Hydrolysis of GTP by p21NRAS, the NRAS protooncogene product, is accompanied by a conformational change in the wild-type protein: Use of a single fluorescent probe at the catalytic site

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ABSTRACT 2'(3')-O-(N-Methyl)anthraniloylguanosine 5'-triphosphate (mantGTP) is a fluorescent analogue of GTP that has similar properties to the physiological substrate in terms of its binding constant and the kinetics of its interactions with p21NRAS, the NRAS protooncogene product. There is a 3-fold increase in fluorescence intensity when mantGTP binds to p21NRAS. The rate constant for the cleavage of mantGTP complexed with the protein is similar to that of GTP, and cleavage is accompanied by a fluorescence intensity change in the wild-type protein complex. A two-phase fluorescence change also occurs when the nonhydrolyzable analogue 2'(3')-O-(N-methyl)anthraniloylguanosine 5'-[β,γ-imido]triphosphate (mantp[NH]ppG) binds to wild-type p21NRAS. The second phase occurs at the same rate as the second phase observed after mantGTP binding. Thus this second phase is probably a conformation change of the p21NRAS-nucleoside triphosphate complex and that the change controls the rate of GTP hydrolysis on the protein. With a transforming mutant, [Asp12]-p21NRAS, there is no second phase of the fluorescence change after mantGTP or mantp[NH]ppG binding, even though mantGTP is hydrolyzed. This shows that an equivalent conformational change does not occur and thus the mutant may stay in a “GTP-like” conformation throughout the GTPase cycle. These results are discussed in terms of the proposed role of p21NRAS in signal transduction and the transforming properties of the mutant.

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MATERIALS AND METHODS

p21NRAS (wild-type [Gly12]p21NRAS or mutant [Asp12]-p21NRAS) was isolated as a complex with GDP from overproducing strains of E. coli (12) and further purified as an apoprotein by hydrophobic interaction HPLC on a Spherogel TSK-phenyl-5PW column (75 × 7.5 mm, Beckman) (7, 13). The protein was used immediately. p21NRAS activity was measured by its ability to bind [3H]GDP by using a filter binding assay (12).

MantGTP, mantGDP, and mantp[NH]ppG were synthesized by reaction of the parent nucleotide with N-methylisatoic anhydride (Molecular Probes) (9) but purified on DEAE-cellulose using a gradient of triethylammonium bicarbonate. Nucleotides were analyzed by anion-exchange HPLC on a Partisil-10 SAX column (250 × 4.6 mm, Whatman) with 0.6 M (NH4)2HPO4, adjusted to pH 4.0 with HCl.

Abbreviations: mantGTP, mantGDP, etc., 2'(3')-O-(N-methyl)anthraniloylguanosine 5'-triphosphate, 2'(3')-O-(N-methyl)anthraniloylguanosine 5'-diphosphate, etc.; p[NH]ppG, guanosine 5'-[β,γ-imido]triphosphate.

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HCl/15% (vol/vol) methanol at 2 ml/min. This showed that the nucleotide analogues contained <1% of the parent nucleotide. To determine purity further and to obtain information about the location of the ester linkage (14), 1H NMR spectra were obtained on a Bruker AM500 spectrometer.

The binding affinity of mantGDP relative to GDP was determined by measuring the competition between mantGDP and [3H]GDP for binding to p21NRAS. Solutions (final volume, 125 μl) containing 5 μM GDP-p21NRAS complex, 10 μM [3H]GDP, 50 mM Hepes, 0.5 mM MgCl₂, 100 mM NaCl, 1 mM dithiothreitol, 0.25 mM adenosine 5’-[β,γ-imido]triphosphate (added to prevent nonspecific binding by [3H]GDP), pH 7.6, were incubated with a range of concentrations of the analogue (1 μM–1 mM) at 37°C for 90 min. Each measurement was done in triplicate. The amount of [3H]GDP remaining bound to p21NRAS was then determined by binding to a cellulose nitrate filter. The data were analyzed as described (15).

To prepare nucleotide-p21NRAS complexes, a 2-fold excess of nucleotide was added to the apoprotein at 0°C. The protein was desalted and the excess nucleotide was removed using a Bio-Gel P4 column in a buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM MgCl₂, and 10 mM dithiothreitol at 4°C. This procedure left the complex intact as shown by the coincidence of nucleotides and protein in the elution profile. All measurements were made in this buffer, unless otherwise indicated.

