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# Conformational effects of nucleotide exchange in ras p21 proteins as studied by fluorescence spectroscopy

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The intrinsic fluorescence properties of the oncogene protein $p21^{N\text{-}ras}$, $p21^{H\text{-}ras}$ and one of its transforming mutants, $p21^{N\text{-}ras}$ (Val12), have been investigated. A mutant containing a single tryptophan at position 28 in $p21^{H\text{-}ras}$ (Trp28) has been specifically engineered to provide a probe of protein conformation on nucleotide binding. The proteins produced essentially similar circular dichroism spectra typical of alpha/beta proteins. A decrease in the intensity of the fluorescence emission spectrum due to tyrosine occurred on GDP/GTP nucleotide exchange in the native and mutant proteins. Selective excitation of the single tryptophan in p21 produced a decrease in fluorescence intensity which was accompanied by a blue shift in the wavelength of maximum emission on nucleotide exchange. A reduction in the residual $Mg^{2+}$ ion concentration enhanced this effect.

p21 ras protein; Guanine nucleotide binding; Tryptophan fluorescence

## 1. INTRODUCTION

The p21 products of the 3 functional ras genes, c.Ha, c.Ki and N-ras bind GTP/GTP, possess intrinsic GTPase activity [1–5] and display some sequence homology with the guanine nucleotide regulatory proteins (G proteins) [6–9]. This has led to speculation that the p21s act as signal transducers modulated by GTP hydrolysis and GDP/GTP exchange [10]. Point mutations at codons 12, 13, 59, 61 or 63 in ras genes have been shown to result in the production of oncoproteins capable of transforming established cell lines [11–13]. Many of these mutated proteins have altered GTPase activities and the amino acids involved occur in a highly conserved nucleotide binding domain [4,5,14–17]. The $p21^{ras} \cdot GTP$ complex represents the active conformation and the GDP bound form, the inactive one. When stimulated by a postulated detector molecule in the membrane, the protein then becomes activated, possibly through a conformational change required to accommodate the phosphate. The altered conformation may be translated into an intracellular signal by interaction with one or more postulated effector molecules (target proteins). Recently, a cytoplasmic protein GAP (GTPase activating protein) has been implicated as an effector protein [19]. It is believed that this protein interacts with ras proteins in the proposed effector binding region of the molecule, residues 32–40, and stimulates the GTPase activity of normal p21 but not its transforming mutants [19,20]. However, mutations in the effector domain have no effect on the intrinsic nucleotide exchange or GTPase activities of p21 [20]. The biological active state of p21 is regulated by the rate of hydrolysis of GTP. Transforming mutations occurring in the guanine dinucleotide beta-phosphate binding loop alter the protein conformation resulting in a stabilised active form and a reduction in the rate of GTP hydrolysis. It is speculated that GAP may serve to orientate this phosphate binding loop in the proposed 'catalytic' domain into a more accessible conformation for the GTPase enzyme [17]. The theory implies that the conformational change would be significant and therefore, would be likely to be translated throughout the molecule. This paper reports on attempts to ascertain whether structural differences between $p21 \cdot GDP$, and $p21 \cdot GTP$ complexes can be detected by fluorescence, a technique that is highly sensitive to changes in protein secondary structure.

The intrinsic fluorescence of p21 is dominated by 9 tyrosine residues in $p21^{N\text{-}ras}$, and 10 in $p21^{H\text{-}ras}$. A single tryptophan residue can act as a useful intrinsic probe of structure in steady-state fluorescence measurements by providing information on the local environment of the fluorophore. A tryptophan residue was specifically engineered to replace phenylalanine at position 28, which was predicted to lie close to the dinucleotide binding domain. This substitution was favoured as it preserves the essential hydrophobic character of the side chain and has minimal effect on the biological activity of the protein. Recent X-ray crystallographic

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evidence has shown that the phenyl side chain lies almost perpendicular to the guanine base [17].

Circular dichroism spectra were recorded in the far UV region of the spectrum (250–200 nm) to detect possible structural differences between the mutants and the effects of guanine nucleotide exchange.

