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Characterisation of the metal-ion – GDP complex at the active sites of transforming and nontransforming p21 proteins by observation of the ^{17}O-Mn superhyperfine coupling and by kinetic methods

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Kinetic studies on the interaction of three Ha-*ras*-encoded p21 proteins with GDP and MgGDP have yielded values for the association ($10^6 - 10^7$ M^{-1} s^{-1}) and dissociation ($10^{-3} - 10^{-5}$ s^{-1}) rate constants at 0 °C. Dramatic differences in the rate constants were not observed for the three proteins. Under non-physiological conditions (absence of Mg^{2+}), the rate constant for GDP release was an order of magnitude faster for the viral protein $p21_v$ than for the cellular form $p21_c$ or the T24 mutant $p21_t$, but this was reduced to a factor of about 3 in the presence of Mg^{2+}. In all cases, there was an increase of about one order of magnitude in the rate of GDP release on removing magnesium. The binding affinities ranged from 5.7×10^{10} M^{-1} for $p21_c$ to 1.3×10^{11} M^{-1} for $p21_v$. Electron paramagnetic resonance (EPR) measurements on Mn^{2+} bound together with stereospecifically ^{17}O-labelled GDP showed direct coordination of a β-phosphate oxygen to the metal ion with a superhyperfine coupling constant of 0.16 – 0.22 mT, but no interaction with the α-phosphate oxygens at the active site of all three proteins. The association constant of Mn(II) to p21 proteins in the absence of nucleotides was estimated to be $> 10^5$ M^{-1}. In agreement with the EPR results, experiments on the metal ion dependence of the binding of thiophosphate analogs of GDP provided further evidence for the absence of direct coordination of the metal ion to the α-phosphate group. These results have been used to construct a model for the interactions of Mg · GDP with the active site of p21 proteins.

A deeper insight into the role of oncogenes during cell transformation can only be obtained by understanding the function of the proteins they encode. The products of the *ras* oncogenes, the p21 proteins (for reviews, see [1, 2]) are thought to be related to the group of guanine nucleotide (G) regulatory proteins such as transducin and elongation factor Tu (EF-Tu), because of significant sequence homology and similar enzymatic properties [3 – 7]. It is characteristic for these proteins that their regulatory function is modulated by GTP and GDP binding or, more precisely, by the binding of the corresponding Mg^{2+} complexes. A common property of all known G proteins is a low but clearly detectable hydrolytic activity in the absence of appropriate physiological stimulators [2, 8 – 10].

The GTPase activity of p21 is lowered in oncogenic forms of the protein [11 – 14]. There are, however, conflicting reportes concerning the relationship of this lower GTPase activity to the transforming properties of the proteins [15 – 17]. Surprisingly, it is generally believed that the nucleotide binding ability is unaltered in the transforming mutant proteins [2, 11, 15, 17 – 20]. However, it is probable that there are structural differences in the nucleotide binding site between the cellular and mutant forms of p21 which are responsible for the altered GTPase activity. Obviously, knowledge of the structural properties of the nucleotide binding site is essential for the understanding of these differences and may even have an impact on the development of cytostatic drugs which could stabilize one of the states of p21 proteins.

Models for the tertiary structure of the nucleotide-binding site of p21 have been built on the basis of partial sequence homologies between EF-Tu and p21 and using the tertiary structure of the GDP-binding domain of trypsinized EF-Tu from *Escherichia coli* [21, 22], but only a few experimental facts relating to the structure of the p21 · Mg^{2+} · nucleotide complex have been reported, e. g. studies using thioanalogs of GDP and GTP [23] or ^{31}P-NMR [24]. We have applied here kinetic and binding measurements to obtain information about the GDP binding site of p21, and the differences between the cellular and mutant forms of p21. Further information on structural details of Mg^{2+}-nucleotide complexes bound to p21 proteins was obtained by EPR spectroscopy after substitution of Mg^{2+} by its analog Mn^{2+}, especially by the observation of the ^{55}Mn-^{17}O superhyperfine interaction after appropiate modification of ligands. Together these results allow us to describe in detail the metal-nucleotide

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Abbreviations. EPR, electron paramagnetic resonance; GDP[αS], guanosine 5′-[α-thio]diphosphate; GDP[βS], guanosine 5′-[β-thio]diphosphate; GTP[βS], guanosine 5′-[β-thio]triphosphate; Ha-*ras*, the human Ha-*ras* gene; $p21_c$, cellular Ha-*ras*-encoded p21 protein containing Gly-12 and Ala-59; $p21_t$, T24 mutant form containing Val-12; $p21_v$, viral Ha-*ras*-encoded p21 protein containing Arg-12 and Thr-59; EF-Tu, bacterial elongation factur Tu.

complexes at the active center of transforming and non-transforming p21 proteins.

