Is There a Rate-Limiting Step before GTP Cleavage by H-*ras* p21?

Hans Rensland, Alfred Lautwein, Alfred Wittinghofer, and Roger S. Goody*
Abteilung Biophysik, Max-Planck-Institut für Medizinsiche Forschung, Jahnstrasse 29, 6900 Heidelberg, FRG
Received May 28, 1991; Revised Manuscript Received August 29, 1991

ABSTRACT: A slow fluorescence change of the complex between *ras* p21 and the fluorescent GTP analogue 2′(3′)-O-(*N*-methylanthraniloyl)guanosine 5′-triphosphate (mGTP) has been postulated to be a signal arising from a step which is rate limiting and precedes the actual GTP hydrolysis reaction [Neal, S. E., Eccleston, J. F., & Webb, M. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3562–3565]. We have now shown that the rate of the fluorescence change is accelerated by GTPase-activating protein (GAP) in the same manner as that of the GTP cleavage reaction. In contrast, a faster fluorescence change of smaller amplitude seen in the complex between p21 and the uncleavable 2′(3′)-*O*-(*N*-methylanthraniloyl)guanosine 5′-*O*-(β,γ-imidotriphosphate) (mGppNHp) is not affected by GAP. The corresponding fluorescent derivative of guanosine 5′-*O*-(γ-thiotriphosphate) (mGTPγS) shows a very slow fluorescence change after binding to p21, and this rate is also accelerated significantly by GAP. Hydrolysis of GTPγS is similarly slow, and it is accelerated by GAP in a similar manner to the fluorescence change. The results are interpreted to indicate that the fluorescence change occurs either at the hydrolysis step or on release of inorganic phosphate or thiophosphate but does not occur in a rate-limiting step preceding hydrolysis.

p21 proteins, the products of the *ras* onco- and proto-oncogenes, are guanine nucleotide binding proteins with a very slow intrinsic rate of GTP hydrolysis. This rate can be accelerated maximally by a factor of ca. 10^5 by GAP (John, 1990; Gideon et al., unpublished results), which appears to be a negative regulator of p21 and possibly the downstream effector of p21 action. It is known that point mutations at certain characteristic sites in the sequence increase transforming activity of the protein. Most of these mutations lead to an increase in the lifetime or the relative concentration of the p21–GTP state, either because of a reduction of the GAP-activated GTPase or because of an increase in the rate of GDP/GTP exchange.

To understand both the mechanism of signal transduction and the mechanism of oncogenic transformation in this system, detailed studies of the structures of its components and of their mechanism of action are needed. These have taken the form of high-resolution structural investigations (Pai et al., 1989, 1990; Tong et al., 1989; Milburn et al., 1990), and attempts are presently being made to extend these with dynamic studies both in solution and in crystals of p21 (John et al., 1990; Schlichting et al., 1990).

It has recently been suggested that a slow, rate-limiting step occurs before the actual GTP hydrolysis step of p21 (Neal et al., 1990). This was based on a slow change in the fluorescence of a GTP analogue, 2′(3′)-(*N*-methylanthraniloyl)-GTP [2′-(3′)-mGTP],[1] after binding to p21. This change occurred at the same rate as the overall rate of GTP hydrolysis on the enzyme. Since a change at a similar rate was also observed using the corresponding fluorescent derivative of the non-hydrolyzable GTP analogue GppNHp, it was concluded that the fluorescence change occurred in a rate-limiting step before the actual cleavage step, which involves attack of water on the γ-phosphate group of GTP. This mechanism implies that the actual GTP cleavage step is much faster than the observed rate of GTP hydrolysis. While this interpretation was consistent with the results obtained, it appeared to us that a crucial test of the suggestion was whether the rate of the slow fluorescence change was accelerated by GAP, in the case of mGTP as well as mGppNHp. In the course of investigating this question, more details on the interaction of the individual isomers of the methylanthraniloyl analogues with p21 were obtained. We show that the interpretation in its original form cannot be upheld.