## 2. MATERIALS AND METHODS

The construction of *E. coli* strains containing normal and mutant N-ras and Ha-ras expression plasmids is described elsewhere [20]. Harvested cells were lysed by sonication and the cell debris was pelleted by centrifugation. Nucleic acids were precipitated by addition of polyethylene imine (1% v/v). The proteins were purified by ion exchange on a DEAE Sephacel column equilibrated with buffer A (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 5 mM dithiothreitol). The protein was eluted with a salt gradient (50 to 500 mM NaCl) and fractions assayed for p21 by an [$^3$H]GTP binding assay [2]. p21-containing fractions were pooled and precipitated with 75% saturated ammonium sulphate. The precipitate was resuspended in buffer B (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 5 mM dithiothreitol) and applied to a G-75 Sephadex column. p21-containing fractions were pooled and stored as an ammonium sulphate precipitate at 4°C. Prior to usage the protein was dialysed against several changes of buffer A.

GTP binding assays were carried out at 37°C. [$^3$H]GTP (2 $\mu$M) was incubated with p21 · GDP (0.5 $\mu$M) in a total reaction volume of 40 $\mu$l containing 10 mM Tris-HCl, pH 7.5, containing 50 mM NaCl, 10 mM $MgCl_2$. Samples were removed at 20-min intervals and quenched with cold buffer (1 ml). After filtration through Millipore HAWP membranes, the [$^3$H]GTP-bound p21 was determined by scintillation counting.

Circular dichroism spectra were recorded on a Jasco J40CS spectropolarimeter. Silica optical cells of path lengths 0.05–0.1 cm were used. The chart record was in degrees of optical polarisation ($\theta$) × MRW (mean residue weight). All spectra were baseline corrected. In each case the control spectrum of the guanine nucleotide in buffer was subtracted. The spectra were recorded at intervals of 30 and 60 min.

Fluorescence measurements were recorded on a Perkin Elmer Luminescence Spectrofluorimeter LS-5. The observed excitation and emission spectra were corrected for variation in monochromator efficiency and photomultiplier sensitivity and expressed in arbitrary units. The appropriate buffer contributions were subtracted and the spectra were corrected for absorbance due to the guanine nucleotide. Quinine sulphate in 1 N $H_2SO_4$ was used as a standard to calibrate the instrument. Optical band widths of 2.5 and 4 nm were used for excitation and emission, respectively. A 2 × 10 mm cuvette was used. All fluorescence measurements were recorded at room temperature (25°C). p21 was incubated with up to 4-fold excess nucleotide (molar ratio) at 37°C prior to the measurements.

Protein concentration was determined using the Bradford reagent [22].

## 3. RESULTS AND DISCUSSION

The circular dichroism spectra of $p21^{N\text{-}ras}$, $p21^{H\text{-}ras}$, $p21^{N\text{-}ras}$ (Val12) and $p21^{H\text{-}ras}$ (Trp28) are essentially superimposable. The data are consistent with similar high alpha-helix and beta-sheet contents in all 4 proteins.

Incubation of a two-fold molar excess of GTP with normal p21 at 37°C resulted in a significant reduction in the negative ellipticity after 30 min. After a total interval of 60 min the signal had further decreased and a shift in the wavelength minimum from 218 to 215 nm was recorded. A similar experiment with GDP also resulted in a significant decrease in the signal. However, in this case, there was no observed shift in wavelength (fig.1). A control experiment in which protein was allowed to remain in the cell compartment at 37°C for 6 h without additional GDP/GTP showed that there was some loss of signal over this period. This could be attributed to partial loss of protein due to denaturation. GTP/GDP exchange rates were determined by filter binding as described in section 2. The rate of exchange is in agreement with results previously reported [23] and was found to be consistent with the rate at which the spectral shift is observed. A reduction in signal intensity which occurs in all preparations may be attributed to partial denaturation of the protein. These results support those of Pingoud et al. [24] who report small differences in the far UV circular dichroism spectra between p21 in the GTP and GDP forms but contradict later results suggesting that differences are not detectable in this region of the CD spectrum [25]. In a similar experiment using a limited amount of $p21^{N\text{-}ras}$ with a point mutant at Val12, the change observed for the normal ras protein was also detected.