MATERIALS AND METHODS

Materials

^{17}O-enriched water was obtained from Monsanto Research Corporation (Miamisburg, USA) and contained 12.5% $H_2{}^{16}O$, 52.4% $H_2{}^{17}O$ and 35.1% $H_2{}^{18}O$. [^{3}H]GDP (400 GBq/mmol) was obtained from Amersham Buchler and was, if necessary, diluted to the desired specific activity with unlabelled GDP (Pharma Waldhof). Nitrocellulose filters, type BA85, 0.45 μm were from Schleicher & Schüll. All reagents were of the highest purity available.

Preparation of ^{17}O-labelled GDP and of thioanalogs

Regio- and stereospecifically labelled GDPs were prepared as described by Wittinghofer et al. [25] using ^{17}O-enriched water. Thiophosphate analogs of GDP were prepared according to Goody and Leberman [26] and Roesch et al. [27]. All nonradioactive nucleotides were passed over Chelex to remove trace amounts of heavy metal ions.

Protein preparation

The p21 proteins $p21_c$, $p21_t$ and $p21_v$ were bacterially expressed and purified as described by Tucker et al. [23]. The GDP binding activity of the proteins used in this study was always higher than 45 nmol/mg using the simple nitrocellulose filter binding assay [23]. Protein concentration was determined using the method of Bradford [28] or the previously determined molar absorption coefficient of $\varepsilon_{280} = 18450\ M^{-1}\ cm^{-1}$ at ambient temperature [23]. Nucleotide-free and metal-free p21 was prepared by HPLC chromatography as will be described in a separate report (Feuerstein et al., unpublished). For kinetic measurements, the protein was prepared in buffer A (64 mM Tris/HCl pH 7.6, 0.5 mM dithioerythritol). For the inhibition studies the protein was prepared in buffer B (100 mM Tris/HCl pH 7.6 and 20 mM ascorbate instead of dithioerythritol to avoid reaction of Cd^{2+} with dithioerythritol [29]). After removal of nucleotide, the protein retained >90% of its GDP binding activity. It was always kept on ice until used.

For EPR measurements the nucleotide-free and metal-free p21 proteins were prepared in Hepps buffer: 33.7 mM Hepps and 0.5 mM dithioerythritol, ionic strength = 0.02 M, pH adjusted to 6.8 with NaOH; this was passed twice over Chelex before use. Protein solutions in this buffer were concentrated to the desired concentration (greater than 1 mM) in an Amicon concentrating cell (Minicon B 15).

Kinetic and binding measurements

The second-order rate constant for the association of p21 and GDP was measured by mixing metal-ion-free and nucleotide-free p21 at a concentration of about 0.1 nM with [^{3}H]GDP at 400 GBq/mmol (≈1 nM) in the presence of 5 mM Mg^{2+} or 2 mM EDTA in buffer A. 5-ml aliquots were removed at 10-s intervals and quickly filtered through nitrocellulose filters, which were washed twice with buffer A with and without 5 mM Mg^{2+}. The rate of dissociation of bound GDP was determined by addition of a large excess of unlabelled GDP (5 μM) to a mixture of 0.5 nM p21 and ≈2 nM [^{3}H]GDP in buffer A, which had been allowed to equilibrate either in the presence of 5 mM Mg^{2+} or 2 mM EDTA. Rate constants were determined using non-linear least-square fits to single exponentials in both cases.

Inhibition of binding of [^{3}H]GDP was measured by adding simultaneously 6.5 μM [^{3}H]GDP and varying concentrations of phosphorothioate analogs of GDP to 1 μM nucleotide-free and metal-ion-free protein in the presence or absence of 1.4 mM Cd^{2+}. This mixture was incubated for 30 min at 0°C in 100 mM Tris/HCl pH 7.6 and 20 mM ascorbate. The incubation mixture was diluted with ice-cold Tris/ascorbate buffer and quickly filtered through nitrocellulose filters, washed, dried and counted in a liquid scintillation counter. The data points were fitted to hyperbolic curves after calculating the free analog concentration for each point.

EPR measurements and spectra simulation

All EPR measurements were performed with a Bruker BER-420 spectrometer operating at *Q*-band frequencies. The sample temperature was kept constant at 274 ± 0.1 K by a nitrogen gas-flow system. Samples were contained in quartz capillaries sealed with a polyethylene plug.

The errors given correspond to a 95% confidence level obtained by applying the *t*-test to the experimental data. The EPR spectra were simulated as described previously (Kalbitzer et al. [30]) assuming an isotropic superhyperfine coupling.

