We have employed ESR spectroscopy using guanine nucleotides that contain a spin label at the 2',3'-position of the ribose to investigate structural changes in the proto-oncogene product p21\(^{ras}\) that are dependent on nucleotide hydrolysis. The three nucleotide analogs used were 2',3'-(2,2,5,5-tetramethyl-3-pyrroline-1-oxyl-3-carboxylic acid ester) SL GTP, SL-GDP, and the non-hydrolyzable analog SL-guanilylimidodiphosphate. SL-GTP was hydrolyzed by p21 with rates similar to those for GDP hydrolysis and appears to be an excellent substrate analog. The ESR spectra of SL-GTP and SL-GDP in complex with p21 differ significantly when acquired at 0 °C or 5 °C indicating different environments (conformations) of the protein-bound radicals depending on the phosphorylation state of the bound nucleotide. We calculated the rate constant for the conformational change as deduced from the changes in the corresponding ESR spectra upon incubation of the p21-SL-GTP complex at 25 °C and compared it to the rate constant of hydrolysis of SL-GTP at the same temperature. The rate constant deduced from the ESR method was similar to that determined by a high performance liquid chromatography technique. The data are in agreement with the idea that a conformational change during GTP hydrolysis by p21 occurs simultaneously with the actual hydrolysis step.

G-proteins (guanosine-binding proteins) play a major role in signal transduction processes in biological systems. In the present work, we investigated a member of the family of monomeric G-proteins, the ras proto-oncogene product p21. The role of p21 lies at the center of intracellular signaling systems that regulate such important functions as cell growth and differentiation (1–6).

One of the most characteristic features of the p21 protein, and of other GTPases involved in signal transduction and regulation, is a molecular switch function due to a conformational change that is coupled to GTP hydrolysis. Many (but not all) of the mutations responsible for the transformation of normal p21 to its oncogenic form result in reduced GTPase activity, or, more importantly, in loss of activation of this intrinsically slow reaction by interaction with a GTPase-activating protein (GAP).\(^1\) GAP can stimulate the GTPase activity of p21 strongly, leading to production of inactivated p21 in the GDP conformation. Impaired binding of GAP to p21 or lack of stimulation of the GTPase activity leads to inhibited GTP hydrolysis and a constantly active p21-GTP complex. This is known to be a key event in malignant transformation. It is therefore of specific importance to study the mechanism of GTP hydrolysis and the corresponding conformational changes to understand the action of p21.

It has been shown previously that hydrolysis of the \(\gamma\)-phosphate of GTP by p21 results in an inversion of the configuration of chiral \(\Pi\), indicative of a direct transfer reaction onto water without a phosphoenzyme intermediate (7). There is also evidence that \(\Pi\) release is not rate-limiting in the GAP-stimulated reaction (8). The question as to the position of the rate-limiting step in GTP hydrolysis, however, remains unclear. Using fluorescence spectroscopy and fluorescent analogs of GTP, GDP, and the non-hydrolyzable analog GMPPNP, Neal et al. (9) and Moore et al. (10) provided evidence of a slow conformational change that precedes the actual GTP hydrolysis. They concluded that the conformational change was the rate-limiting step and that the actual hydrolysis of GTP was rapid with respect to this step. Rensland et al. (11), however, using the same technique, showed that the observed conformational change upon hydrolysis of p21-GTP was accelerated upon addition of GAP, whereas no effect was observed when instead of GTP the non-hydrolyzable GMPPNP was used. They concluded that the conformational change detected by structural methods (12, 13) and the actual GTP hydrolysis step occur simultaneously.

ESR spectroscopy using spin-labeled nucleotides has been successfully applied to investigate biochemical systems (for reviews, see Refs. 14–16). In addition to simple binding studies, where the amount of free substrate analog can be directly measured, one of the major advantages of ESR spectroscopy as a biophysical technique include its high sensitivity for conformational changes within the environment of the protein-bound radical.

In this paper, we present the first application of ESR spectroscopy using spin-labeled guanine nucleotides to study conformational changes within p21\(^{ras}\) during the GTP hydrolysis step. We show conformational differences within the nucleotide binding sites, depending on the phosphorylation state of the nucleotide bound (GTP, or GMPPNP versus GDP) and the conformational transition during hydrolysis.

**MATERIALS AND METHODS**

*Synthesis of Nucleotide Analogues—GMPPNP was synthesized directly from guanosine according to the method by Ludwig (17) for natural guanosine triphosphate, replacing pyrophosphate in the second step by imidodiphosphate. The esterification of GTP, GMPPNP, and GDP with the spin label moiety was achieved essentially as described (18, 19) with C2' and the C3' of the ribose moiety; SL-GDP, the corresponding GDP analog; SL-GMPPNP, guanylylimidodiphosphate, the corresponding, non-hydrolyzable spin-labeled analog of GTP; HPLC, high performance liquid chromatography.*
modifications in the purification procedure, where an anion exchange column, Sephadex DEAE A25, and a 0–0.6 M gradient of triethyl ammonium bicarbonate buffer, pH 7.5, was used. The procedure yielded 8–15% of the corresponding nucleotide analogs.

