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PROTON NMR STUDIES OF THE GDP.MG^{2+} COMPLEX OF THE HA-RAS ONCOGENE PRODUCT P21

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Summary: Two-dimensional proton NMR studies were performed on the c-Ha-ras encoded proto-oncogene product $p21_c$. COSY and NOESY spectra of the $p21_c$.GDP.Mg^{2+} complex show that the ribose H1' proton of the bound GDP is in close proximity to the aromatic side chain of a phenylalanyl residue. From sequence homology with the bacterial elongation factor Tu (EF-Tu) and the known X-ray structure of the EF-Tu.GDP.Mg^{2+} complex it may be inferred that the Phe residue in question is either Phe^{78} or Phe^{82} in the p21 sequence.

The gene products of the ras family are highly related proteins of molecular weight 21 kDa termed p21 with a chain length of 189 amino acids [1,2]. They bind guanine nucleotides with high affinity and specificity and exhibit low GTPase activity [1-3]. Since the X-ray structure of p21 is not yet available, the method of choice to obtain information on structural features of p21.nucleotide complexes in solution is proton NMR spectroscopy, in particular NOESY-spectroscopy [4].

We have applied this technique to the study of highly purified bacterially expressed cellular p21 ($p21_c$) in the $p21_c$.GDP.Mg^{2+} complex in order to elucidate details of protein-substrate interactions.

Abbreviations: COSY: correlated spectroscopy, EF-Tu: bacterial elongation factor EF-Tu, NOESY: nuclear Overhauser enhancement spectroscopy, NMR: nuclear magnetic resonance, $p21_c$: the Ha-ras-gene encoded cellular p21, RELAY: Relayed coherence transfer spectroscopy

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Material and methods

Sample Preparation: p21 was prepared as its p21.GDP.Mg^{2+} complex and activity checked with the radioactive GDP binding assay as described in [5]. Activity was found to be higher than 90 % of the theoretical maximum. H_2O was exchanged for D_2O by freeze-drying and redissolving the samples twice in D_2O. This results in no loss of activity.

NMR-Spectroscopy: NMR experiments were performed on a commercial Bruker AM 500 spectrometer working at a proton resonance frequency of 500 MHz. Sample temperature was kept at 303 K with a precooled stream of dry air which was temperature-regulated with a standard Bruker VT1000 unit. Standard procedures and commercially available software was used throughout. The residual HDO resonance was suppressed by permanent (execpt acquisition) selective irradiation at the HDO frequency. Quadrature detection and its phase-cycle generated ω_1-analog were used in all experiments. COSY and NOESY experiments were performed as phase sensitive experiments. All spectra were referenced to internal sodium 4,4-dimethyl-4-silapentanesulfonate (DSS).

Experimental data: Double-quantum filtered COSY-experiment: time-domain matrix size 512w x 4k, sweep-width 4716 Hz in either dimension, relaxation delay 1.2 sec, 256 scans per time increment; frequency-domain matrix 2k x 2k; an unshifted sine filter was applied before transformation in both dimensions. NOESY-experiment: time-domain matrix size 1k x 1k, relaxation delay 1.4 sec, mixing time 0.15 sec, random variation of mixing time 15 %, number of scans per time increment 128; a shifted sine filter ($\pi/32$) was applied in both dimensions.

Results

The resonances in the one-dimensional proton NMR spectrum of the p21.GDP.Mg^{2+} complex appear rather ill-resolved (fig. 1a). Only very few multiplets originating from aromatic side chains can be observed (fig. 1b). The singulets at 7.70 ppm and 7.55 ppm represent two and one of the C2-H resonances of the three imidazole rings, respectively, in the p21 sequence. The resonance at 6.04 ppm of intensity one proton as calibrated against the intensity of the histidyl imidazole ring singulet resonances originates from the H1' proton of the ribose of the bound GDP. Even in the p21 bound state, it shows the typical doublet splitting due to the coupling to the ribose H2' - proton.