RESULTS AND DISCUSSION

We have previously described the construction of bacterial expression vectors from which high amounts of authentic soluble p21 proteins with high activity could be obtained [23]. The p21 isolated contains, as has been observed also by Poe et al. [31], one mole of bound nucleotide per mole of protein. We have shown that the bound nucleotide can be exchanged against external nucleotides and that the reaction rate is strongly inhibited by increasing Mg^{2+} concentrations. In analogy to other nucleotide-binding proteins, it seems likely that the guanosine nucleotide is bound as its Mg^{2+} complex, but this must be proved rather than assumed. For studies related to this question, we needed p21 free of nucleotide and metal, since already bound nucleotide dissociates extremely slowly from the protein. We have developed an HPLC method (Feuerstein et al., unpublished) for completely removing these ligands under native conditions. The protein thus isolated is much more labile against denaturation, as is nucleotide-free EF-Tu, but still retains ⩾90% of the starting nucleotide-binding activity over a period of several hours if it is stored at 0°C. This protein was used to study association and dissociation rate constants of guanosine nucleotide to p21. Fig 1 shows examples of the kinetics of GDP binding and dissociation. The data were analysed assuming the kinetic mechanism of binding to be

$$p21 + GDP\ (\text{or } Mg \cdot GDP) \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} p21 \cdot GDP\ (\text{or } p21 \cdot MgGDP).$$

As long as pseudo-first-order conditions pertain, i.e. [GDP] ⩾ [p21], the observed rate constant of binding (k') is given by

$$k' = k_{+1}[GDP] + k_{-1}.$$

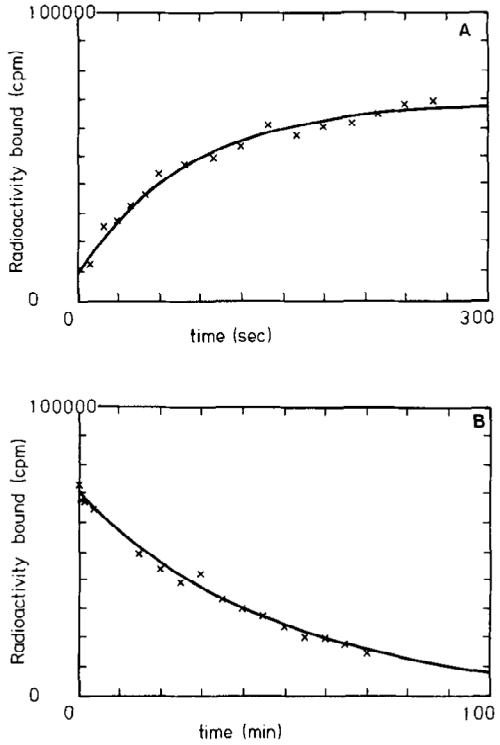

Fig. 1. *Kinetics of the interaction of GDP with p21$_c$.* (A) Time course of association of GDP with p21$_c$ in the absence of Mg^{2+}. Conditions: 0.44 nM p21, 2.2 nM [^{3}H]GDP, 0°C (see Methods). (B) Time course of displacement of [^{3}H]GDP from its complex with p21 in the absence of Mg^{2+}. Conditions: 0.44 nM p21, 4.4 μM GDP. Solid lines show best non-linear least-square fits to the data. Rate constants as in Table 1

Table 1. *Kinetic constants for the reaction of GDP with p21 proteins at 0°C in the presence and absence of Mg^{2+}*
Values were measured as described in Methods and in the legend to Fig. 1

Protein	EDTA/Mg^{2+}	$k_{+1} \times 10^{-6}$	$k_{-1} \times 10^{4}$	$K_1 \times 10^{-10}$
		$M^{-1} s^{-1}$	s^{-1}	M^{-1}
p21$_c$	EDTA	5.2	6.0	0.87
	+Mg^{2+}	1.7	0.3	5.7
p21$_t$	EDTA	3.7	3.3	1.1
	+Mg^{2+}	2.6	0.20	13
p21$_v$	EDTA	7.8	28	0.28
	+Mg^{2+}	5.7	0.8	7.1

Since k_{-1} could be determined independently in displacement experiments, it was possible to calculate k_{+1} from the observed kinetics of association. The results are shown in Table 1. The values obtained for k_{+1} are all between $10^6 - 10^7\ M^{-1}\ s^{-1}$. These are typical for association reactions between proteins and nucleoside polyphosphates, e.g. myosin [32], actin [33], and EF-Tu [34, 35] and are significantly lower than those expected for a diffusion-controlled reaction, suggesting the possibility of the involvement of more than one step in the binding process, as has been demonstrated for myosin [32]. The dissociation rates are extremely low, as already indicated by the slow rate of exchange of bound nucleotides. The binding constants calculated from the ratio of on and off rates of GDP or Mg · GDP are very high (all between $10^9 - 10^{11}\ M^{-1}$), explaining why p21 isolated under native conditions contains one mole of bound nucleotide, mostly GDP, per mole of protein [23, 31].

