Kinetic Analysis of the Hydrolysis of GTP by p21<sup>N-ras</sup>

THE BASAL GTPase MECHANISM*

Susan E. Neal, John F. Eccleston, Alan Hall†, and Martin R. Webb§

From the Division of Physical Biochemistry, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 IAA and †The Institute of Cancer Research, Chester Beatty Laboratories, Fulham Road, London SW3 6JB, United Kingdom

The rate constants have been determined for elementary steps in the basal GTPase mechanism of normal p21<sup>N-ras</sup> (Gly-12) and an oncogenic mutant (Asp-12): namely GTP binding, hydrolysis, phosphate release, and GDP release. By extrapolation from data at lower temperatures, the GTP association rate constant at 37 °C is 1.4 × 10<sup>8</sup> M<sup>-1</sup> s<sup>-1</sup> for the normal protein and 4.8 × 10<sup>8</sup> M<sup>-1</sup> s<sup>-1</sup> for the mutant. Other rate constants were measured directly at 37 °C, and three processes have similar slow values. GTP dissociation is at 1.0 × 10<sup>4</sup> s<sup>-1</sup> (normal) and 5.0 × 10<sup>4</sup> s<sup>-1</sup> (mutant). The hydrolysis step is at 3.4 × 10<sup>4</sup> s<sup>-1</sup> (normal) and 1.5 × 10<sup>4</sup> s<sup>-1</sup> (mutant). GDP dissociates at 4.2 × 10<sup>4</sup> s<sup>-1</sup> (normal) and 2.0 × 10<sup>4</sup> s<sup>-1</sup> (mutant). GDP association rate constants are similar to those for GTP, 0.5 × 10<sup>8</sup> M<sup>-1</sup> s<sup>-1</sup> for normal and 0.7 × 10<sup>8</sup> M<sup>-1</sup> s<sup>-1</sup> for mutant. Both hydrolysis and GDP release therefore contribute to rate limitation of the basal GTPase activity. There are distinct differences (up to 5-fold) between rate constants for the normal and mutant proteins at a number of steps. The values are consistent with the reduced GTPase activity for this mutant and suggest little difference between normal and mutant proteins in the relative steady-state concentrations of GTP and GDP complexes that may represent active and inactive states. The results are discussed in terms of the likely role of p21<sup>N-ras</sup> in transmembrane signalling.

p21<sup>N-ras</sup> is a 21,000 molecular weight protein with a single polypeptide chain. When expressed in eukaryotic cells, it is palmitoylated at a cysteine near the C terminus probably to facilitate membrane association (Willumsen et al., 1984). Although p21 is expressed in many cells, its function is not known, but it is likely to have a role in transmembrane signal transduction associated with cell growth with a mechanism analogous to G proteins (reviewed by Stryer and Bourne, 1986; Gilman, 1987). Certain single point mutants are found in many cancer cells (reviewed by Barbacid, 1987), but the molecular basis is not known by which these mutations effect the presumed signal transduction, and so cell growth.

With G proteins (and hence by analogy, p21<sup>N-ras</sup>) the change from inactive to active states with respect to signal transduction is induced by GTP binding. The GTP-bound state can then activate an effector protein, although, at present, effectors in the p21 system have not been clearly identified. Because of this nucleotide-dependent activation, we wish to understand the importance of various nucleotide complexes and how they interconvert. Scheme 1 shows the minimal number of steps for the GTPase activity, with p21<sup>N-ras</sup> represented by R.

\[
P + GTP \rightleftharpoons R \cdot GTP \rightleftharpoons R \cdot GDP \cdot P \rightleftharpoons P \cdot GDP \rightleftharpoons R + GDP
\]

**Scheme 1**

Each step is numbered so that the forward and reverse rate constants for step i are \( k_i \) and \( k_i^\text{−} \), respectively. The hydrolysis occurs with inversion of configuration and so is unlikely to be via a phosphoenzyme: each process of nucleotide binding, hydrolysis, and product release is shown as a single step. The GTPase mechanism may be more complex than this scheme because, for example, no protein conformation changes are included, although such changes have been found with other transducing nucleoside triphosphatases. However, this scheme is sufficient, based on current knowledge of the system. We describe here the measurement of rate constants for nucleotide binding and release, and the hydrolysis step, for both the normal (Gly-12) protein and also the transforming single point mutant (Asp-12) for p21<sup>N-ras</sup>, produced from expression in *Escherichia coli*. A complete description of the kinetic scheme of this basal GTPase is important for understanding differences between normal and mutant proteins and as a basis for understanding which steps are modified by interaction with receptors or effectors.