Materials and Methods

p21 was prepared as previously described using an *Escherichia coli* expression system (Tucker et al., 1986). Nucleotide-free p21 was prepared as described (John et al., 1990). *N*-Methylanthraniloyl derivatives of GDP, GTP, dGDP, dGTP, GppNHp, and dGppNHp were prepared essentially as described (John et al., 1990). The corresponding derivatives of 3′,5′-cyclic GMP and of GTPγS were prepared in the same manner.

The C-terminal domain of human GTPase-activating protein (GAP), corresponding to amino acids 714–1047 and which is referred to here as GAP_{334} (F. McCormick, unpublished results), was expressed in *E. coli* from the expression vector ptrc 99A (Amman et al., 1988). It was purified using a two-column procedure involving DEAE-Sepharose and gel filtration (Gideon et al., unpublished results).

Filter-binding assays to monitor the interaction of [^{3}H]GDP with p21 were performed as previously described (Feuerstein et al., 1987a,b). Kinetic data were analyzed by nonlinear regression, normally using a single-exponential function, with the program Enzfitter (Elsevier Biosoft) or Grafit (Erithacus Software).

Static and slow time scale dynamic fluorescent measurements were done using an SLM 8000 spectrophotometer. Excitation and emission wavelengths were normally 370 and 440 nm, respectively. Nucleotides were separated analytically by reversed-phase HPLC as described previously (John et al., 1990) using a UV absorption detector or a fluorescence detector for methylanthraniloyl nucleotides with excitation and emission wavelengths of 370 and 440 nm, respectively.

[1] Abbreviations: p21, the protein product of the human c-Ha-*ras* protooncogene; GppNHp, guanosine 5′-(β,γ-imidotriphosphate); GTPγS, guanosine 5′-(γ-thiotriphosphate); 2′(3′)-mGDP/GTP, 2′(3′)-(*N*-methylanthraniloyl)guanosine di/triphosphate; 3′-mdGDP/GTP, 3-(*N*-methylanthraniloyl)-2′-deoxyguanosine di/triphosphate.

0006-2960/91/0430-11181$02.50/0

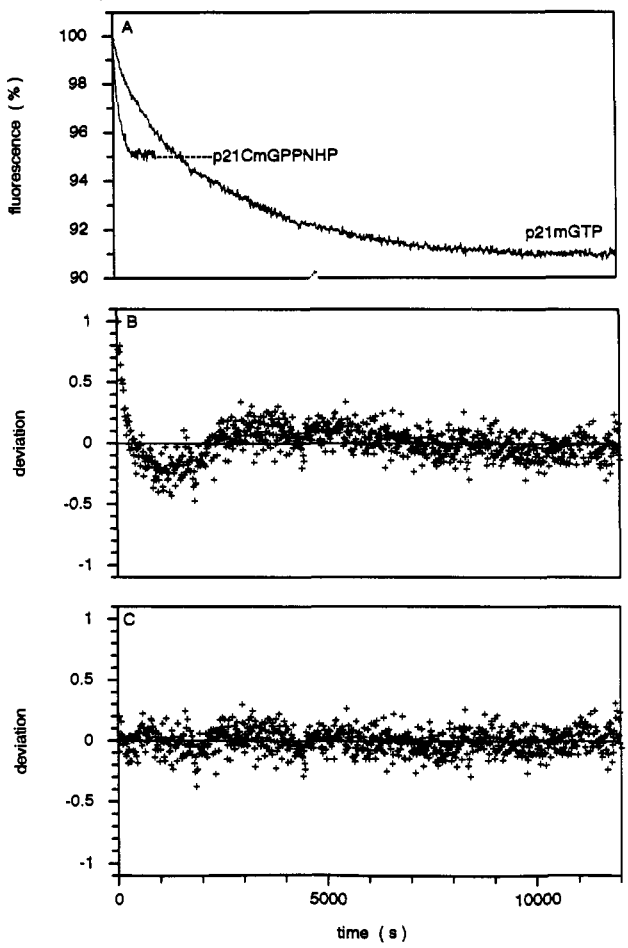

FIGURE 1: (A) Slow fluorescence transient seen on increasing the temperature of solutions of p21–mGTP and p21–mGppNHp from 0 to 37 °C. Excitation wavelength: 370 nm; emission wavelength: 440 nm. p21–mGTP concentration: 5 μM. (B) Residuals (i.e., the difference between each data point and the fitted curve) for a single-exponential fit to the mGTP curve with $k = 4.1 \times 10^{-4}$ s^{-1}. (C) Residuals for a double-exponential fit with $k_1 = 2.7 \times 10^{-3}$ s^{-1} and $k_2 = 3.8 \times 10^{-4}$ s^{-1}.