We performed COSY- and NOESY-experiments in order to identify some of the aromatic spin systems in the spectrum and to obtain spatial information on the location of the GDP in the p21.GDP.Mg^{2+} complex. In spite of the rather ill-resolved one dimensional spectrum the two-dimensional spectra were of surprisingly good quality. They corroborate the assignment of the 6.04 ppm resonance to the ribose H1'-proton by the detection of a COSY crosspeak between this proton and a resonance at 4.75 ppm originating from the H2' proton of the ribose, and of a NOESY crosspeak (6.04 ppm - 8.0 ppm) origi-

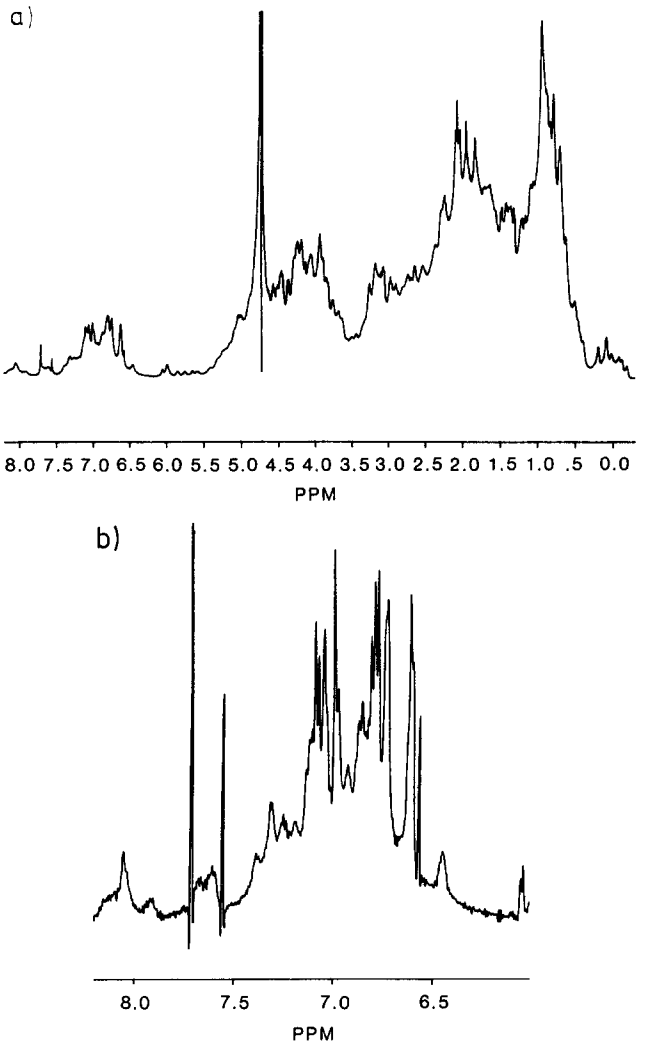

Fig. 1. (a) Proton NMR spectrum of the p21$_c$.GDP.Mg^{2+} complex; protein concentration: 1 mM; buffer: 50 mM sodium borate, pH 8, 10 mM $MgCl_2$, 1 mM DTE, 2 mM NaN_3. (b) aromatic part of (a), Gaussian multiplication (GB^2 = 0.1) before transformation.

nating from the 8 proton of the guanine base of the bound GDP (data not shown). The NOESY-spectrum clearly shows two crosspeaks between the H1' of the bound GDP and two resonances in the aromatic region (fig. 2). These two resonances can be shown to belong to the spin system of the aromatic side chain of a single phenylalanyl residue, termed F^A in fig. 2. The chemical shift values of the F^A resonances are 6.45 ppm (meta), 6.63 ppm (ortho or para), and 6.88 ppm (para or ortho), which means that the order of resonances is changed as compared to those found for the free amino acid (7.30 ppm/ortho, 7.34 ppm/para and 7.39 ppm/meta respectively [4]). Ortho

