The Mechanism of Guanosine Nucleotide Hydrolysis by p21 c-Ha-ras

THE STEREOCHEMICAL COURSE OF THE GTPase REACTION*

Jürgen Feuerstein‡, Roger S. Goody‡, and Martin R. Webb§†

From the 2Max-Planck Institut für Medizinische Forschung, Abteilung Biophysik, Jahnstrasse 29, 6900 Heidelberg, West Germany and the §Division of Physical Biochemistry, National Institute for Medical Research, Mill Hill, London NW7 1AA, United Kingdom

The use of guanosine 5'-O-(γ-thio)triphosphate as a substrate for p21 c-Ha-ras was established. By using chirally labeled [γ-3H,18O]guanosine 5'-O-(γ-thio)triphosphate, the stereochemical course of the GTPase reaction was determined. The analysis shows that the hydrolysis occurs with inversion at the γ-phosphorus. This shows that the most likely mechanism is a single step, in-line transfer, without a phosphoenzyme or other phosphorylated intermediate.

p21ras is a single polypeptide protein, molecular weight 21,000, expressed in many different types of cells. Its function is unknown, but it is thought to have a role in regulation of cell growth, with a mechanism analogous to G proteins. The best defined biochemical property relates to its ability to bind guanosine nucleotides very tightly and to hydrolyze GTP. There are three distinct human ras genes (c-H, c-Ki, and N), although several closely related genes occur (Marshall, 1986). A variety of single point mutants are oncogenic, found in many cancerous cells (reviewed by Barbacid, 1987). The molecular basis for this change is not understood, although recent work has shown the existence of a factor that modulates ras, is that the tightly bound GTP and GDP (as M+ complexes) confer active and inactive states, respectively, in the proteins. (For recent reviews, see Stryer and Bourne, 1987.) It is therefore important to determine how these states interconvert, both by nucleotide exchange and by hydrolysis, and whether there are any significant intermediates. One approach to these questions is to determine the stereochemical course of GTP hydrolysis catalyzed by p21. This provides an important test for the existence of a phosphoenzyme intermediate on the GTPase reaction pathway. Each enzymic phosphotransfer occurs with inversion of configuration at the phosphorus. Thus if the overall reaction is inversion, there is most probably a single transfer from GTP to water. If the overall reaction takes place with retention, this is probably the result of two inversions, namely the formation and breakdown of a phosphorylated intermediate. In the case of ATPases, and also for kinases, examples of both types of result are known (Webb, 1982; Frey, 1982; Gerlt et al., 1983; Eckstein, 1983).

For GTP hydrolysis, it is necessary to distinguish all 4 atoms on the phosphorus of the hydrolysis product, P. Since only three stable oxygen isotopes are available, sulfur is used to label the fourth position.

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\begin{align*}
\text{GTP} & \quad \text{H}_2\text{O} \\
\text{GDP} & \quad \text{P} + \text{S} \\
\text{GTP}^+ & \quad \text{P} + \text{S} \\
\end{align*}
\]

The substrate is therefore isotopically labeled GTP(S). We show that this is a reasonable substrate for the p21 GTPase activity. Work on other systems has shown that although the rates of reactions may alter, the chemical mechanism is not altered on changing from phosphate to thiophosphate. In particular, the stereochemical course is unaffected by substitution by sulfur (Eckstein 1983), so that the result found for GTP(S) should also apply for the natural substrate.

EXPERIMENTAL PROCEDURES

The cellular form of p21 H-ras was prepared from Escherichia coli RRI M15 cells harboring a plasmid containing the H-ras gene under the control of the tac promoter as described by Tucker et al. (1986). The isolated protein was stored as its GDP complex at -70 °C in 50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, and 1.4 mM dithioerythritol. Bound GDP was removed immediately prior to incubations with GTP(S) by high performance liquid chromatography on a TSK-phenyl-5PW column (21.5 x 150 mm) (Beckman Instruments) as described by Feuerstein et al. (1987b).