RESULTS

Slow Fluorescent Transient after Binding of Methylanthraniloyl Nucleotides to p21. In our earlier work on the interaction of methylanthraniloyl nucleotides with H-*ras* p21 (John et al., 1990), we had not observed a slow decrease of fluorescence after the initial large increase associated with binding of the nucleoside triphosphates to p21 as reported by Neal et al. (1990). Our studies were done with methylanthraniloyl derivatives of the 2′-deoxynucleotides, since 2′-O- and 3′-O-acylated ribonucleotides are known to equilibrate with each other (Trentham & Reese, 1965), and it seemed preferable to avoid potential problems caused by working with such a mixture. The work of Neal et al. (1990) prompted us to reinvestigate this question, and we were able to confirm that no such fluorescence change occurs when p21 interacts with 3′-(*N*-methylanthraniloyl)-2′-dGTP. Experiments with 2′(3′)-mGTP, in which nucleotide-free p21 was mixed with an equimolar ratio of the fluorescent nucleotide, showed a significant slow fluorescence change, but a larger and more easily observable change was seen using the protocol of Neal et al.(1990), which involves incubation of an excess of 2′(3′)-mGTP with p21 at low temperature, followed by rapid gel filtration to remove excess nucleotide and to isolate the 1:1 protein–nucleotide complex. The reaction is then started in a fluorescence cuvette by increasing the temperature to 37 °C

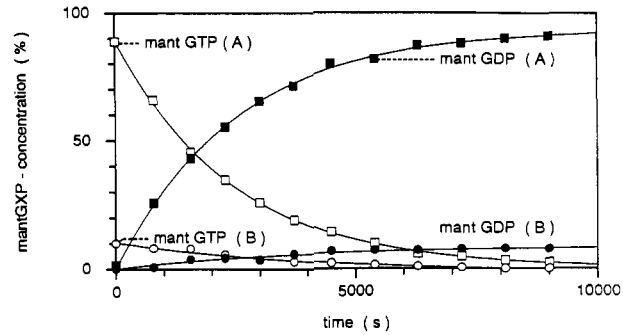

FIGURE 2: Time course of cleavage of the isomers of mGTP after increasing the temperature from 0 to 37 ° C. Samples were analyzed by HPLC at the times shown.

(Figure 1A). Using the same protocol with 2′(3′)-mGppNHp also leads to a slow change in fluorescence, but the amplitude is significantly smaller than for 2′(3′)-mGTP, and the rate is significantly higher (Figure 1A).

Structural Assignment of the 2′- and 3′-Isomers of 2′-(3′)-mGTP and Interaction of the Individual Isomers with p21. Since there was a difference in the behavior of mGTP and 3′-mdGTP with p21, it was thought that this might be due to the possibility of isomerization of the 2′- and 3′-acylated isomers of the ribose derivative. This was checked using reversed phase HPLC. The isomers could be separated on an analytical scale and were found to be present at a ratio of 60:40 (A:B) in the absence of p21 at neutral pH (isomer A is that eluting earlier from the HPLC column). mGTP bound to p21 after incubation of the nucleotide-free protein with excess nucleotide at low temperature tended to show a higher ratio than 60:40 of the A and B isomers. This ratio was found to vary according to the exact details of the conditions used for preparing the complex. In particular, low pH and high ammonium sulfate concentrations during the last stage of removal and degradation of nucleotide bound to the protein led to a high value (up to ca. 90:10), as shown by HPLC. If a more nearly neutral pH and less ammonium sulfate were used, this ratio was nearer to that of 60:40 found in solution. These effects are understandable in terms of the behavior of the individual isomers, as discussed below. Both isomers are hydrolyzed by p21 at similar rates, as shown in Figure 2, and the ratio of the corresponding isomers of mGDP after the loss of the γ-phosphate group was also 9:1 in favor of isomer A in this experiment.