The binding constants determined here are approximately 100 – 500-fold higher than has been found by other authors [20, 36 – 38]. At higher temperatures (25°C), further measurements show that the dissociation rates increase by a factor of about 4 and the association rates by a factor of 1.5 – 2, so that the binding constants in the presence of Mg^{2+} are still greater than $10^{10}\ M^{-1}$. We can offer several explanations which, alone or in combination, may account for the discrepancies between our measured values and those given in the literature.

a) Some authors used bacterially expressed proteins which were found in the insoluble fraction of the bacterial cell extract. These p21 proteins have been purified under denaturing conditions such as 7 M urea or guanidinium hydrochloride. They did not show full GDP binding activity and had to be stored in guanidinium hydrochloride in order to be kept in solution.

b) In some cases, binding constants were determined using immunoprecipitates from crude extracts, and not with purified proteins.

c) p21 should, even with a binding constant of $10^8\ M^{-1}$ (like EF-Tu), always contain one mole of bound nucleotide per mole of protein, which in fact we [23] and Poe et al. [31] find for protein isolated under native conditions. This makes a determination of the binding constant by equilibrium methods impossible, since only exchange of labelled for unlabelled nucleotide is observed, which is independent of the absolute magnitude of the binding constant. Also the determination of the specific radioactivity of labelled ligands in the presence of unlabelled, bound ligand is difficult. If, on the other hand, the protein is isolated without equimolar amounts of guanosine nucleotide it is thermally unstable (Feuerstein et al., unpublished results).

d) The p21 · GDP complex, in the presence of excess Mg^{2+} ions, exchanges its bound nucleotide extremely slowly against externally added nucleotides and takes several hours even at 37°C to completely exchange.

These errors have been avoided in the determination of the binding constants from the kinetics of binding and release of GDP. It is of interest to note that in the one case where the dissociation rate constant has been determined with native cellular protein ($3.3 \times 10^{-4}\ s^{-1}$ at 37°C) [38], it agrees reasonably well with our data.

In the presence of EDTA, the rate constants for dissociation of the p21 · GDP complexes are greater by a factor of more than 10 than those of the corresponding Mg^{2+} complexes (Table 1). The association rate constants are also higher by a factor of about 1.5 – 3. Since the off-rate is the rate-limiting step of the exchange reaction, it is obvious that the rate of this reaction is increased in the presence of EDTA, as has been observed previously [23]. The ratio of binding constants in the presence and absence of Mg^{2+} is of the order of 10, as for elongation factor Tu [39], to which p21 is believed to be structurally related. The absolute numbers for the binding constants are approximately 100-fold higher for p21 than for EF-Tu [34, 39 – 41].

There are no dramatic differences in the kinetics of GDP interaction with p21$_c$ and its p21$_t$ mutant, which has a single point mutation of position 12 (Gly → Val). The binding constant to p21$_t$ is slightly higher than to p21$_c$ in the presence of Mg^{2+} due to a small increase in the association rate constant and a small decrease in the dissociation rate constant. In models which have been presented for the structure of

p21 · GDP complexes, Gly-12 is near, but not in, the GDP-binding site. The small differences detected in the GDP interaction kinetics are perhaps due to structural changes in the so-called phosphoryl binding loop, as suggested previously [21]. $p21_v$, which has two mutations (Gly-12 → Arg, Ala-59 → Thr), shows a higher rate of association than either of the other two forms, but the largest difference lies in the dissociation rate constant, which is 3–4-fold higher than for the other two forms in the presence of Mg^{2+} and 5-fold higher than for $p21_c$ (8-fold than for $p21_v$) in the absence of Mg^{2+}. This accounts for our observation that nucleotide exchange is faster for $p21_v$ than for the other two forms. The higher rate of association could be due to an additional electrostatic interaction of nucleotide with Arg-12 during the binding process and it is feasible that this effect also explains the more rapid dissociation if a weakly bound initial binding state is stabilized by this residue.

The results reported here agree qualitatively with the findings of other authors, i.e. that the binding ability of GTP/GDP is basically unaltered between the cellular and activated forms of p21 [2, 11, 15, 17–20]. However, the more sensitive methods used show that there are quantitative differences in the kinetics of the interaction, which must be due, directly or indirectly, to the amino-acid exchanges. Since the effects seen on the binding kinetics are not dramatic, it seems unlikely that the residues involved are in direct interaction with the nucleotide in the p21 · GDP complexes. We have measured the binding and kinetic constants only for GDP, but since different GTPase activities have been shown for the p21 mutants, we would expect differences for the GTP constants as well. Our inhibition studies [23] with cellular p21 have shown that GTP binds with slightly higher affinity than GDP, in agreement with published observations [2, 20, 36–38]. The absolute number for the binding constant of GTP must, however, be higher than that calculated previously; preliminary results indicate that there are also characteristic differences between cellular and mutant p21 proteins with respect to GTP binding.