Upon excitation at 278 nm, the intrinsic fluorescence emission spectrum of normal $p21^{N\text{-}ras}$ and $p21^{H\text{-}ras}$ is dominated by tyrosines as there are no tryptophans present. The spectra recorded after incubation of $p21^{H\text{-}ras}$ and p21 (Val12) with GTP at 37°C for 30 min shows a decrease in intensity (fig.2) whilst the wavelength of maximum emission at 306 nm ($\lambda_{em}$) is unchanged. This is characteristic of tyrosine fluorescence. A 10% decrease in intensity was obtained in a control experiment which was incubated at 37°C in buffer A only. Adjusting the pH of p21 to 2.0, at which the protein is inactive, produces an increase in fluorescence intensity. Decreased tyrosine fluorescence in the native state frequently originates from H-bonding of the tyrosyl groups. In $p21^{H\text{-}ras}$ (Trp28) the single tryptophan was selectively excited at 295 nm. An emission spectrum with a maximum at 342 nm was observed (fig.3). Preincubation with GTP for 30 min resulted in a reduction of approximately 20% in fluorescence intensity and a shift in the emission maximum to 338 nm. A control experiment in which protein was incubated at 37°C showed an approximate 10% increase in fluorescence intensity but no shift in wavelength maximum. The presence of a 4-fold molar excess of the non-hydrolysable analogue of GTP, guanosine-5′-*O*-(3-thiotriphospate) (GTP$\gamma$S) with $p21^{H\text{-}ras}$ (Trp28) causes a similar effect on fluorescence intensity relative to GDP. It is thus evident that the substitution at phenylalanine 28 does not impair GTP/GDP exchange. A time course study of these changes correlates with the rate of exchange of GTP with p21 · GDP and the rate of association of the non-hydrolysable analogue

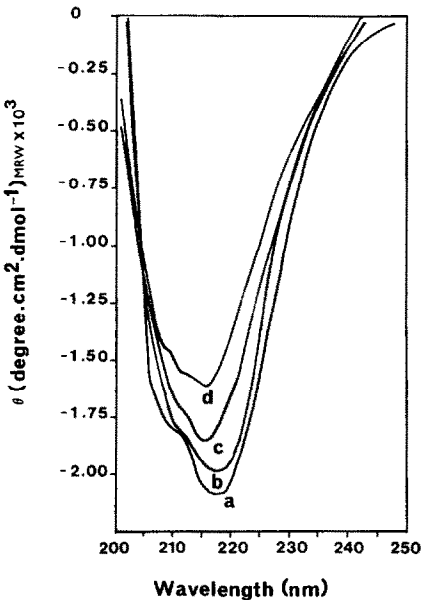


Fig.1. Circular dichroism spectra of $p21^{H\text{-}ras}$. (a) $p21^{H\text{-}ras}$ (20 $\mu$M) in buffer A. (b) $p21^{H\text{-}ras}$ (20 $\mu$M) incubated in buffer A + GDP (100 $\mu$M) for 30 min. (c) $p21^{H\text{-}ras}$ (20 $\mu$M) incubated in buffer A + GTP (100 $\mu$M) for 30 min. (d) $p21^{H\text{-}ras}$ (20 $\mu$M) incubated in buffer A + GTP (100 $\mu$M) for 60 min.

GTP$\gamma$S, and therefore, for $p21^{H\text{-}ras}$ (Trp28), fluorescence can be used to assess quantitative binding of guanine nucleotides and their analogues (data not shown). In the presence of residual $Mg^{2+}$ and GTP, p21·GDP exists as a stable complex with a half-life of approximately 20 min [23]. It is reported that the affinity of p21 for GTP and GDP are approximately the same at residual concentrations of 5 mM $Mg^{2+}$ whereas at 0.5 $\mu$M $Mg^{2+}$ p21 has a 10-fold greater affinity for GTP [21,23]. To bias the binding in favour of GTP and thereby reduce the incubation periods, the nucleotide exchange experiments were carried out in the presence of 10 mM EDTA. It was calculated that this would reduce the $Mg^{2+}$ to below 0.5 $\mu$M. The presence of