Since there is no change in the ratio of the isomers on the hydrolysis of mGTP, the slow change in fluorescence cannot be caused by isomerization of the nucleotide analogue. It is, however, of interest to assign the structures of the isomers and to determine whether their interaction with p21 differs. The structures were assigned in the following manner. 3′,5′-Cyclic mGMP was treated with an amount of beef liver 3′,5′-cyclic nucleotide phosphodiesterase which was sufficient to degrade it in a few minutes. This led initially to the formation of predominantly one isomer of mGMP, which must be the 2′-isomer, and which equilibrated over a period of ca. 20 min at room temperature to a mixture of the two at a ratio of ca. 40:60, but this time in favor of the fraction eluting more slowly from the HPLC column. To check whether the order of elution of the isomers from the column changes between mGDP and mGMP, a small amount of mGDP was rapidly separated into its isomers by HPLC, and the isomers, which reequilibrate relatively slowly at 0 °C, were treated separately with alkaline phosphatase. When this was mainly the A isomer, the resulting GMP showed a similar ratio of peaks, but the order of elution from the column was reversed. The same

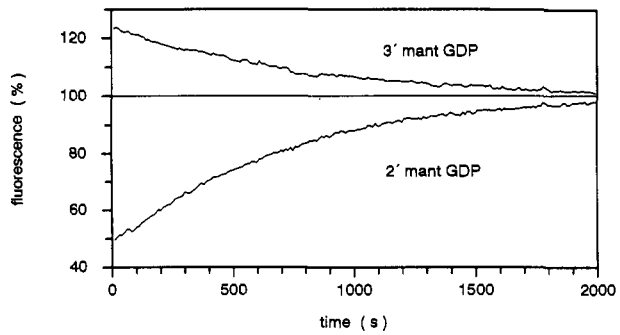

FIGURE 3: Equilibration of the mGDP isomers at 37 °C and pH 7.0. Upper curve: 3'-mGDP. Lower curve: 2'-mGDP. Middle curve: equilibrium mixture.

applied to the (*N*-methylanthraniloyl)guanosine peaks. When the B isomer fraction of mGDP was used (data not shown), a similar effect was seen; i.e., the ratio of the isomers was not changed significantly after degradation, but the order of elution was reversed. By knowing which of the mGMP peaks corresponds to the 2'-isomer from the degradation of cyclic 2'-(*N*-methylanthraniloyl)-GMP by phosphodiesterase, it is apparent that the 3'-isomer of 2'(3')-mGDP is the one eluted more rapidly from the HPLC column and bound preferentially to p21. From experiments using separated isomers of 2'-(3')-mGTP with p21 and from HPLC analysis of the degradation of the separated isomers with alkaline phosphatase, it is clear that the same order of elution and ratio of affinities to p21 also apply to 2'(3')-mGTP.

The observation that a slow fluorescence change was seen in the complex between p21 and 2'(3')-mGTP but not with 3'-(*N*-methylanthraniloyl)-2'-deoxy-GTP raises the question of the behavior of the 2'-isomer. Starting from 3'-deoxy-GTP, small amounts of 2'-(*N*-methylanthraniloyl)-3'-deoxy GTP were prepared. Surprisingly, after mixing this analogue with p21, a much larger fluorescence change was seen (amplitude 30%; data not shown). The rate of this change was the same as the rate of hydrolysis for this analogue (4.6×10^{-4} s^{-1}). The studies reported here and future work on the system would probably be significantly easier to perform using this analogue, due to the larger fluorescence change and the lack of the complicating acyl transfer reaction. This is prohibited at present by the low availability of 3'-deoxyguanosine nucleotides.