Fluorescence measurements were made using an SLM 8000 photon-counting spectrophotometer. Kinetic measurements were at 37°C under the identical conditions described (7). Kinetic data were fitted to single exponential curves by using a nonlinear least squares procedure (16).

RESULTS

To assess how closely mantGTP and mantGDP mimic GTP and GDP in their interaction with p21NRAS, the binding affinity of mantGDP for p21NRAS and the rate constants for mantGTP and mantGTP dissociation and for mantGTP cleavage step were measured under the identical conditions used (7) for GTP and GDP. MantGDP binds to wild-type p21NRAS 1.8 times weaker than GDP (association constant, 1 x 10¹¹ M⁻¹; ref. 7), as determined by a competitive binding experiment with [3H]GDP.

The rate constant for the cleavage of mantGTP-p21NRAS was determined by following the time course of mantGTP formation after the addition of a substoichiometric amount of mantGTP to p21NRAS (Fig. 2). The rate constant for the dissociation of mantGTP from p21NRAS was determined by a similar experiment in which a large excess of GDP was added to the mantGTP-p21NRAS complex and the proportions of nucleotide were determined by HPLC. The rationale for these experiments has been described (7). The rate constant for the dissociation of mantGDP from p21NRAS was measured using the large enhancement of fluorescence that occurs on the binding of mantGDP to p21NRAS (see below). After the addition of a large excess of GDP to a solution of mantGDP-p21NRAS complexes, the fluorescence intensity was followed with time.

The results of these experiments are summarized in Table 1 and show that all of the rate constants determined for the nucleotides are within a factor of 2 of those for the physiological nucleotides with both wild-type and mutant proteins.

On binding to wild-type p21NRAS, mantGTP shows a 3.2-fold enhancement of fluorescence intensity and mantGDP shows a 2.8-fold enhancement (Fig. 3). When either mantGTP or mantGDP bind to the mutant protein [Asp₁₂]p21NRAS, a 3.2-fold fluorescence enhancement is observed (data not shown). We have monitored this change in fluorescence intensity for the wild-type protein by incubating mantGTP-p21NRAS complexes over the time course of the hydrolysis to mantGDP-p21NRAS complexes (Scheme I, steps 2 and 3) (Fig. 4A). The fluorescence intensity decreases by 10% for the wild-type protein with a rate constant of 3.1 x 10⁻⁴ s⁻¹, which is not significantly different from the rate constant in Table 1 controlling cleavage of mantGTP-p21NRAS or GTP-p21NRAS. By contrast, there is no detectable change in the fluorescence intensity of the mutant mantGTP-[Asp₁₂]-p21NRAS complex over the time course of mantGTP cleavage (compare Figs. 2 and 4A).

Two controls were carried out to show that the complexes are resistant to nucleotide dissociation and protein denaturation throughout the time course. The fluorescence of wild-

Table 1. Rate constants for the p21NRAS GTPase scheme, obtained using either [3H]guanine nucleotide or mant-nucleotide at 37°C for wild-type [Gly₁₂]p21NRAS or a mutant, [Asp₁₂]p21NRAS.

<table>
<thead>
<tr>
<th>Reactants</th>
<th>Rate constant (x10⁶) s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guanine nucleotide + [Gly₁₂]p21NRAS</td>
<td>1.0 3.4 &lt;0.2 4.2</td>
</tr>
<tr>
<td>Guanine nucleotide + [Asp₁₂]p21NRAS</td>
<td>5.0 1.5 &lt;0.1 2.0</td>
</tr>
<tr>
<td>Mant-nucleotide + [Gly₁₂]p21NRAS</td>
<td>1.7 3.0 &lt;0.2 2.8</td>
</tr>
<tr>
<td>Mant-nucleotide + [Asp₁₂]p21NRAS</td>
<td>3.0 2.3 &lt;0.1 1.6</td>
</tr>
</tbody>
</table>

Rate constants refer to steps in the GTPase mechanism (Scheme I). Data for guanine nucleotide binding are from ref. 7. Data for mant-nucleotide binding are from this work.
**DISCUSSION**

MantGTP and mantGDP closely mimic GTP and GDP in their interactions with p21^{NRAS}, both binding affinity and the measured rate constants in the GTPase mechanism are within a factor of 2 for the two types of nucleotide. This small effect of the modification of the ribose moiety of the nucleotides is consistent with the crystal structure of p21^{NRAS}, which shows the 2',3'-hydroxyl groups of the nucleotide project away from the protein toward the solvent (17–19).