The rate constants for GTP(S) association with p21 and for dissociation of the analog from its complex with the protein were determined as previously described for GDP (Feuerstein et al., 1987a, 1987b) using [35S]GTP(S). These determinations were carried out at 0 °C to allow easier measurement of the association rate constant. Buffer conditions for these and all other experiments were 64 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM dithioerythritol.

The rate of GTP(S) hydrolysis by p21 at 37 °C (otherwise under the same conditions as described above) was determined by measuring the initial rate of production of GDP after mixing GDP-free p21 with a 3-fold excess of GTP(S). The nucleotide was analyzed by ion-pair reversed-phase chromatography on a Shandon RP C₈ column eluted with 50 mM potassium phosphate, pH 6.5, 0.2 mM tetraethylammonium bromide, 3% acetonitrile. The measured rate was corrected for the rate of spontaneous hydrolysis of GTP(S) measured under identical conditions.

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\text{(y-S)}-\text{[35S]GTP-S} \quad \text{(y-S)}-\text{[35S]GTP} \quad \text{GTP(S)} \quad \text{GTP}\]

1 The abbreviations used are: GTP(S), guanosine 5'-O-(γ-thio)triphosphate; ATP(S), adenosine 5'-O-(β-thio)triphosphate.
For preparation of chiral inorganic thiophosphate as the product of GTPγS hydrolysis by p21, the following conditions were used: 6.3 μmol of p21 were incubated for 15 h at 37 °C with 20 μmol of labeled GTPγS. Ion pair reversed-phase chromatography as described above showed that ~90% hydrolysis of GTPγS had occurred after this incubation.

31P NMR spectra were obtained at 201 MHz on a Bruker AM500 spectrometer. The pulse width was 5 μs, acquisition time 4 μs, with a spectral width 1000 Hz. A line broadening of 0.1 Hz was applied to the spectrum prior to Fourier transformation.

RESULTS AND DISCUSSION

GTPγS is a good analog of GTP in terms of its interaction with the binding site of p21. This can be seen from the kinetic parameters for association and dissociation in comparison with the corresponding values for GTP (Table I). The association rate constant is about a factor of 2 smaller for GTPγS than for GTP, and the dissociation rate constant is somewhat larger. These two effects lead to a factor of ~3 difference in the association constants for the two nucleotides to p21, in good agreement with the data obtained from competition experiments by Tucker et al. (1986).

As expected from data obtained with other systems, the rate of hydrolysis of GTPγS by p21 is considerably slower than that of GTP. Since the latter reaction is already very slow, it was important to determine whether the rate of GTPγS hydrolysis by p21 is fast enough to compete with spontaneous decay of the analog under the conditions used for the incubation of chiral-labeled GTPγS. The rate constant for spontaneous hydrolysis was found to be 0.00017 min⁻¹ (37 °C, pH 7.6, 10 mM MgCl₂), corresponding to a half-life of 57.5 h. This can be compared with the rate constant for p21-catalyzed hydrolysis of the analog, which was found to be 0.0030 min⁻¹. In the incubation mixture which was used for producing the chiral thiophosphate for analysis, 90% of the GTPγS was hydrolyzed. Using the measured rate constant for spontaneous decay, it can be calculated that 14% of the thiophosphate arises by this route, the rest from p21-catalyzed hydrolysis. This calculation is likely to lead to an overestimate of the contribution from spontaneous decay, since under the conditions used (3-fold excess of GTPγS over protein), one-third of the analog will be immediately bound to the enzyme. Presumably this can only be hydrolyzed by reaction with a water molecule which is the one normally involved in catalysis. Thus we conclude that between ~9 and ~14% of the inorganic thiophosphate produced is the product of spontaneous decay of GTPγS under the conditions used. This amount is too small to influence the determined stereochemistry of phosphotransfer.