**EXPERIMENTAL PROCEDURES AND RESULTS**

**DISCUSSION**

We have defined a minimal mechanism for the hydrolysis of GTP by p21<sup>N-ras</sup> and measured most of the elementary rate constants of this mechanism for both the normal (Gly-12) protein and a transforming single point mutant (Asp-12). The values of these rate constants at 37 °C together with the GTP and GDP association equilibrium constants calculated from \( k_i/k_i^\text{−} \) and \( k_i^\text{−}/k_i \), respectively, are shown in Table 1. The missing rate constants relate to P<sub>i</sub> release and rebinding which are discussed below.

An essential feature of this work is that experiments were performed using the apoprotein of p21<sup>N-ras</sup> prepared by the method of Feuerstein et al. (1987a) combined with the use of substoichiometric amounts of nucleotide (i.e., single turnover

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† To whom correspondence should be addressed.

1 J. Feuerstein, R. S. Goody, M. R. Webb, unpublished results.

2 Portions of this paper including "Materials and Methods," "Results," "Tables" 2-4, and Figs. 1-5) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

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The abbreviations used are: EF-Tu, elongation factor Tu; HPLC, high performance liquid chromatography.

1 J. Hunter and M. R. Webb, unpublished results.

2 These are obtained from values at 0 and 10 °C by extrapolation of an Arrhenius plot.

3 Limit from the measurement of the equilibrium constant for the hydrolysis step 2 being completely (>95%) in favor of products.

Table I

| Summary of rate constants of p21 \(^{He}\) GTPase scheme, at 37 °C |
|-------------------------|-------|-------|
|                        | Normal | Mutant |
| \(k_+1\) (M\(^{-1}\) s\(^{-1}\)) \(^p\) | 1.4 \times 10\(^8\) | 4.8 \times 10\(^8\) |
| \(k_+4\) (s\(^{-1}\)) | 1.8 \times 10\(^4\) | 5.0 \times 10\(^4\) |
| \(k_+2\) (s\(^{-1}\)) | 3.4 \times 10\(^4\) | 1.5 \times 10\(^4\) |
| \(k_+3\) (s\(^{-1}\)) | <0.17 \times 10\(^4\) | <0.08 \times 10\(^4\) |
| \(k_-3\) (s\(^{-1}\)) | 4.2 \times 10\(^4\) | 2.0 \times 10\(^4\) |
| \(k_+5\) (M\(^{-1}\) s\(^{-1}\)) \(^p\) | 0.51 \times 10\(^5\) | 0.70 \times 10\(^5\) |
| GTP association constant (M\(^{-1}\)) | 1.0 \times 10\(^{-3}\) | 3.8 \times 10\(^{-3}\) |
| GDP association constant (M\(^{-1}\)) | 1.2 \times 10\(^{-3}\) | 2.5 \times 10\(^{-3}\) |

\[ \frac{[R.GTP]}{[R.GDP]} = \frac{k_+4}{k_-4} \]

As mentioned in the Introduction, Scheme 1 only includes intermediates with different nucleotide states. Currently, there is no evidence for significant protein conformation changes, although such changes are likely to occur, in order to alter the p21 from active to inactive states in terms of signal transduction. An example of a nucleoside triphosphatase transducing system with well-recognized conformation changes is myosin (reviewed by Trentham et al., 1976; see also Sleep and Hutton, 1980). With p21, the large free energy change going from R-GTP to R-GDP, and R-GDP might well include a protein conformation change. We are probing this using fluorescent-nucleotide analogues and oxygen-exchange studies.