EPR spectra of manganese-p21 complexes

In order to obtain more structural information concerning the metal · guanine nucleotide · p21 binding site, we have used EPR spectra of Mn^{2+} complexed to p21 and p21 · nucleotide complexes. Mn^{2+} has been found to be a good analogue of Mg^{2+} in such complexes (for reviews, see [41, 42]) just as in the case of the well characterised EF-Tu [25, 30]. The EPR spectra of all manganese complexes of p21 studied, i.e. p21 · Mn, p21 · Mn · GDP, and p21 · Mn · GTP, show the characteristic six-line hyperfine pattern caused by the interaction of the nuclear spin 5/2 of manganese with its electron spin. The hyperfine coupling constant of 9.17 mT is indicative of hexacoordination of manganese in these complexes [43], a coordination scheme typically found also in magnesium complexes. Outside the range centered around $g = 2$ no additional signals could be detected.

The two lowest-field hyperfine lines for the different manganese-p21 complexes are depicted in Fig. 2. The EPR spectrum of p21 · Mn · GDP shows the smallest linewidth, that of p21 · Mn is slightly broader, whereas that of p21 · Mn · GTP is much broader than the others (Table 2). The relative differences in linewidth correspond qualitatively to those observed in EF-Tu · Mn · GDP, EF-Tu · Mn and EF-Tu · Mn · GTP [30]. The absolute values are clearly different, the EPR resonance lines of the p21 complexes being much narrower.

The spectrum of p21 · Mn · GTP shown in Fig. 2 is actually a superposition of two components, a sharper one and a broader one. The sharper component probably represents the spectrum of p21 · Mn · GDP due to residual

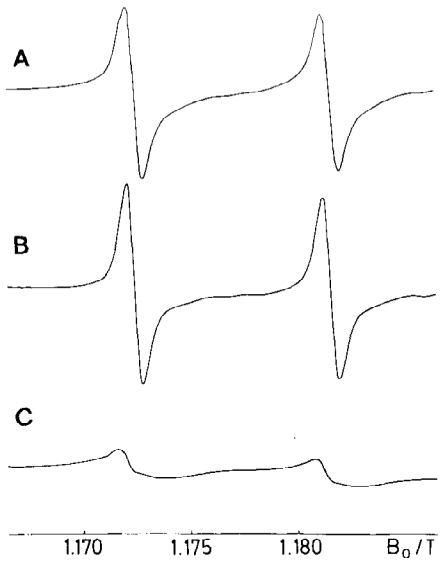

Fig. 2. *Q-band EPR spectra of manganese complexes with p21 protein.* (A) $p21_t$ · Mn; (B) $p21_t$ · Mn · GDP; (C) $p21_t$ · Mn · GTP. All samples contained 2.4 mM $p21_t$, 2 mM $MnCl_2$, 0.5 mM dithioerythritol in 33.7 mM Hepps buffer, pH 6.8. In addition, sample (B) contained 2.6 mM GDP and sample (C) 3.0 mM GTP. All spectra were recorded at 274 K. Only the two lowest-field hyperfine lines are shown

Table 2. *EPR linewidth and superhyperfine coupling constant of various manganese complexes with transforming and nontransforming p21 proteins*

Results of measurements using different protein preparations. Experimental conditons as described in Figs 2 and 3. The superhyperfine coupling constant was determined as described in Materials and Methods

Complex	Linewidth of the lowest field hyperfine line		
	$p21_c$	$p21_t$	$p21_v$
	mT		
p21 · Mn	0.96 ± 0.01	0.95 ± 0.01	0.81 ± 0.01
p21 · Mn · GDP	0.81 ± 0.01	0.79 ± 0.01	0.77 ± 0.01
p21 · Mn · (*S*)-[α-^{17}O]GDP	0.79 ± 0.01	0.81 ± 0.02	0.80 ± 0.02
p21 · Mn · (*R*)-[α-^{17}O]GDP	0.80 ± 0.01	0.80 ± 0.01	0.81 ± 0.03
p21 · Mn · [α-$^{17}O_3$]GDP	0.81 ± 0.03	0.82 ± 0.02	0.76 ± 0.02
p21 · Mn · [β-$^{17}O_4$]GDP	0.95 ± 0.02	1.01 ± 0.01	0.92 ± 0.01
p21 · Mn · GTP	2.26 ± 0.03	2.1 ± 0.2	2.23 ± 0.02
	Superhyperfine coupling constant		
	$p21_c$	$p21_v$	$p21_t$
	mT		
p21 · Mn · [β-$^{17}O_4$]GDP	0.16	0.22	0.16