Fluorescence Yields and Equilibration of 2'(3')-Methylanthraniloyl Isomers. Finally, the behavior of the separated isomers of 2'(3')-mGTP was examined. Small amounts of these were isolated by HPLC (purity in each case ca. 90%, i.e., with 10% contamination by the other isomer). On mixing with p21, it was noticed that the fluorescence enhancement due to binding to p21 was different with the two isomers. Whereas the 3'-isomer showed an enhancement of 140%, the corresponding value for the 2'-isomer was 350%. This was not a result of a higher fluorescence yield for the p21–2'-mGTP complex than for the p21–3'-mGTP complex, but rather of a lower yield for 2'-mGTP than 3'-mGTP free in solution. This could be demonstrated directly by following the fluorescence intensity of the individual isomers free in solution as a function of time after their separation. As is apparent from Figure 3, the fluorescence decreases with time in the case of the 3'-isomer but increases in the case of the 2-isomer, whereas the equilibrium mixture stays constant. The rate constant for equilibration is 1.4×10^{-3} s^{-1} at 37 °C. This rate constant is the sum of the rate constants in both directions. By knowing that the ratio of the rate constants is 60:40 (from the equilibrium distribution), the individual rate constants can be calculated to be 8.4×10^{-4} s^{-1} in the 2' → 3' and 5.6×10^{-4} s^{-1} in the 3' → 2' direction.

Equilibration of the isomers after binding of 2'(3')-mGDP to p21 was found to be much slower than in solution, the ratio of concentrations being stable over a period of at least 6 h at 37 °C, independent of the starting ratio on the enzyme. Because of this, it was possible to determine the rate constants for release of the individual isomers from their complexes with p21 after HPLC separation of the isomers and mixing with nucleotide-free enzyme at 37 °C and in the absence of ammonium sulfate at neutral pH. These were found to be 4.4 $\times 10^{-3}$ s^{-1} for the 2'-isomer and 5.4×10^{-4} s^{-1} for the 3'-isomer. Mixing an excess of the 60:40 equilibrium mixture with p21 under these conditions led to a 60:40 distribution on the enzyme, suggesting that the association rate constants for the two isomers are similar. Thus, the difference in dissociation rate constants alone determines the difference in affinities, so that the 3'-isomer is seen to bind ca. 10-fold more tightly than the 2'-isomer. Assuming a similar difference in the affinities of the mGTP isomers, this observation provides the key to understanding the higher but variable ratio of 3' to 2' isomer bound to p21 when the enzyme was incubated with the equilibrium mixture of the mGTP isomers at relatively low pH and in the presence of ammonium sulfate (introduced with the phosphatase used for degrading GDP). Changing the pH from 7 to 6 increased the dissociation rate of 2'(3')-mGDP by a factor of 3, while increasing the ammonium sulfate concentration led to a linear increase in the dissociation rate constant by a factor of 3 for a 10 mM concentration increase. These factors allow the higher affinity of the 3'-isomer to be expressed, since the increased dissociation rates allow equilibration of the bound forms. In contrast, in the absence of ammonium sulfate and at neutral pH, the extremely slow dissociation rates lead to lack of "selection" of the isomers, since equilibration does not occur at the active site of p21.

Fluorescent Analogues and the GTPase Reaction. After characterizing the details of the binding of the individual isomers of the methylanthranilyl analogues to p21, we returned to an examination of the GTPase reaction. Since GTPγS is a better analogue of GTP than GppNHp in terms of its binding affinity to p21 (Feuerstein et al., 1989; this is true in general for the γ-thiophosphate analogues of GTP and ATP in their interactions with a large number of enzymes), it seemed appropriate to test 2'(3')-mGTPγS in the fluorescence assay used above. A very slow decrease of fluorescence of 2'(3')-mGTPγS bound to p21 was seen. This change was so slow that it was difficult to measure accurately, but it was of the order of a factor of 10–20 slower than that for GTP. This compares well with the factor of 18 found for the p21-catalyzed hydrolysis of GTPγS compared to that of GTP measured previously using an HPLC method (Feuerstein et al., 1989). This agreement is not the result expected from the mechanism suggested by Neal et al. (1990), since all available experience with the γ-thiophosphate analogues of GTP and ATP indicates that events involved in binding of the analogues to enzymes are identical or nearly identical to those for the natural triphosphates but that the rate of transfer of the thiophosphate group to its acceptor (water or other second substrate) is significantly slower than the transfer of phosphate. Thus, if the slow fluorescence change represents a step preceding hydrolysis, we would expect to see a fluorescence transient of a rate similar to that seen for GTP but a slower rate of hydrolysis.