Data from amino acid sequences and crystallographic studies indicate similarities between the nucleotide binding sites of p21 and elongation factor Tu (EF-Tu) from E. coli (Haliday, 1984; Jurnak, 1985; McCormick et al., 1985; de Vos et al., 1988). The two proteins probably have a similar common chemical mechanism for the hydrolysis: direct, in-line phospho transfer from GTP to water, with no phosphoenzyme intermediate (Eccleston and Webb, 1982). However, the kinetic data reported here for p21\(^{He}\) show that there are significant differences between the interaction of the two proteins with guanosine nucleotides. Both GTP and GDP bind to p21\(^{He}\) at similar rates which at 37 °C would be close to those expected for diffusion controlled reactions (Gutfreund, 1987), whereas the association rate constants of GTP and GDP to EF-Tu at 0 °C are 1 \times 10\(^4\) M\(^{-1}\) s\(^{-1}\) and 2.6 \times 10\(^4\) M\(^{-1}\) s\(^{-1}\), respectively (Fasano et al., 1978). This difference between the rate constants of GTP and GDP binding to EF-Tu has been confirmed at physiological concentrations of reactants using chromophoric analogs of GTP and GDP with rapid-reaction techniques (Eccleston, 1981). The results were interpreted as showing a two-step binding mechanism, that is, binding itself followed by a conformational change of the complex. Distinctive differences in solution structure between EF-Tu-GTP and EF-Tu-GDP have been reported (Kaziro, 1978), but no such differences have yet been documented for p21. The rate constant for the cleavage step of EF-Tu is 5 \times 10\(^{4}\) s\(^{-1}\) at 24 °C (Kalbitzer et al., 1984) although when the EF-Tu-GTP complex with aminoacyl-tRNA binds to the A site of programmed ribosomes, this rate is accelerated to 50 s\(^{-1}\) (Eccleston et al., 1985). The rate of GDP release from EF-Tu \textit{in vivo} is also accelerated by interaction with another protein factor, EF-Ts (Chau et al., 1981). It remains to be seen whether other cellular components can effect the rate of the cleavage step of p21 or of the rate of release of nucleotide.

Recently, there has been reported a widespread, cytosolic protein GAP ("GTPase activating protein"), which interacts with normal p21\(^{He}\); increasing GTPase activity, but does not seem to affect transforming mutants (Trabey and Mc-
Cormick, 1987; Cales et al., 1988; Adari et al., 1988). There is evidence in this work that GAP is an effector for the ras signal transducing system. It will be of great interest to see how interaction with GAP affects individual rate constants of the p21 GTPase mechanism. For example, if the GTPase stimulation is preferentially in step 2, this may affect the quasiequilibrium of Scheme 2 and cause deactivation of p21. Conversely, interaction with activated receptor, by accelerating step 4, would induce the postulated active GTP-bound conformation via nucleotide exchange.

REFERENCES

Preparation of nucleotide-free p21GTP

Normal (Olig) and mutant (Appl) p21GTP were isolated as their guanine-nucleotide complexes from an overproducing strain of E. coli (Hall and Sef, 1987). The protein stored in liquid nitrogen in buffer A [50 Tris-HCl (pH 8.0), 50 mM MgCl2, 10% glycerol, 200 mM sodium succinate, 0.5 mg/ml bovine serum albumin] showed no loss of activity over a period of at least 1 month. The activity was determined by the ability of the protein to bind [3H]GTP (6000 cpm/mg) from an acid-precipitable filter binding assay (Hall and Sef, 1987). The incubation conditions were 2°C, 60 min for the nucleotide-bound protein and 60°C for the guanine nucleotide exchange experiments. To check for the presence of dithirolactate and its products, the reaction mixtures were stopped by the addition of EDTA and the protein precipitated with trichloroacetic acid.

The ATPase activity was obtained by hydrophobic interaction high performance liquid chromatography (HPLC) on a Sepharose 28, 5X75 cm column, 15 mM Tris-HCl (pH 8.0), 150 mM NaCl, 200 mM sodium succinate, 0.5 mg/ml bovine serum albumin. The ATPase activity was measured by the amount of ATP released from ATP in buffer A, and incubated at 37°C for 15 min, before injection onto the column. The ATPase activity was measured by the amount of ATP released from ATP in buffer A, and incubated at 37°C for 15 min, before injection onto the column. The ATPase activity was measured by the amount of ATP released from ATP in buffer A, and incubated at 37°C for 15 min, before injection onto the column.

Fig. 1 shows a typical elution profile obtained from p21GTP. The protein was eluted with a 0-100% gradient of NaCl in buffer A, and incubated at 37°C for 15 min, before injection onto the column. The ATPase activity was measured by the amount of ATP released from ATP in buffer A, and incubated at 37°C for 15 min, before injection onto the column. The ATPase activity was measured by the amount of ATP released from ATP in buffer A, and incubated at 37°C for 15 min, before injection onto the column. The ATPase activity was measured by the amount of ATP released from ATP in buffer A, and incubated at 37°C for 15 min, before injection onto the column.
For the single-turnover and cold-chase experiments, samples (125 µl) from the reaction mixture were taken at timed intervals after addition of nucleotide and assayed by isocratic reverse-phase HPLC. The samples were immediately adjusted to pH 7 by the addition of 0.1 M sodium phosphate. GDP or GTP was added at a concentration of 0.8 moles/mole protein and the reaction was initiated by the addition of 0.1 M NaOH in 10% glycerol. The reaction was followed using high performance liquid chromatography to analyze nucleotides. The results are presented in Table 1, which shows that there is a rapid equilibrium of the cleavage step: such that the formation of nucleotide over protein. For GTP binding, the observed rate of binding is shown in Fig. 2B, which gives the best fit to a single exponential. A similar equation applies for GDP.