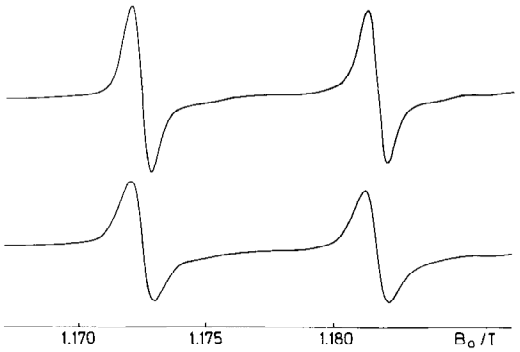

Fig. 3. *The effect of GDP labelled at β-position by ^{17}O on the EPR spectrum of the p21 · Mn · GDP complex.* Q-band EPR spectrum of $p21_t$ · Mn · GDP (upper trace) and of $p21_t$ · Mn · [β-$^{17}O_4$]GDP (lower trace). Only the two lowest-field hyperfine lines are shown. The samples contained 2.4 mM $p21_t$, 2 mM $MnCl_2$, 0.5 mM dithioerythritol in 33.7 mM Hepps buffer, pH 6.8 and 3 mM GDP or [β-$^{17}O_4$]GDP. All spectra were recorded at 274 K

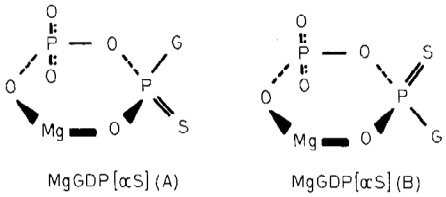

Fig. 4. *Structures of the α,β-bidentate Mg^{2+} complexes of the diastereomers (S)-GDP[αS] (A) and (R)-GDP[αS] (B).* On substituting Cd^{2+}, which binds preferentially to sulfur, for Mg^{2+}, a structure analogous to Mg · (*S*)-GDP[αS] should be formed with Cd · (S)-GDP[αS] (see also [44, 45]) and vice versa, so that if this bidentate complex is recognized by p21, a reversal of stereospecificity should then be observed

Table 3. *The binding of thioanalogs of GDP to p21 proteins in the presence of Cd^{2+}* ions
The relative affinity of analogs is defined as the inverse of the ratio of concentration [analog]/[GDP] which causes 50% inhibition of GDP binding (for details see Materials and Methods). Temperature 0 °C. The values for the affinities in presence of Mg^{2+} ions are from Tucker et al. [23]

Analog	Me^{2+}	Relative affinities for the analog of		
		$p21_c$	$p21_t$	$p21_v$
(*S*)-GDP[αS]	none	3.3	—	—
	Mg^{2+}	1.3	0.65	2.0
	Cd^{2+}	0.92	0.43	0.64
(*R*)-GDP[αS]	none	1.8	—	—
	Mg^{2+}	0.2	0.2	0.16
	Cd^{2+}	0.094	0.15	0.09
(*S*)/(*R*)	none	1.8	—	—
	Mg^{2+}	6.5	3.1	13
	Cd^{2+}	9.8	2.9	7.2
GDP[βS]	none	0.6	—	—
	Mg^{2+}	0.4	0.55	0.18
	Cd^{2+}	0.05	0.04	0.03

amounts of GDP bound to the protein (see Materials and Methods) which vary somewhat from preparation to preparation.

In general, the EPR spectra of the different manganese complexes of the transforming and nontransforming p21 proteins are very similar, suggesting that the environment of the metal ion (and probably the residues coordinated) are the same in all p21 proteins studied here. Even the absolute values of linewidths are almost identical (Table 2), a fact that is not insignificant, since in many cases the EPR linewidth of manganese-protein complexes is highly dependent on the quality of the preparation. In our experience, highly reproducible EPR spectra together with the smallest linewidth obtained for a given complex coincide with a highly purified and biologically highly active preparation.

An exception to the uniform nature of the spectra is that of the manganese complex with $p21_t$, the product of the T24 mutant of the *ras* oncogene, which exhibits a clearly smaller linewidth than the spectra of the manganese complexes with $p21_c$ and $p21_v$. The smaller linewidth could be caused by a higher residual amount of GDP (which is characterized by a smaller linewidth) but could also be a real intrinsic effect arising from differences in the metal coordination.

A lower limit for the association constant between the p21 proteins and Mn^{2+} can be calculated from the EPR results to be 10^5 M^{-1}.