At this point, the contradictory results obtained using the two GTP analogues prompted us to compare the fluorescent

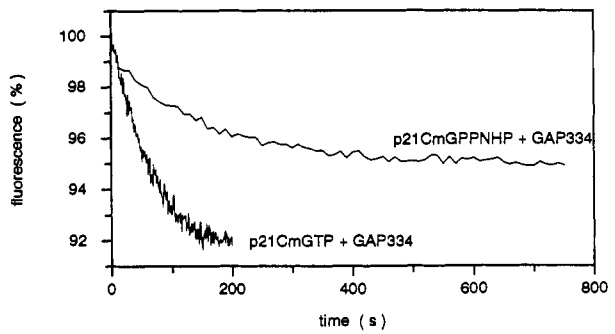

FIGURE 4: Slow fluorescence transients of p21–2′(3′)-mGTP (lower curve) and p21–2′(3′)-mGppNHp (both at 5 μM) in the presence of GAP_{334} (ca. 50 nM).

transients seen with 2′(3′)-mGTP and 2′(3′)-mGppNHp more carefully. As described above, the rate was considerably faster for 2′(3′)-mGppNHp than for 2′(3′)-mGTP. In addition, we noticed a tendency for the transient with 2′(3′)-mGTP to deviate from monoexponential behavior. As shown in Figure 1B, a plot of the residuals (i.e., the difference between the data and the theoretical curve as a function of time) after fitting a single-exponential function to the data showed marked systematic deviation from the zero value. The data could be well fitted using the sum of two exponential terms, as shown in Figure 1C. The rate of the slower reaction was similar to that obtained using a simple first-order analysis, whereas the faster component was similar in rate to that seen for the p21–2′(3′)-mGppNHp complex. This behavior was only observed if the reaction was started by forming the 2′(3′)-mGTP complex at low temperature and starting the reaction by increasing the temperature to 37 °C. If nucleotide-free p21 was mixed directly with 2′(3′)-mGTP in a 1:1 ratio, two differences were noted. First, the amplitude of the signal was less than that obtained when the cleavage reaction was induced by raising the temperature. Second, the data were very well fitted by a single-exponential function. A similar experiment with 2′(3′)-mGppNHp showed that, under these conditions, no fluorescence transient was seen on the time scale of the change seen using the "temperature jump" method. Thus, the change seen with mGppNHp, and presumably the faster component with 2′(3′)-mGTP, appears to be attributable to a transition in the system induced by raising the temperature from 4 to 37 °C. This could, in theory, occur before the cleavage step or be independent of it. The latter would imply that it is not a mandatory step in the ordered series of events involved in GTP hydrolysis. The results presented in the next section provide a test of this point.

Effect of GAP on the Rate of the Fluorescence Transient. If the slow fluorescence change seen in the work of Neal et al. (1990) and in the present work is indeed due to a rate-limiting isomerization occurring before the GTP cleavage step, it should be accelerated by GAP in the same manner as that observed for the hydrolysis step. This is the case for 2′(3′)-mGTP, as shown in Figure 4. There is good agreement between the rate of the fluorescence change and the rate of the GTPase reaction at increasing GAP_{334} concentrations. A value of 4.2×10^5 M^{-1} s^{-1} can be calculated for the k_{cat}/K_m value for the GAP p21–mGTP interaction using the fluorescence data. This is ca. 10-fold lower than that reported for full-length GAP (John, 1990; Gideon et al., unpublished results) but agrees well with the value we have obtained from measurements of the p21–GTPase reaction in the presence of GAP_{334}. If the slow fluorescence change seen with 2′(3′)-mGppNHp also arises from the same conformational change, a similar acceleration by GAP should be observable. However,