In the case of nucleotide binding to p21, this approach is limited by the fact that high concentration of nucleotide or at high temperatures, the rate of binding becomes too fast to measure by the manual sampling techniques. However, we have measured the rate of binding over a 1D range of nucleotide concentrations at 0°C. As with nucleotide binding to p21, it was observed that the rate of binding is limited by the association constant and the time-course of COP formation observed are summarized in Table 3.

Values of rate constants at 0°C were obtained from slopes of linear regression at different concentrations of GDP or GTP and mutant protein at 37°C, which were plotted as a function of GDP concentration. The rate constants are given in Table 2, and show little difference between GDP and mutant protein.

In order to test if the single turnover rate is limited by the release of GDP, a stock solution of [3H]GTP was incubated at 37°C with p21N to form a complex. The formation of GDP was followed using HPLC analysis of a aliquot by filtration. The results are presented in Table 1, which shows that the formation of GDP is limited by the association constant and the time-course of COP formation observed are summarized in Table 3.

An important feature of these single-turnover experiments is that the reaction is limited by the rate of GDP release. This can be assessed by the measurement of GDP or GTP, which is then plotted as a function of GDP concentration. The rate constants are given in Table 2, and show little difference between GDP and mutant protein. The results are presented in Table 1, which shows that the formation of GDP is limited by the association constant and the time-course of COP formation observed are summarized in Table 3.

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Kinetics of GTP Hydrolysis by p21

After 5 min, the solution was made 1 ml in unlabeled GDP and incubated at 0°C. A substoichiometric amount of $[^3H]GTP$ was added to the apoprotein at 0°C. After 5 min, a large excess of unlabeled GDP was added to the reaction mixture. The amount of $[^3H]GDP$ remaining bound to the mutant protein was measured at various times using the filter assay. The results of one experiment are shown in Fig. 1 and the rate constants are shown in Table 1. It was found that GDP release was sensitive to ammonium sulfate concentration, so that at -100 mM ammonium sulfate, $k_2$ was 8-fold larger. This type of dependence has been found with α proteins (Ferguson et al., 1986). We also found that GTP release was sensitive to ammonium sulfate, so all measurements were done under conditions when ammonium sulfate was reduced to less than 8 mM. Table 1 shows that the dissociation of GDP from mutant is about 2-fold slower than from the normal protein.

Extracellular data

Figs. 2-5 show that good fits to single exponentials are obtained in all experiments. Table 1 shows the rate constants for each individual experiment using the same or different preparations of p21 and differed by less than $k_1$.

Table 1

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_{obs}$</th>
<th>$[^3H]GDP[^3H]GDP$</th>
<th>$k_1$</th>
<th>$k_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>$4.5 \times 10^{-7}$</td>
<td>$3.0\times10^{-4}$</td>
<td>$1.93 \times 10^{-3}$</td>
<td></td>
</tr>
<tr>
<td>Mutant</td>
<td>$6.6 \times 10^{-6}$</td>
<td>$0.32$</td>
<td>$9.02 \times 10^{-4}$</td>
<td>$1.40 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

Values for $k_{obs}$ and $k_2$ were calculated from data as shown in Fig. 6 and described in the method section.

GTP dissociation kinetics (Measurement of $k_{obs}$)

The rate constant for GDP dissociation was measured by displacing $[^3H]GDP$ from the complex with p21 using a large excess of GTP. After $k_2$, a large excess of unlabeled GDP was added to the reaction mixture. The amount of $[^3H]GDP$ remaining bound to the protein was measured at various times using the filter assay. The results of one experiment are shown in Fig. 1 and the rate constants are shown in Table 1. It was found that GDP release was sensitive to ammonium sulfate concentration, so that at -100 mM ammonium sulfate, $k_2$ was 8-fold larger. This type of dependence has been found with α proteins (Ferguson et al., 1986). We also found that GTP release was sensitive to ammonium sulfate, so all measurements were done under conditions when ammonium sulfate was reduced to less than 8 mM. Table 1 shows that the dissociation of GDP from mutant is about 2-fold slower than from the normal protein.