The interaction of manganese with ^{17}O-labelled GDP

Replacing normal GDP by GDP labelled with ^{17}O at the β-phosphate group leads to an approximately 20% broadening of the resonance lines of Mn^{2+} at the active site of p21 proteins (Fig. 3), which is indicative of an interaction between the nuclear spin of the oxygen isotope and the electron spin of manganese. The magnitude of this broadening can only be explained by an unresolved splitting due to the superhyperfine coupling to an oxygen atom in the first coordination sphere of manganese [42, 47]. Line simulation results in a superhyperfine coupling constant of between 0.16 mT and 0.22 mT (Table 2), a value which appears to be typical for the ^{55}Mn-^{17}O coupling to one oxygen of a phosphate group [30, 46].

GDP, regiospecifically or stereospecifically labelled at the α-phosphate group shows only a small effect on the linewidth of the manganese EPR spectrum (Table 2) which is not significant at our confidence level. It is conceivable from theory, but unlikely, that the superhyperfine coupling constant could be too small to be detected even though the manganese ion is coordinated to a ^{17}O atom. Since there is no experimental evidence for such an effect [41, 42], we conclude that GDP is bound as its β-monodentate metal ion complex at the active center of all p21 proteins studied.

Inhibition studies with thiophosphate analogs of GDP

A further test for this proposal can come from inhibition studies with phosphorothioate analogs of GDP. Since the predominant form of Mg · GDP in solution probably has the bidentate structure (Fig. 4), it might be assumed initially that it should also be bound to p21 in this form. It is possible to test this model by using the *S* and *R* diastereomers of GDP[αS] (the α-phosphorus being the chiral centre) and as divalent ion Cd^{2+} which has a higher tendency to complex with sulfur than Mg^{2+}. This would lead to the reversal of affinity for the GDP[αS] diastereomers as explained in the legend to Fig. 4 and has been observed for different nucleotide-binding proteins (for a review see [44]). We have reported earlier on the inhibition of GDP binding by phosphorothioate analogs of

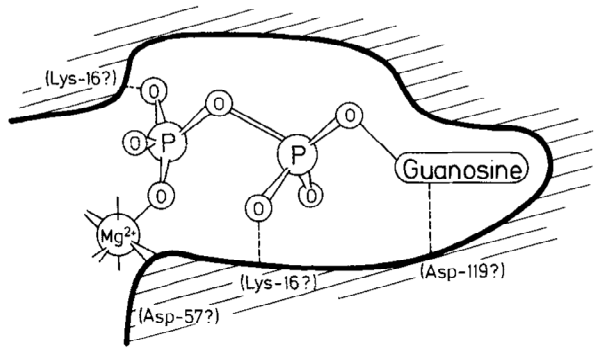

Fig. 5. *Proposed coordination scheme for Mg · GDP at the active site of p21 proteins.* The continous black line represents the active site of the protein. It would be consistent with our data that more than one oxygen atom of the β-phosphate group would interact with the protein and that the metal-ion coordinates to more than one funcional group of the protein

GDP in the presence of Mg^{2+}, which showed a preference for the *S* isomer of GDP[αS] [23]. Here we have used nucleotide-free and metal-ion-free p21 to perform similar studies with Cd^{2+} and (*S*)-GDP[αS], (*R*)-GDP[αS] and GDP[βS]. The experiments were done in the presence of 1.4 mM Cd^{2+}, under which conditions the protein still retains 90% of its GDP binding ability. Table 3 summarizes the results with the three different proteins and compares them with the results obtained in the presence of Mg^{2+} [23]. The data show that the (*S*)-isomer of GDP[αS] is preferred with both Cd^{2+} and Mg^{2+}, i.e. that there is no reversal of stereospecificity. This is in agreement with the EPR results and confirms our conclusion that there is no coordination between the α-phosphate group and the metal ion at the active site of p21. The data with Cd^{2+} also show that the relative affinities of both isomers of GDP[αS] are somewhat reduced and that the *S*/*R* ratios, the ratios of affinities of the diastereomers, are similar in the presence of Mg^{2+} and Cd^{2+}. Cd^{2+} does, however, drastically reduce the relative affinity of GDP[βS] to all three p21 proteins, an effect which we cannot explain a present.

Inhibition studies were also performed for the cellular p21 in the presence of EDTA to probe the binding site of p21 in the absence of metal ions. From the results shown in Table 3 it is apparent that the affinities of both GDP[αS] isomers are increased and (*S*)-GDP[αS] now binds 3.3-fold better than GDP. It can also be seen that the *S*/*R* ratio as a measure of specificity is decreased. This indicates that the metal ion, although it coordinates directly only to the β-phosphate atom, also influences the interaction of the α-phosphorus with the protein.

Although the same general pattern has been found for all p21 proteins studied here, significant quantitative differences in the relative binding constants and *S*/*R* ratios are observable. Especially the preference for the *S*-isomer of GDP[αS] is more pronounced for the viral protein. This may imply that, in spite of the same general coordination pattern, the functional groups interacting with the α-phosphate group or more probably their spatial arrangement may be different in the viral and transforming p21 proteins.

