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^{31}P-NMR Spectra of the Ha-ras p21.Nucleotide Complexes

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Summary: Phosphorus nuclear magnetic resonance spectra of the Ha-ras oncogene product p21 and its nucleotide complexes have been obtained. It is shown that the ^{31}P nuclear magnetic resonance spectra of a number of nucleotide-enzyme complexes show some common features. In particular, the chemical shift values of the β-phosphorus resonance of enzyme-bound NTP and NDP (N=A,G) of hydrolases exhibit a downfield shift virtually identical for myosin, elongation factor Tu, and the Ha-ras oncogene product p21. This suggests that the stereochemistry around the β-phosphorus might be similar in these compounds.

Introduction: Much speculation prevails currently concerning structural similarities of nucleotide-binding enzymes such as hydrolases and phosphokinases. This discussion has been stimulated in particular by the discovery of similarities in primary structure between the nucleotide-binding ras oncogene products and other nucleotide-binding proteins like elongation factor Tu (EF-Tu), the signal-transducing G-proteins, and adenylate kinase (AK) [1-4]. Recently, tertiary structure predictions of the ras-oncogene product p21 have suggested that there may be significant similarities between the nucleotide-binding sites of p21 and EF-Tu from E.coli as determined by X-ray studies of the GDP-complex of trypsin treated protein [5,6].

Unfortunately, all these speculations lack direct experimental evidence. A method particularly suited to investigate structural similarities of proteins is nuclear magnetic resonance (NMR). In particular phosphorus NMR is a method most suitable for probing directly the surroundings of the phosphate moiety of enzyme-bound nucleotides [7]. Studies of this kind have been performed on the nucleoside diphosphate as well as on the nucleoside triphosphate complexes of

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the proteins. In the case of kinases, such as adenylate kinase, these complexes could be observed with or without the ongoing catalytic reaction [8,9]. In the case of the GTP hydrolase EF-Tu, the triphosphatase activity in the absence of effectors (the ribosome) is so slow that the GTP and GTP.Mg^{2+} complexes and even an intermediate state complex could be observed by means of NMR as well as EPR [10,11,12].

Until recently, NMR studies on p21 have been impossible due to the prohibitively low amount of material available from normal or retrovirus infected cells. We have expressed the authentic viral Ha-ras encoded p21 in E.coli, and were thus able to obtain gram quantities of the viral protein in a soluble form [13]. Therefore a comparison of the p21-nucleotide complexes with other protein-nucleotide complexes by NMR is now possible.

Materials and Methods: The viral Ha-ras gene was cloned into an E.coli expression vector containing the tac promoter such that the authentic p21 was expressed in high amounts in a soluble form. The construction of the expression vector and purification of $p21_v$ will be described elsewhere [13]. The samples for the NMR experiments were prepared by freeze drying and redissolving the protein to give a final concentration of 1 mM p21 in 50 mM Tris buffer, pH 7.4, and 2 mM DTE. 10% D_2O was added. All NMR measurements were performed on a Bruker CXP 360 spectrometer with a ^{31}P-NMR frequency of 145.78 MHz. Temperature was controlled with a standard Bruker temperature control unit. 10 mm sample tubes containing 2 ml solution were used throughout. Chemical shifts are referenced to external 85% H_3PO_4 as usual.

Results and Discussion: Figure 1 shows the spectrum of the p21.GDP.Mg^{2+} complex as it resulted from the protein preparation. The α-phosphate and β-phosphate resonances of the bound GDP are clearly visible as the only resonances appearing in the spectrum. The good signal-to-noise ratio indicates that $p21_v$ contains approximately equimolar concentrations of bound GDP.

The most remarkable feature of this spectrum is the value of the chemical shift of the β-phosphorus resonance at -1.3 ppm, which is 4 ppm downfield from unbound GDP.Mg^{2+} [14]. Removal of the metal ion from the sample by addition of EDTA resulted in the spectrum shown in the lower trace. The most prominent change in the spectrum is the shift of the β-phosphate resonance towards higher field, i. e. from -1.3 ppm to -2.8 ppm, closer to the position where the corresponding resonance of metal-free GDP were to be expected (6.3 ppm) [14]. Another most remarkable feature of the lower spectrum in the figure is