Protein—p21ras was prepared using an Escherichia coli expression system as described (20). Nucleotide-depleted p21 was prepared according to Ref. 21. Protein concentrations were determined according to Bradford (22) using defatted bovine serum albumin as a standard.

Spin-labeled Nucleotide Binding to p21—When nucleotide-depleted p21 was used for investigations, 40–50 μM protein were dissolved in 50 mM Tris-Cl, pH 7.5, and incubated with 38–45 μM spin-labeled nucleotide in the presence of 7.5 mM MgCl2. For nucleotide exchange, a 20-fold excess of spin-labeled nucleotide was incubated at room temperature with GDP-containing p21 in 50 mM Tris-Cl, 20 mM EDTA, pH 7.5. Unbound nucleotides were then removed by passage through a PD10 column (5 × 1.5 cm, Bio-Rad) eluted with 50 mM HEPES, pH 7.5.

p21-mediated Hydrolysis of SL-GTP—To determine the rate constants for p21-mediated SL-GTP hydrolysis, p21 in complex with SL-GTP was incubated at various temperatures for the times indicated in Fig. 1. Samples were injected onto a C18 reversed phase column (Shandon) under ion pairing conditions. The nucleotide analogs were eluted using a gradient of acetonitrile in a buffer containing 100 mM KH2PO4/K2HPO4, 10 mM tetrabutyl ammonium bromide, pH 6.5. The peaks were detected at 254 nm, and the peak areas were calculated using the 300E spectrometer operating the X-band mode. The spectra were recorded at 12.6 milliwatts microwave power and a peak to peak modulation amplitude of 0.8–2 G. The settings for signal gain, conversion constants for p21-mediated SL-GTP hydrolysis, p21 in complex with SL-GTP was incubated at various temperatures for the times indicated in Fig. 1. Samples were injected onto a C18 reversed phase column (Shandon) under ion pairing conditions. The nucleotide analogs were eluted using a gradient of acetonitrile in a buffer containing 100 mM KH2PO4/K2HPO4, 10 mM tetrabutyl ammonium bromide, pH 6.5. The peaks were detected at 254 nm, and the peak areas were calculated using the System Gold Software by Beckman Instruments.

ESR Spectroscopy—ESR spectra were recorded using a Bruker ESP 300E spectrometer operating the X-band mode. The spectra were recorded at 12.6 milliwatts microwave power and a peak to peak modulation amplitude of 0.8–2 G. The settings for signal gain, conversion times, and scan numbers varied with the different samples.

RESULTS

Fig. 1 shows the temperature dependence of p21-mediated hydrolysis of SL-GTP in the presence of excess Mg2+ ions. The data were obtained by determining the amounts of SL-GDP formed at the different times and temperatures indicated in the figure using a reversed phase, ion-pairing HPLC technique as described (Materials and Methods). Although hydrolysis was very slow at 0 °C, significant hydrolysis of the analog was observed at 25 °C. The rate constants calculated from the curves in Fig. 1 are summarized in Table I.

In Fig. 2 the ESR spectra of the corresponding non-hydrolyzable spin-labeled GTP analog, SL-GMPPNP, in complex with p21 are shown. When the spectra were recorded at room temperature (21 °C), three rather sharp signals dominate the ESR spectra (see Fig. 2, midfield region) that suggest a high amount of non-protein-bound, freely tumbling radicals. Increase of the signal gain, however, showed that at 21 °C the protein-bound radical species also showed rather high mobility (Fig. 2, low and high field region, 21 °C), resulting in signals of the bound component that are similar to the signals of the free radicals. Decrease of temperature led to drastic changes within the shapes of the ESR spectra, best seen in the increase of the hyperfine splitting (2Azz value) of the protein-immobilized radical species. The 2Azz values of the three different guanine nucleotide analogs in complex with p21 at the different temperatures are summarized in Table II. The data indicate significant differences of the relative environment of the different labels when bound to p21 (different conformations of the protein). The corresponding ESR spectra of SL-GTP, SL-GTP, and SL-GMPPNP as complexes with p21 also show significant differences in shape when recorded at 0 °C, as can be seen in Fig. 3.