CONCLUSION

From the results presented above we can draw a schematic model of the nucleotide-binding site of p21 (Fig. 5) which has the following characteristics. The Mg^{2+} ion is depicted as being accessible to the solvent water since it can be easily removed from p21 · GDP · Mg^{2+} by EDTA. From the EPR data it can be concluded that the metal ion is directly coordinated to a β-phosphate oxygen, but not to the α-phosphate. This agrees with the lack of reversal of stereospecifity for the binding of the diastereomers of GDP[αS] and the large high-field shift of the β-phosphorus resonance in the ^{31}P-NMR spectrum of the p21 · MgGDP complex [24]. The metal ion is most probably also coordinated to the protein itself, as suggested by the observation that the association constant of the metal ion to p21 · GDP is higher than to free GDP and that p21 has a metal-binding site even in the absence of GDP. A possible candidate for the binding would be Asp-57 which is highly conserved in all G proteins [6] and should be near the GDP-binding site [21, 22]. There is probably in additional interaction between the protein and at least one of the oxygens of the β-phosphate moiety because the relative affinity for GDP[βS] is decreased in the presence and absence of metal ions. The *pro-R* oxygen of the α-phosphate seems to be coordinated to the protein, since its replacement by sulfur is not easily tolerated by the protein in the presence of Mg^{2+} and Cd^{2+}. The contacting residue of the *pro-R* oxygen could be the side chain of Lys-16, since Sigal et al. [38] have presented evidence that its replacement by Asn reduces the affinity of the protein by a factor of 100. This lysine residue is well conserved between the *ras* proteins and G proteins and is found near the phosphate residues of GDP in the three-dimensional structure of EF-Tu [21, 22]. A similar lysine residue is also conserved in the phosphate binding loops of ATP-binding proteins such as adenylate kinase [3], strengthening the notion that it is involved in phosphate binding. Surprisingly, in the absence of metal ions both diasteromers of GDP[αS] bind better to p21 than the natural substrate GDP. The charge density at the sulfur atom can be expected to be smaller than at an oxygen atom in the phosphate moiety, so that the electrostatic repulsion by a negative charge on the protein should be diminished in a sulfur derivative. A possible candidate is the above-mentioned negatively charged group of the protein (Asp-57), which has been suggested to interact with the Mg^{2+} ion and whose charge would be physiologically screened by the positively charged divalent ion. There is a hint from the EPR spectroscopy that the metal ion is near (but not coordinated to) both oxygens of the α-phosphate group. As in the case of EF-Tu [25], the data suggest a small line-broading by the *R* as well as by the *S* isomer of [α-^{17}O]GDP which could be the effect of a dipolar 'through-space' interaction between the manganese electron spin and the ^{17}O nuclear spin.

Apart from Lys-16, it is likely that there are many more binding contacts between the protein and the nucleotide to generate an interaction with a binding constant of over 10^{10} M^{-1}. One of these residues could be Asp-119, which is in a well conserved Asn-Lys-Cys-Asp sequence, which in EF-Tu forms part of the binding site for the guanine base, and whose replacement by Ala has been found to reduce the specificity of GDP binding [38].

We have shown here with our measurements that there are slight structural differences between the active sites of cellular and mutant p21 proteins. Since the EPR spectra of Mn^{2+}-nucleotide complexes with the three proteins are very similar, it must be concluded that the nature of the ligands and their spatial arrangement are very similar in the three proteins investigated. The protein ligands around the phosphate oxygens, however, have a somewhat different spatial arrangement, as can be seen from the difference in specificity of analog

binding, the metal ion dependence of this specificity and the different kinetics of GDP binding both in the presence and absence of metal ions. Of the two p21 mutants $p21_v$ and $p21_t$, the difference is more pronounced for $p21_v$, with two amino acid replacements, than for the T24 protein which has only a single point mutation [Gly-12 → Val). There is, however, no reason to believe that in these three proteins the overall structure of the metal-nucleotide binding site shown schematically in Fig. 5 is altered. Thus, at present there is no hint from these relatively detailed studies of the interaction of Mg-GDP with the active site of p21 proteins of an effect which might be responsible for the transforming properties of the mutant forms. Such information might be obtained from an extension of similar studies to the corresponding GTP complexes and by more detailed investigation of the kinetics of GTP hydrolysis. For example, since the simple correlation between rate of GTP hydrolysis and transforming properties does not appear to hold, it is conceivable that the answer lies in a different distribution of protein-nucleotide species (at least three, namely p21 · GTP, p21 · GDP · P_i and p21 · GDP, are expected) during the steady state. Our present efforts are directed towards answering these questions.

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