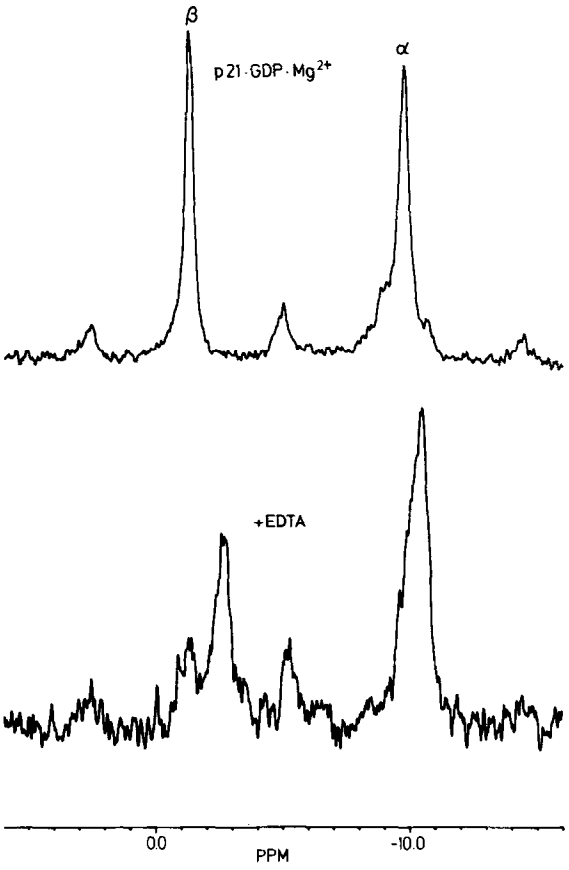

Figure 1: Upper spectrum: ^{31}P-NMR spectrum of the p21.GDP.Mg^{2+} complex; additional peaks are attributed to the presence of some p21.GTP.Mg^{2+}.
Lower spectrum: as above, 8-fold excess of EDTA added.

the linebroadening of the β-phosphate resonance still present at an 8-fold excess of EDTA. This can only be explained as indicating a binding constant of the metal ion to the enzyme.nucleotide complex of the same order of magnitude as the EDTA.Mg^{2+} binding constant under these experimental conditions.

Addition of 3 mM GTP to a sample containing the p21.GDP.Mg^{2+} complex in 1 mM concentration in the presence of an 8-fold excess of magnesium chloride resulted after 2 hrs incubation in the spectrum shown in the top trace of figure 2. Most prominent are the resonances of the α-, β-, and γ-phosphate groups of GTP.Mg^{2+} free in solution [14]. In addition, resonances at the position of the α- and β-resonances of uncomplexed GDP.Mg^{2+} appear [14]. Of the remaining resonances, the ones originating from bound GDP.Mg^{2+} are readily assigned by their position as in figure 1. The last three resonances are

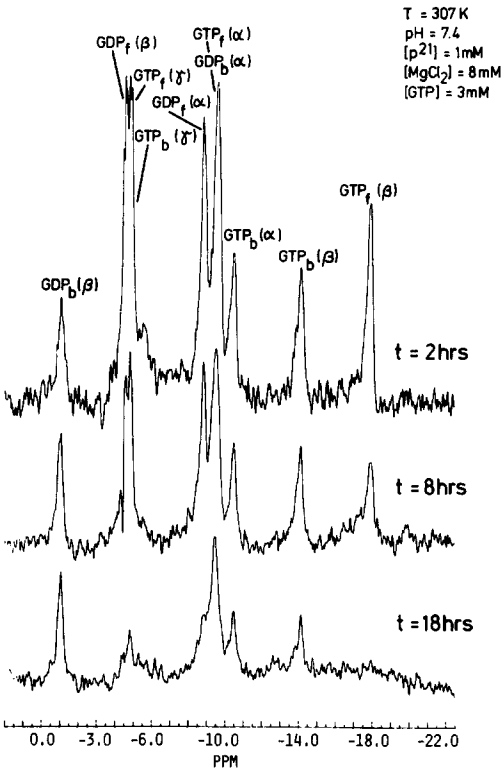

Figure 2: ^{31}P-spectra of 1mM p21.GDP.Mg^{2+} complex 2 hrs, 8 hrs, and 18 hrs after addition of 3 mM GTP.

assigned to the phosphate groups of enzyme-bound GTP.Mg^{2+}, which means that protein-bound GDP has been exchanged against GTP.

These assignments are corroborated by following the time course of the peak intensities (middle and bottom trace of fig. 2). The peaks corresponding to free GTP.Mg^{2+} are markedly reduced after 8 hrs of incubation and disappear completely after 18 hrs. A similar time course is followed by the resonances corresponding to free GDP.Mg^{2+}. The disappearance of the GTP resonances is most probably due to the intrinsic GTPase activity of p21. The GTPase activity of viral p21 has been reported to be much lower than that of cellular p21 [15,16,17], but at 1mM concentration it is apparently high enough to completely hydrolyze the added GTP within the experimental time. The reduction of the GDP resonances is not readily explained and may be attributed to some GTP:GMP phosphotransferase activity within the sample, either caused by the p21 itself or, more likely, by some contaminating enzyme. In any case, the only major remaining peaks after 18 hrs incubation originate from the bound

metal.nucleotide complexes. (The resonances of P_i and GMP are outside the displayed spectrum.) No phosphothreonine peak was observed during the GTP hydrolysis reaction.