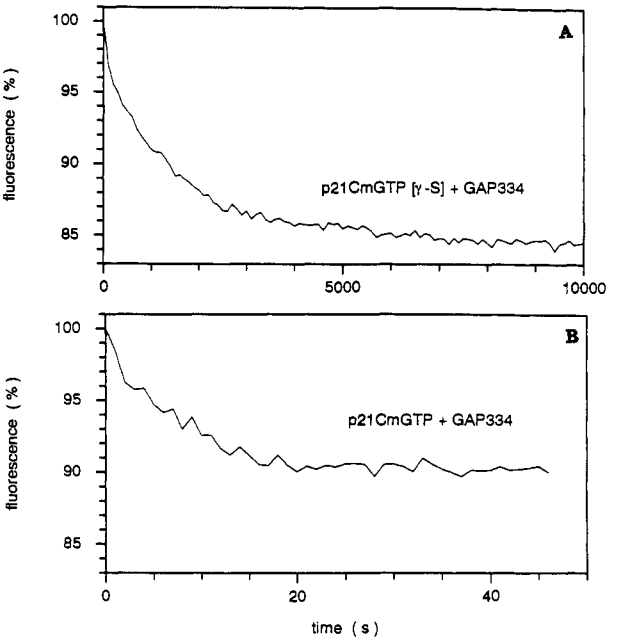

FIGURE 5: (A) Slow fluorescence transient of p21–2′(3′)-mGTPγS in the presence of GAP_{334} (300 nM). In the absence of GAP_{334}, no significant change in the fluorescence is seen on this time scale. (B) Transient seen with p21–2′(3′)-mGTP at the same GAP_{334} concentration (lower curve).

as shown in Figure 4, this is not the case. In the absence of GAP, the rate of the fluorescence change is considerably faster with 2′(3′)-mGppNHp than with 2′(3′)-mGTP but is of smaller amplitude, as mentioned above. The rate stays constant as the GAP_{334} concentration is increased. At a concentration of GAP_{334} which accelerated the fluorescence transient with p21–2′(3′)-mGTP by a factor of 1000, the rate for p21–2′(3′)-mGppNHp was unchanged.

Figure 5A shows the effect of GAP_{334} on the fluorescence transient with p21-2′(3′)-mGTPγS. It can be seen that the rate is accelerated significantly, although considerably higher concentrations of GAP are needed than in the case of mGTP. The apparent second-order rate constant (k_{cat}/K_m) calculated for the GAP activation of the fluorescent transient was calculated to be 2×10^3 M^{-1} s^{-1}, which is about a factor of 100 less than for mGTP. It was of interest to determine whether the cleavage reaction is accelerated by GAP_{334} in a similar manner. This was shown to be the case using an HPLC method, and more quantitatively using the filter binding method with [γ-^{35}S]GTPγS. These data led to a value of 9×10^3 M^{-1} s^{-1} for k_{cat}/K_m. The slightly higher value obtained in this experiment could be due to a less efficient interaction of p21–mGTPγS than p21–GTPγS with GAP.

These results can be readily explained by assuming that the fluorescence change seen with p21–2′(3′)-mGTPγS occurs at the hydrolysis step.

CONCLUSIONS

The most straightforward interpretation of the evidence presented here is that the slow fluorescence change seen after initial binding of mGTP to p21 occurs concomitantly with the phosphate cleavage step or perhaps with the release of inorganic phosphate from the p21–GDP–P_i complex. The results do not provide definitive evidence against a slow isomerization reaction prior to the GTP cleavage reaction in the p21–GTP complex. They do, however, show that the fluorescence signal from the methylanthraniloyl group bound to the sugar moiety in the analogues used does not provide evidence of or information on such a step and this raises the question of the

validity of kinetic models incorporating such a step, unless other independent evidence is found which supports its existence. It seems appropriate at present to work on the simplest possible model of the kinetic mechanism which is consistent with the available evidence. This is shown in Scheme I. This

Scheme I

$$p21 + GTP \rightleftharpoons p21{-}GTP \rightleftharpoons p21^{*}{-}GTP \rightleftharpoons p21{-}GDP{-}P_i \rightleftharpoons p21{-}GDP \rightleftharpoons p21^{\#}{-}GDP \rightleftharpoons p21 + GDP$$

model includes two step-binding mechanisms of both substrate binding and product (GDP) release, as we have demonstrated earlier (John et al., 1990), and the rate-limiting step is hydrolysis of GTP, as argued in the present work.