The mant-nucleotides are environmentally sensitive (9) and on binding to p21^{NRAS}, there is a large enhancement of their fluorescence. With the wild-type protein, this is 3.2-fold for mantGTP and 2.8-fold for mantGDP. This suggests that the mant-fluorophore is in a slightly different environment in the two complexes although small changes in total fluorescent intensities are difficult to measure between different solutions. However, we have been able to measure the decrease in fluorescent intensity with time when mantGTP-p21^{NRAS} hydrolyzes to mantGDP-p21^{NRAS}, and in this case solution and instrumental parameters are constant for all measurements. We have shown that this hydrolysis is accompanied by an exponential decrease in fluorescence with a first-order rate constant, the same within experimental error as that measured for the cleavage step. There are three possible interpretations of this result.

(i) A conformation change of the protein–nucleotide complex occurs during the cleavage step that changes the local environment of the fluorophore.

(ii) A conformation change occurs during the release of P_i from the mantGDP-p21^{NRAS} complex.

(iii) The rate of the cleavage step is limited by a conformation change of the mantGTP-p21^{NRAS} complex, which precedes cleavage.

The decrease in fluorescence intensity of the complex of p21^{NRAS} with mantp[NH]ppG, which is not hydrolyzed and so does not undergo step 2 (Scheme 1), suggests that the third situation occurs. In this case the measured value of k_{42} reported here for mantGTP and that for GTP reported previously (7) is actually the rate of the isomerization step.
and the subsequent cleavage step is controlled by this rate. The smaller fluorescence decrease with mantp[NH]ppG relative to mantGTP is due to slightly different local environments of the fluorophore in the two complexes. Alternatively, there could be approximately equal fluorescence changes with mantGTP on both the proposed conformation change and on either the cleavage or phosphate release steps.

For the mutant protein, no change in fluorescence intensity occurs during the conversion of mantGTP[Asp^{12}]p21NRAS to mantGDP[Asp^{12}]p21NRAS or on incubation of the mantp-[NH]ppG[Asp^{12}]p21NRAS complex. Either the proposed isomerization step does not occur with the mutant protein or it does occur but the environment of the fluorophore does not change in the same way as in the wild-type protein. With the wild-type and mutant proteins, the GDP complexes undergo oxygen exchange between $[^{18}O_4]$P, and water faster than would be expected for the forward and reverse rate constants of the cleavage step (ref. 7; J. Hunter and M. R. W., unpublished data). This result lends support to the argument that a conformation change occurs prior to the cleavage step in wild-type and mutant protein.

It is possible that the proposed conformation change is important in determining the biologically active state of the protein. In the wild-type protein, this process results in the formation of an "inactive" state of the protein but with the mutant protein, although hydrolysis occurs, the protein stays in an "active" or GTP-like conformation.

There is evidence (19–23) of conformation differences, either between GTP- and GDP-bound forms or between wild-type and mutant proteins. Conformational differences between p21NRAS-GDP and p21NRAS-GDP have been shown using circular dichroism measurements (20). The measurement suggests a change in the percent $\alpha$-helix, although differences between the wild-type protein and mutants are small. Structural differences between p21NRAS (as the GDP complex) and a mutant (Val^{12})p21NRAS have been observed in the crystal structure (21). In particular a change in protein conformation about the GDP $\beta$-phosphate is seen. NMR data also suggests small structural differences between wild-type p21NRAS and the mutant [Asp^{12}]p21NRAS in the GDP states (22, 23). The crystal structure has now been obtained with p[NH]ppG bound (19), and this will be important in comparing different structural states due to different bound nucleotides.

The proposed conformation change requires a minimum of one other conformation change in the GTPase cycle to re-form the original conformation as in R-GTP (Scheme I). This could occur elsewhere in the GTPase mechanism or in nucleotide-free p21NRAS, which could exist in two interconvertible states—one capable of binding GDP and the other capable of binding GTP. Further studies are required to determine where this second conformation change occurs. It will be of interest to determine which steps the GTPase-activating protein (GAP) modifies and whether its interactions with individual complexes are consistent with its postulated role as part of the effector system. The proposed conformation change is consistent with a simple model of GAP interaction with p21NRAS. GAP can interact with wild-type or mutant proteins, but only with the former does the conformation change allow GAP to accelerate the hydrolysis and so deactivate the p21NRAS.

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