required to accelerate nucleotide exchange. This would cause an increase in ionic strength, which in turn decreases the effect of GAP. Experiments show that increasing the ionic strength from 25 mM to 185 mM by addition of NaCl causes a 13-fold decrease in the second-order rate of R·GTP hydrolysis catalysed by GAP.

The coupling of phosphate release to the MESG/phosphorylase system has provided a way to measure the interaction of GAP with $p21^{ras}$. The data are treated here as following Michaelis–Menten kinetics. This assumes that the rate-limiting transformation of R·GTP is accelerated by GAP at all substrate concentrations. In this case, this assumption appears to be valid. The limitations of this model for GAP activation become apparent for the EDTA-dependent GTPase activity, which includes slow nucleotide release (see below). The basic kinetic data have been obtained, showing a K_m of 30–40 μM for wild-type $p21^{N\text{-}ras}$ in the presence of Mg^{2+} at low ionic strength and 30 °C. The $k_{cat.}$ of 2 s^{-1} relates to P_i release and shows that GAP can accelerate the GTP hydrolysis of R·GTP ~ 10^4-fold relative to the basal rate (Neal *et al.*, 1988). The current data do not indicate which step controlling P_i release is rate limiting. It has previously been shown that a protein conformation change of R·GTP precedes hydrolysis and limits the rate of hydrolysis (Neal *et al.*, 1990). This step is the potential location of GAP activation. The value of $k_{cat.}$ obtained here is 5–10-fold lower than other estimates (John *et al.*, 1990; Antonny *et al.*, 1991) and so may reflect the possibility that P_i release becomes rate limiting at very high GAP activation.

This method allows the continuous assay of P_i release over the complete time course, and so provides an exact measure of GAP activation, or inhibition by a mutant. Using this technique, we showed that the GTP complex of a mutant H-ras (Leu-61,Ser-186) binds to GAP ~ 10-fold more tightly than does wild-type $p21^{ras}$, although its GTP was not hydrolysed under the conditions of this experiment. Replacing GTP with p[NH]ppG did not affect the steady-state interaction of $p21^{ras}$ with GAP. An Asp-12 mutant bound GAP with similar affinity to the wild-type $p21^{N\text{-}ras}$.

The GTPase activity of $p21^{ras}$ + GAP with EDTA provides a novel way of obtaining multiple turnovers of both proteins. Either GAP can accelerate the GTP hydrolysis without co-ordination of Mg^{2+} to the nucleotide, or R·GTP binds Mg^{2+} sufficiently tightly to compete with the EDTA. Experiments at 10-fold higher concentrations of EDTA still show GTP hydrolysis, with only the rate decrease expected for the increase in ionic strength. If Mg^{2+} remains bound to the nucleotide complex with $p21^{ras}$, it must be in relatively rapid exchange with solution (Reinstein *et al.*, 1991) to allow acceleration of nucleotide release.

Addition of 10 mM-ammonium sulphate greatly accelerated the observed rate of hydrolysis at high GAP concentrations, presumably due to acceleration of nucleotide release. These represent better conditions for an assay for GAP activity. The rate is proportional to GAP concentrations over a wide range. There is steady-state GTP hydrolysis and hence linear absorbance change with time, directly related to the added GAP. This is much easier to measure than the exponential absorbance rise observed in the single-turnover conditions in the presence of high concentrations of free Mg^{2+}. A set of conditions for the GAP activity assay are given in the Experimental section.

In summary, this paper demonstrates the value of measuring P_i release in the micromolar range in reactions in biological systems. This allows measurement of the concentration of R·GTP complex, by measuring the total phosphate release on hydrolysis. Rate measurements give information about the interaction of $p21^{ras}$ with GAP. The GTPase activity in the presence of EDTA and ammonium sulphate provides a way to assay GAP.

We thank Dr. A. Hall (Institute of Cancer Research, London) for the gift of expression systems and Dr. D. R. Trentham (N. I. M. R., London) for helpful discussions. This work was supported by the Medical Research Council, the European Community Twinning Scheme, and the Human Frontiers Science Program.

REFERENCES

Antonny, B., Chardin, P., Roux, M. & Chabre, M. (1991) Biochemistry **30**, 8287–8295

Broom, A. D. & Milne, G. H. (1975) J. Heterocycl. Chem. **12**, 171–174
de Vos, A. M., Tong, L., Milburn, M. V., Matias, P. M., Jancarik, J., Noguchi, S., Nishimura, S., Miura, K., Ohtsuka, E. & Kim, S. H. (1988) Science **239**, 888–893
Gibbs, J. B., Schaber, M. D., Allard, W. J., Sigal, I. S. & Scolnick, E. M. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 5026–5030
Hall, A. (1990) Cell **61**, 921–923
Hall, A. & Self, A. J. (1986) J. Biol. Chem **261**, 10963–10965
Hart, P. A. & Marshall, C. J. (1990) Oncogene **5**, 1099–1101
Hoshino, M., Clanton, D. J., Shih, T. Y., Kawakita, M. & Hattori, S. (1987) J. Biochem. (Tokyo) **102**, 503–511
John, J., Sohmen, R., Feuerstein, J., Linke, R., Wittinghofer, A. & Goody, R. S. (1990) Biochemistry **29**, 6058–6065
Krengel, U., Schlichting, I., Scherer, A., Schumann, R., Frech, M., John, J., Kabsch, W., Pai, E. & Wittinghofer, A. (1990) Cell **62**, 539–548
Manne, V., Yamazaki, S. & Kung, H.-F. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 6953–6957
Marshall, M. S., Hill, W. S., Ng, A. S., Vogel, U. S., Schaber, M. D., Scolnick, E. M., Dixon, R. A. F., Sigal, I. S. & Gibbs, J. B. (1989) EMBO J. **8**, 1105–1110
Neal, S. E., Eccleston, J. F., Hall, A. & Webb, M. R. (1988) J. Biol. Chem. **263**, 19718–19722
Neal, S. E., Eccleston, J. F. & Webb, M. R. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 3562–3565
Reinstein, J., Schlichting, I., Frech, M., Goody, R. S. & Wittinghofer, A. (1991) J. Biol. Chem. **266**, 17700–17706
Schlichting, I., Almo, S. C., Rapp, G., Wilson, K., Petratos, K., Lentfer, A., Wittinghofer, A., Kabsch, W., Pai, E., Petsko, G. A. & Goody, R. S. (1990) Nature (London) **245**, 309–315
Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985) Anal. Biochem. **150**, 76–85
Trahey, M. & McCormick, F. (1987) Science **238**, 542–545
Webb, M. R. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 4884–4887

Received 6 April 1992; accepted 30 April 1992