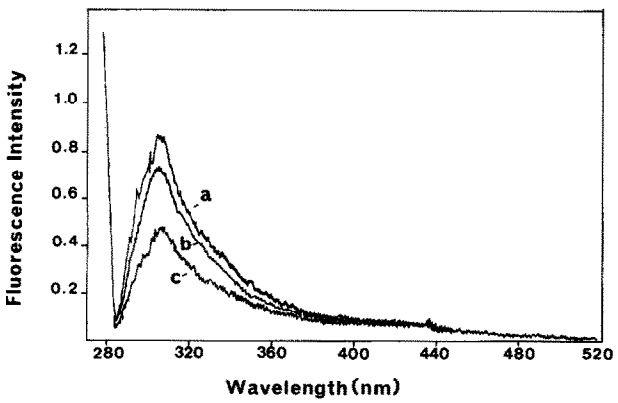


Fig.2. Emission spectra of $p21^{H\text{-}ras}$ excited at 278 nm. (a) $p21^{H\text{-}ras}$ (0.5 $\mu$M) in buffer A. (b) $p21^{H\text{-}ras}$ (0.5 $\mu$M) incubated at 37°C for 30 min in buffer A. (c) $p21^{H\text{-}ras}$ (0.5 $\mu$M) + GTP 2 M incubated at 37°C in buffer A.

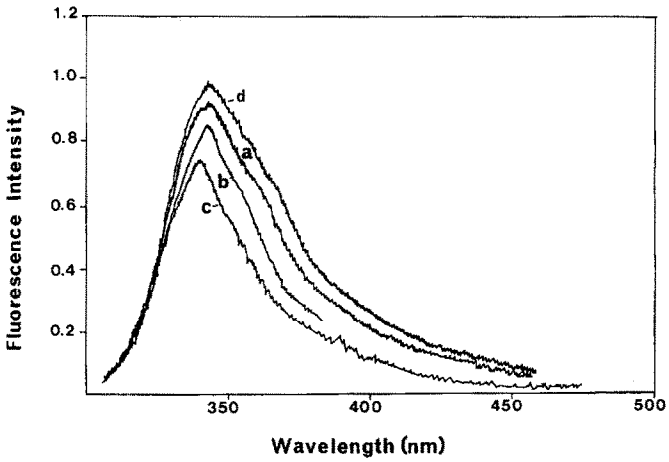


Fig.3. Emission spectra of $p21^{H\text{-}ras}$ excited at 295 nm. (a) $p21^{H\text{-}ras}$(Trp28) (0.5 $\mu$M) in buffer A. (b) $p21^{H\text{-}ras}$(Trp18) (0.5 $\mu$M) incubated for 30 min in buffer A + 2 $\mu$M GDP. (c) $p21^{H\text{-}ras}$(Trp28) (0.5 $\mu$M) incubated for 30 min in buffer A + 2 $\mu$M GTP. (d) $p21^{H\text{-}ras}$(Trp28) (0.5 $\mu$M) in buffer A + 10 mM EDTA.

EDTA alone was shown to have no effect on the fluorescence spectrum of $p21^{N\text{-}ras}$ and $p21^{H\text{-}ras}$. However, excitation of $p21^{H\text{-}ras}$ (Trp28) at 295 nm in 10 mM EDTA resulted in a significant increase in fluorescence due to the single tryptophan residue. This is presumably caused by displacement of a dinucleotide from the guanine nucleotide binding site due to chelation of $Mg^{2+}$ which is essential for effective nucleotide binding, thereby permitting Trp28 to become more accessible to solvent. The fluorescence was instantly quenched by the subsequent addition of GDP and/or GTP. The slight differences between the fluorescence spectra of $p21^{H\text{-}ras}$(Trp28)·GDP and $p21^{H\text{-}ras}$(Trp28)·GTP may reflect conformational differences between them. Similar experiments to confirm these results are being carried out using preparations of nucleotide free protein.

These preliminary results suggest that the GDP and GTP p21s are conformationally distinct in both the normal and transforming mutant Val12. The distinct forms can be detected both by circular dichroism and intrinsic fluorescence spectroscopy. The genetically-engineered mutation at Phe28 will provide an important probe in further studies of structural and dynamic aspects of the mechanism of nucleotide exchange and hydrolysis which are currently in progress. The fact that substitution of this phenylalanine residue by tryptophan does not impair GDP/GTP binding suggests that it is not directly involved in nucleotide binding. In conclusion, the results of this study are consistent with the current hypothesis for the mechanism of action of p21 which suggests that the protein can adopt two conformations. The implication of this awaits a full comparison of the X-ray structures of the GDP- and GTP-bound forms.

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