Due to the differences within the spectral shapes and the differences in the corresponding 2Azz values of SL-GTP in complex with p21 versus SL-GDP in complex with p21 when the spectra were acquired at 0 °C, it was possible to study the conformational transition of the protein upon hydrolysis of the bound SL-GTP. This was achieved by incubating the p21-SL-GTP complex at 25 °C for various times and then acquiring the appropriate ESR spectra at 0 °C. The results are shown in Fig. 4. Even though there are distinct differences within the 2Azz values of the GTP and the GDP conformation of p21 and the transition from the GTP to the GDP form is clearly visible, the ESR spectra can not be resolved during the transition period due to superposition of the respective spectra. To be able to calculate a rate constant for the conformational transition, we therefore set the 2Azz value of the GDP form of p21 (see also Fig. 3B) to represent 100% formation of Ras-bound SL-GDP, while the 2Azz value of the GTP form (see also Fig. 3A) was set as 0% Ras-bound SL-GDP. The differences of the 2Azz values of the beginning or end points (GTP or GDP form) and the 2Azz value of the respective transition forms of p21 (which represent a superposition of both conformations at different ratios) were then normalized and plotted over the time of incubation at 25 °C. The error in determining the 2Azz values was within 2%.

| Temperature | 0 °C | 5 °C | 10 °C | 25 °C |
|-------------|-----|-----|-------|------|
| k (min⁻¹)  | 0.07 × 10⁻³ | 0.33 × 10⁻³ | 0.6 × 10⁻³ | 5.5 × 10⁻³ |
| Half-time (h) | 164 | 35 | 20 | 2.05 |

Fig. 1. p21-catalyzed hydrolysis of SL-GTP at different temperatures. p21 was incubated at concentrations ranging from 35 to 50 μM with equimolar concentrations of SL-GTP in the presence of 6.5–7.5 mM MgCl2 in 50 mM Tris-HCl, pH 7.5. Diamonds, the generation of SL-GDP after incubation at 0 °C; circles, generation of SL-GDP after incubation at 5 °C; inverted triangles, generation of SL-GDP after incubation at 10 °C; triangles, generation of SL-GDP after incubation at 25 °C. The curves were fitted using first order nonlinear least square fitting procedures.
The obtained data showed that SL-GTP is hydrolyzed by the corresponding GTP analog with p21 (Fig. 1). The rates calculated to test the formation of SL-GDP upon interaction of the corresponding protein and the non-hydrolyzable analog SL-GMPPNP (18, 19). To be able to study G-proteins using ESR spectroscopy as a direct indicator of the different conformations of the protein when either GTP or GDP are bound.

**DISCUSSION**

To be able to study G-proteins using ESR spectroscopy as a technique, it was necessary to synthesize spin-labeled guanine nucleotides that bind readily to the proteins. In the fluorescence spectroscopy studies mentioned in the Introduction, guanine nucleotide derivatives were employed where the fluorescence probe, an N-methyl anthraniloyl group, was attached to the 2’ and 3’-hydroxyls of the ribose moiety via an ester bond, similar to the 2’,3’-spin-labeled adenine nucleotides used to study F$_2$-ATPases from different species (19, 23–26) and the human Hsp90 (27). It is known that 2’,3’-modified guanine nucleotides bind to p21$^{ras}$ protein and are hydrolyzed with rate constants similar to GTP hydrolysis. We employed a synthesis procedure analogous to that for the adenine nucleotides to synthesize the corresponding 2’,3’-SL-GTP (SL-GTP), the GDP derivative, and the derivative of the non-hydrolyzable GMP-PNP analog (18, 19).

**21-mediated Hydrolysis of SL-GTP**—We determined the rate constant of hydrolysis of SL-GTP using HPLC techniques to test the formation of SL-GDP upon interaction of the corresponding GTP analog with p21 (Fig. 1). The rates calculated from the obtained data showed that SL-GTP is hydrolyzed by p21 with a rate constant ($5.5 \times 10^{-3}$ min$^{-1}$) at 25 °C similar to that of normal GTP ($6.8 \times 10^{-3}$ min$^{-1}$ at 20 °C (28)). SL-GTP can therefore be viewed as an excellent substrate analog of GTP for this protein (Table I).

**ESR Spectra of p21 in Complex with Spin-labeled Guanine Nucleotides**—ESR spectra of p21 protein in complex with the non-hydrolyzable analog SL-GMPPNP show that at 21 °C the signal of the protein-immobilized spin-labeled nucleotide is almost overlapped by the signals of the remaining free, non-enzyme-bound radical component, indicative of rather high mobility of the protein-bound radical (Fig. 2). To be able to observe structural changes within the protein, it is required that the signals of the protein-bound and the free radical components be well separated. We therefore decreased the temperature of the ESR experiments progressively to investigate the effects of lower temperatures on the signals of the bound versus the free components. We found that at 5 °C and 0 °C the signals of the protein-bound spin-labeled nucleotides were well separated from those of the free nucleotides. Therefore, and to exclude slow GTP hydrolysis during the ESR experiments, all the following ESR experiments were carried out at 0 °C.