The stereochemical analysis uses the 31P NMR spectrum of the thiophosphate product, after its stereoselective incorporation into ATPβS. The procedures for purification of inorganic thiophosphate and synthesis of ATPβS via a series of enzymic reactions are described by Webb (1982). During this synthesis, one oxygen atom is lost from the thiophosphate moiety: one-third of the molecules lose 16O, one-third 18O, and one-third 14O. Molecules that retain 18O will give a very broad NMR signal due to the quadrupole moment of 18O. If 17O is lost, the ATPβS has a bridging or non-bridging 18O, depending on the enantiomer of inorganic thiophosphate (Scheme 2).

The spectrum in Fig. 1 shows the signal due to the β-phosphorus of ATPβS. It is a triplet (due to the α- and γ-phosphorus atoms) and each member of the triplet is a series of peaks due to the presence of 18O in some of the molecules, causing an upfield shift. The isotopic labeling pattern giving rise to each peak is indicated in this figure, based on comparison with known compounds (Webb and Trentham, 1980). The major species present has a β-phosphorus 18O indicating that the main pathway is inversion (Scheme 2). The other labeled species arise due to the lack of 100% isotopic enrichment, especially the 18O which is less than 50% enriched, and also contains 16O. Table II shows the relative proportions of each isotopically enriched species, based on the enrichment of the ATPβS. The spectrum corresponds closely to what is predicted for 100% inversion.

No exception is known to the rule that each enzymic transfer of a phosho group occurs with inversion at phosphorus. However, there is one example of an enzyme, adenosuccinate synthetase, which has a phosphorylated intermediate.
mediate, but overall inversion (Webb et al., 1984). In that case, the phosphorylated intermediate formed during GTP hydrolysis, is an activated adduct with IMP. This adduct is subsequently dephosphorylated by C—O bond cleavage, not P—O, so that the configuration about phosphorus is unaltered. There is no evidence for such a mechanism with p21, and it is therefore unnecessary to propose such complications.

The possibility needs considering that the catalyzed reaction is not due to p21ras, but a small percent of a more active impurity. We can effectively rule out the presence of a non-specific phosphatase, because the major product is GDP and this would be degraded rapidly by such an enzyme. Phosphatases act on normal phosphates much more rapidly than thiophosphates. We cannot completely exclude the presence of proteins, the eukaryotic elongation factors G and Tu (Webb and Eccleston, 1981; Eccleston and Webb, 1982). Neither protein has sufficient activity to account for the internal kinetics of this form of p21ras used here. Work with p21 N-ras, from p21ras with baseline resolution on the final column of the preparation, which is the maximum impurity compatible with our preparation. In any case, any remnant elongation factor Tu, the major GTPase in E. coli, is separated from p21ras with base line resolution on the final column of the preparation. Other GTPases are expressed in E. coli at much lower levels, and would very unlikely be at 5% of the p21 level. p21 N-ras has a similar GTPase and GTPαSase activity to the p21 H-ras used here. Work with p21 N-ras, purified similarly but using single turnover conditions, has shown that the internal kinetics of this form of p21ras are compatible with the observed GTPase activity and therefore not due to impurities (Neal et al. 1989). We are therefore confident that the observed stereochemical course is due to p21.

We therefore conclude that GTPαS, and therefore also GTP, is hydrolyzed by ras with a direct, in-line transfer of the terminal phosphate from GTP to water. There is no phosphoenzyme intermediate on this pathway. This same result is obtained for two other guanosine nucleotide-binding proteins, the eukaryotic elongation factors G and Tu (Webb and Eccleston, 1981; Eccleston and Webb, 1982). The putative

nucleotide binding site of Tu and of p21 show considerable sequencing homology. The crystal structure of Tu for this region of the molecule (Jurnak, 1985; McCormick et al., 1985) and that for c-H-ras p21 are published (de Vos et al., 1988).

The result presented here provides direct evidence for a similar hydrolysis mechanism, for the two proteins, in keeping with the similar protein structure around the GTPase catalytic site.

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