The mechanism of GAP activation of the p21 GTPase activity is a subject of current discussion. Two extreme classes of mechanism are imaginable. In one of these, the basic mechanism of catalysis is the same in p21 and in the p21–GAP complex. The role of GAP in the activation of the GTPase reaction would then be to stabilize a particular configuration or constellation of side chains at the active site of p21, so that the attack of a water molecule on the γ-phosphate group is favored. We have recently made a specific suggestion as to how this may occur (Pai et al., 1990). In the other type of mechanism, the involvement of one or more side chains of the GAP molecule in the catalytic reaction is envisaged. This would imply that the mechanism of GTP hydrolysis by GAP-activated p21 is different from that in p21 alone. The mechanism of Neal et al. (1990) would favor the first class of mechanism, since in its simplest form, only the rate-controlling conformational change must be accelerated by GAP. The easiest interpretation of our results is that it is the rate of the catalytic reaction itself which is modified, which could be the result of either type of activation mechanism. However, further evidence will be needed on events occurring before and during GTP cleavage to establish whether other steps must also be affected by GAP.

Acknowledgments

We thank Marija Matuska for excellent technical assistance, Jacob John for performing some preliminary experiments, and Kenneth C. Holmes for continued support.

Registry No. 2′-mGppNHp, 136749-23-0; 3′-mGppNHp, 97141-01-0; 2′-mGTP(γS), 136749-24-1; 3′-mGTP(γS), 136749-26-3; 2′-mGTP, 128422-70-8; 3′-mGTP, 85287-57-6; GTP(γS), 37589-80-3; GTPase, 9059-32-9; 3′-(*N*-methylanthraniloyl)-2′-deoxy-GTP, 124615-99-2; 2′-(*N*-methylanthraniloyl)-3′-deoxy-GTP, 136749-25-2.

References

Amman, E., Ochs, B., & Abel, K.-J. (1988) *Gene 69*, 301–305.
Feuerstein, J., Kalbitzer, H. R., John, J., Goody, R. S., & Wittinghofer, A. (1987a) *Eur. J. Biochem. 162*, 49–55.
Feuerstein, J., Goody, R. S., & Wittinghofer, A. (1987b) *J. Biol. Chem. 262*, 8455–8458.
Feuerstein, J., Goody, R. S., & Webb, M. R. (1989) *J. Biol. Chem. 264*, 6188–6190.
John, J. (1990) Ph.D. Thesis, University of Heidelberg, Heidelberg, FRG.
John, J., Frech, M., & Wittinghofer, A. (1988) *J. Biol. Chem. 263* (24), 11792–11799.
John, J., Schlichting, I., Schiltz, E., Rösch, P., & Wittinghofer, A., (1989) *J. Biol. Chem. 264*, 13086–13092.
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. F., & Wittinghofer, A. (1990) *Cell 62*, 539–548.
Milburn, M. V., Tong, L., deVos, A. M., Brünger, A., Yamaizumi, Z., Nishimura, S., & Kim, S.-H. (1990) *Science 247*, 939–945.
Neal, S. E., Eccleston, J. F., & Webb, M. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3562–3565.
Pai, E. F., Kabsch, U., Krengel, U., Holmes, K. C., John, J., & Wittinghofer, A. (1989) *Nature 341*, 209–214.
Pai, E. F., Krengel, U., Petsko, G. A., Goody, R. S., Kabsch, W., & Wittinghofer, A. (1990) *EMBO J. 9*, 2351–2359.
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. (1990b) *Nature 345*, 309–315.
Stryer, L., & Bourne, H. R. (1986) *Annu. Rev. Cell Biol. 2*, 391–419.
Tong, L., Milburn, M. V., & Kim, S.-H. (1989) *Science 245*, 244.
Tong, L., deVos, A. M., Milburn, M. V., & Kim, S.-H. (1991) *J. Mol. Biol. 217*, 503–516.
Reese, C. B., & Trentham, D. R. (1965) *Tetrahedron Lett.* 2467–2472.
Tucker, J., Sczakiel, G., Feuerstein, J., John, J., Goody, R. S., & Wittinghofer, A. (1986) *EMBO J. 5* (6), 1351–1358.