The difference of at least 3.5 G (SL-GDP compared with SL-GTP) in the 2A$_{zz}$ values of the various nucleotide analogs in complex with p21 (Table II) is indicative of a significantly different environment of the radical component and is therefore a direct indicator of the different conformations of the protein when either GTP or GDP are bound.

In Fig. 3, the ESR spectra of the three different spin-labeled nucleotides (SL-GTP, SL-GDP, and SL-GMPPNP) in complex with p21 at 0 °C are shown. In addition to the differences in the outermost splitting of the signals, which are presented in Table II, there are also distinct differences in the line-shapes of the signals of the bound components. Although the spectra of SL-GDP and SL-GMPPNP in complex with p21 are rather sharp and are therefore probably indicative of one defined environment of the radicals, the spectrum of SL-GTP in complex with p21 shows a pronounced shoulder, most easily seen in the low field signal. This may indicate that in the GTP form of the protein various conformations co-exist, leading to different micro-environments of the bound radicals and therefore to slightly different ESR-signals.

The data presented strongly suggest that at least two significantly different conformations of p21 protein exist when p21 is in complex with either a GTP or a GDP, and that these conformations are discernible using spin-labeled nucleotides and...
ESR spectroscopy. The next step was to investigate whether the transition of the GTP form into the GDP form due to hydrolysis by p21 was also detectable using the ESR technique. It should then become possible to compare the rate constants of the conformational changes that occur during hydrolysis to the actual rate constants of hydrolysis as determined independently by SL-GDP formation using HPLC.

A typical ESR experiment for the time dependence of the conformational changes is shown in Fig. 4. To allow hydrolysis, the reaction mixture was incubated at 25 °C for the times indicated in the figure. The samples were then cooled to 0 °C, and the ESR spectra were taken. A change in the outermost splitting (2Azz value) and the shape of the spectra was observed (Fig. 4). To determine the rate constant for the conformational change, we set the predetermined 2Azz values of SL-GDP and SL-GTP in complex with p21 as the limits (GTP form resulted in a 2Azz value of 63 G; GDP form resulted in a 2Azz value of 59 G) and calculated the conformational change as percentages of the resulting 2Azz values at the different times. In Fig. 5, the results of two individual experiments are presented. Least square line fitting procedures resulted in a rate constant for the conformational change of (13.4 ± 5) × 10⁻³ min⁻¹. Control experiments showed that, in the presence of SL-GMPPNP in complex with p21, no time-dependent change in the shape of the ESR spectra was observed in identical experiments, indicating that no detectable conformational change took place under such conditions in the absence of hydrolysis.

The rate constants for the hydrolysis that were directly measured by GDP formation are in good agreement with the rate constants for the conformational changes that we obtained using ESR spectroscopy. This, together with the lack of a detectable isomerization of the p21-SL-GMPPNP complex, indicates that the results can be interpreted on the basis of a simple mechanism in which the rate-limiting step is the hydrolysis reaction itself, as suggested by Rensland et al. (11). Despite this conclusion, we cannot, of course, rule out that, at some level of interpretation or definition, a structural change may control the rate at which GTP hydrolysis occurs. Indeed, it could be maintained that this must be the case. The question at hand, however, is whether this structural change can be detected in the absence of GTP hydrolysis using the uncleavable GTP analog, and whether this is the step (possibly the only step) that can be accelerated by GAP.

We conclude that the results presented here show that there is no evidence for a pre-cleavage isomerization, even though the label is at a part of the molecule that appears to be sensitive to structural changes at or near the active site. Taken together with recent evidence that residues from GAP are needed to reach the transition state for rapid GTP hydrolysis (29), this further strengthens the hypothesis that the cleavage reaction
The correlation coefficient of the curve was 0.9875, and the standard deviation was 0.466.

The error to the rate constant refers to an estimate of the standard deviation.

The correlation coefficient of the curve was 0.9875, and the standard deviation was 0.466.

itslef is rate-limiting and that GAP alters the chemistry of the GTPase reaction.

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FIG. 5. Nucleotide hydrolysis-dependent conformational changes in p21 as observed using ESR spectroscopy. The 2Azz value for the GDP form of p21ras- was determined from the end point of the experiments described in Fig. 4 and independently from the experiments described in Fig. 3B that was set to represent 100% of the total conformational change observed, while the 2Azz value for the corresponding GTP form of p21ras- (determined from the start point of the experiments from Fig. 4 and from Fig. 3A) was set to represent 0% conformational change. The normalized changes of the 2Azz values were then plotted over time. Solving for the first order rate constants by nonlinear least square fitting procedures resulted in the line shown and the rate constant of (13.4 ± 5) × 10−3 min−1. The error to the rate constant refers to an estimate of the standard deviation.

The correlation coefficient of the curve was 0.9875, and the standard deviation was 0.466.