Thus a shift of the β-phosphate resonance of the p21-bound metal.GTP complex of -14.4 ppm was observed, as compared to -19.0 ppm for the free GTP.Mg^{2+} complex and -21.3 ppm for the free GTP [14]. The changes of chemical shifts of the α-phosphate and γ-phosphate resonances of GTP on enzyme and metal ion complexation were comparatively small, as was the change for the α-phosphorus resonance of GDP.

A similar pattern was observed for the β-phosphate chemical shift with other enzyme.nucleotide complexes. Most remarkable was the similarity between the nucleotide diphosphate complexes of p21, EF-Tu, and myosin S1 shown in Table 1. It shows that the hydrolases and kinases can be grouped separately according to NMR β-resonance shift data. The shift of the kinases, with one exception, is in the -3.3 ppm to -5.7 ppm region, that of the hydrolases in the -1.3 ppm to -1.7 ppm region. It also appears that the magnitude of the chemical shift of the β-phosphate resonance can possibly be correlated to the magnitude of enzyme.nucleotide.Mg^{2+} binding constants, which are much higher for the hydrolases. In the case of the β-resonance of the nucleotide triphosphate complexes of these proteins, the hydrolases show a 2 ppm - 4 ppm shift of unbound versus bound form, whereas the kinases show no such shift (with one exception). Interestingly, in the GTP form, p21 and bacterial

Table 1: ^{31}P chemical shifts of nucleoside diphosphates and nucleoside triphosphates in their protein.complexes in the presence of Mg^{2+}

	NDP*Mg^{2+}		NTP*Mg^{2+}			
	α	β	α	β	γ	
free	- 9.5	- 5.4	-10.4	-19.0	- 5.4	[14]
Elongation Factor Tu	- 9.6	- 1.3	- 9.8	-17.1	- 5.1	[10]
Elongation Factor Tu	- 8.9	- 1.5				[11]
Ha-ras p21	- 9.7	- 1.4	-10.9	-14.4	-5.3	[10]
Myosin S1		- 1.7				
Arginine Kinase	-11.0	- 3.3	-11.0	-19.4	- 5.6	[8]
Creatine Kinase	-11.0	- 3.8	-10.9	-19.0	- 5.4	[8]
Adenylate Kinase	-10.2	- 2.8	-10.7	-14.8	- 6.1	[8]
Phosphoglycerate Kinase	-11.0	- 7.5	-11.0	-19.4	- 6.0	[8]
Pyruvate Kinase	-10.0	- 5.7	-10.9	-19.2	- 5.5	[8]

elongation factor Tu show a large difference in the shift for the β-phosphorus.

From the respective intensities of the β-phosphate resonances of protein-bound GTP.Mg^{2+} and GDP.Mg^{2+} the relative concentration of the two species follows directly. The triphosphate and the diphosphate complexes seem to have a stability constant of the same order of magnitude, as was concluded earlier from binding studies [19,20]. This feature already proved very useful in the course of further NMR studies, because it makes observation of the triphosphate complex much simpler than, for example, with the EF-Tu.GTP.Mg^{2+} complex. In the latter, a difference of two orders of magnitude in the stability constants of the GDP.Mg^{2+} and GTP.Mg^{2+} complexes in favor of the diphosphate form necessitated the use of a recycling system in order to obtain observable concentrations of the triphosphate complex [10].

It has been noted earlier that GTP-dependent autophosphorylation of threonine at position 59 of the viral Ha-ras p21 occurs [21]. We could not detect any sign of a phosphothreonine resonance in our spectra, perhaps due to overlap with the strong resonances caused by P_i and GMP, or because the concentration of autophosphorylated p21 was to low.

The experiments reported here give a direct support to the suggestion by Jurnak and Cormick et al. for similarities between the β-phosphate binding moieties of EF-Tu and p21 [5,6]. In previous studies it has been shown that the EF-Tu.GDP complex binds the metal ion in the β-monodentate form [11,22]. This was also shown for some, but not all, kinases by stereochemical methods and electron paramagnetic resonance spectroscopy. Kinetic and EPR studies lead to the same conclusion for the myosin S1.ADP.Mg^{2+} complex (for a recent review see [23]). Thus it can be concluded from the presented data that the GDP in the p21.GDP.Mg^{2+} complex might also be in a similar environment, perhaps also bound as a monodentate metal-nucleotide complex. It seems, however, from the NMR shift data that the similarities between EF-Tu and p21 might not pertain to the protein.GTP complex. This is also corroborated by the fact that for EF-Tu there is a drastic change in binding constant between EF-Tu.GTP and EF-Tu.GDP [24] and a local conformational change in the nucleotide-binding site,

as seen by EPR-measurements [12] and other methods [24]. For p21, there is only a slight difference in affinity for GTP/GDP ([19,20] and above), and EPR measurements of manganese complexes of p21 with GDP/GTP do not show this drastic change in line shape (Kalbitzer, Feuerstein and Wittinghofer, unpublished).

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