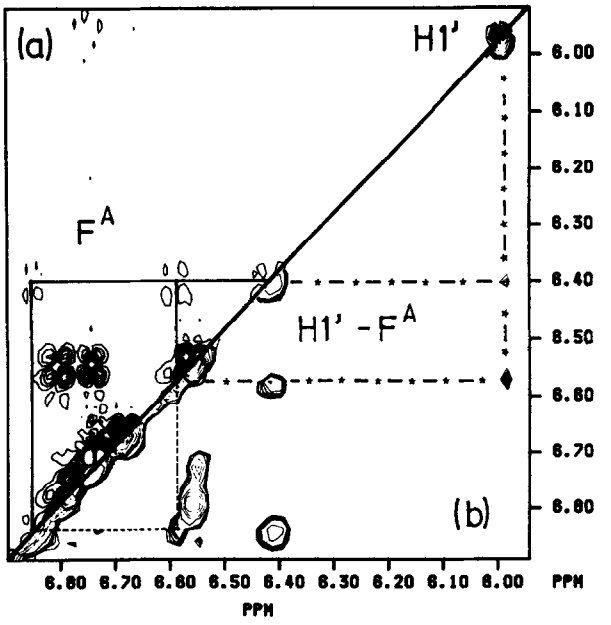

Fig. 2. Part of the COSY- (a) and NOESY- (b) spectrum centered around the H1' resonance of the bound ribose in the $p21_t$.GDP.Mg^{2+} complex; protein concentration: 4 mM; buffer: 50 mM potassium phosphate, pH 6.5, 10 mM $MgCl_2$, 1.5 mM DTE, 3 mM NaN_3.

——: COSY-, - - - -: RELAY- and - * - * -: NOESY-connectivities.

and para resonances cannot be distinguished on the basis of our current experiments. Crosspeaks between the H1' and H8 resonances of the nucleotide and other protein resonances in the aromatic region could not be observed.

Discussion

The presence of high field shifted methyl resonances in the one-dimensional NMR spectrum indicates that our preparation procedure conserves a well defined tertiary structure of the p21.GDP.Mg^{2+} complex. This correlates well with the finding that the proteins purified under native conditions have high GDP/GTP binding and GTPase activity [3,5]. Our two-dimensional COSY and NOESY spectra of the metal.nucleotide complex of p21 show that the meta and ortho or para protons of a phenylalanyl ring are close to the H1' ribose-proton of the p21 bound GDP. Since intensities of NOESY-crosspeaks are proportional to the inverse sixth power of the distance, the results of our NMR experiments may be taken as evidence that the protons of the H1'

ribose and the aromatic ring of one phenylalanyl residue in p21 are within ca. 0.5 nm of each other [4].

Several reports [6,7] have pointed to sequence homologies between p21 proteins and other nucleotide binding proteins, such as the bacterial elongation factor EF-Tu. If the weak but suggestive sequence homology between p21 and EF-Tu implies structural homology between these two GDP binding proteins, then a tentative assignment of the Phe residue in contact with the ribose H1' can be made. Since the three-dimensional structure of EF-Tu is known from X-ray crystallography [8,9] one can infer the positioning of the p21 residues based on the known positions of homologous amino acids in the EF-Tu structure. Only amino acids homologous to two of the five phenylalanyl residues in p21, namely Ala^{101}, which is homologous to Phe^{78}, and Val^{105}, which is homologous to Phe^{82}, are close enough to the GDP molecule in the three-dimensional structure of EF-Tu.GDP.Mg^{2+} as proposed by Jurnak [8] to give rise to the observed effects in p21. Since, from the X-ray data [8], Val^{105} in EF-Tu is closer to the H1' ribose than Ala^{101}, we favour identification of Phe^{A} as Phe^{82}.

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References

1. Gibbs,J., Sigal,I.S. and Scolnick,E.M. (1985) Trends Biochem. Sci. 10, 350-353
2. Barbacid,H. (1987) Ann. Rev. Biochem. 56, 779-827
3. Feuerstein, J., Kalbitzer, H.R., John, J., Goody, R.S. and Wittinghofer, A. (1987) Europ. J. Biochem. 162, 49-55
4. Wüthrich, K. (1986) in: NMR of Proteins and Nucleic Acids, Wiley Interscience, New York
5. Tucker, J., Sczakiel, G., Feuerstein, J., John,J., Goody, R.S., and Wittinghofer, A. (1986) EMBO J. 5,6 1351-1358
6. Lebermann, R. and Egner, U. (1984) EMBO J. 3, 339-341
7. Halliday, J.R. (1983) J. Cyclic. Nu. 9, 435-448
8. Jurnak, F. (1985) Science 230, 32-36
9. LaCour, T.F.M., Nyborg, J., Thirup, S. and Clark, B.F.C. (1985) EMBO J. 4,9 2385-2388