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# Binding of Nucleotides to Guanylate Kinase, p21$^{ras}$, and Nucleoside-diphosphate Kinase Studied by Nano-electrospray Mass Spectrometry*

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**The binding of nucleotides to three different nucleotide-binding proteins and to a control protein was studied by means of nano-electrospray mass spectrometry applied to aqueous nondenaturing solutions. The method leads to unambiguous identification of enzyme complexes with substrates and products but does not allow the determination of dissociation constants or even stoichiometries relevant to the binding in solution. For guanylate kinase (EC 2.7.4.8), the transfer of $HPO_3$ between nucleotides was observed whenever a ternary complex with adenylate or guanylate nucleotides was formed. Guanosine 5′-tetraphosphate was generated after prolonged incubation with GDP or GTP. $Mg^{2+}$ binding was considerably enhanced in functional high affinity complexes, such as observed between guanylate kinase and its bisubstrate inhibitor $P^1$-(5′-guanosyl)-$P^5$-(5′-adenosyl) pentaphosphate or with the tight nucleotide-binding protein p21$^{ras}$ and GDP. Nucleoside-diphosphate kinase (EC 2.7.4.6) itself was phosphorylated in accordance to its known ping-pong mechanism. All nucleotide-binding proteins were shown to bind sulfate ($SO_4^{2-}$) with presumably high affinity and slow exchange rate. The binding of phosphate ($PO_4^{3-}$) could be inferred indirectly from competition with $SO_4^{2-}$.**

Nano-electrospray mass spectrometry (1) is a simple modification of electrospray mass spectrometry, in which the diameter of the nanospray capillary is reduced to a few micrometers. The method is relatively tolerant toward salts because liquid droplets formed by nanospray off small capillaries are smaller than droplets formed by standard pneumatically assisted electrospray methods (2). Smaller droplets lead to less total amount of salt/droplet and, at protein concentrations of approximately 1 molecule/droplet, to less salt on the protein after evaporation of the droplet. Therefore, this method has been successfully applied to the study of proteins and peptides where salt adducts are a common obstacle. It has been shown that complexes formed between ligands and proteins could be detected by means of electrospray mass spectrometry (3–7). We have employed nano-electrospray mass spectrometry to test its suitability for systematic studies of nucleotide binding to proteins in aqueous solutions.

Three different nucleotide-binding proteins were studied: 1) guanylate kinase (GMPK)[1] (EC 2.7.4.8), which belongs to a class of enzymes that catalyze the ATP-dependent phosphorylation of monophosphonucleotides into diphosphonucleotides. The crystal structure of GMPK from *Saccharomyces cerevisiae* complexed with its substrate GMP is known (8). 2) p21$^{ras}$, which belongs to a well investigated class of small GTP-binding proteins that are essential for the regulation of a wide variety of cellular processes (9). Its structure had been determined in the presence of a GTP analogue (10). 3) nucleoside-diphosphate kinase (NDK) (EC 2.7.4.6), which exhibits a much lower substrate specificity than the above enzymes. It is involved in the synthesis of all nucleoside triphosphates other than ATP. It acts via a ping-pong mechanism in which a histidine residue is phosphorylated by transfer of the terminal phosphate group from ATP (11). 4) lysozyme from chicken egg white was used as a control.

This contribution consists of three parts. First, the recombinant purified proteins are characterized by means of nano-electrospray mass spectrometry. Second, the enzymatic activity of GMPK is studied in detail. Third, the limits of the method are shown when it is applied to systematic binding studies.

## EXPERIMENTAL PROCEDURES

Nano-electrospray mass spectrometry was performed with a Finnigan LCQ mass spectrometer equipped with a micromanipulator for the correct positioning of the nanospray needle. The needles and the ion source were made following the original design of the EMBL group (1). In short, capillaries (GC120F from Clark Instruments, Reading, United Kingdom) were pulled using the DMZ-universal puller (Zeitz-Instrumente GmbH, München, Germany). They were coated with gold using a Polaron SC7610 Sputter Coater (VG Microtech, Uckfield, United Kingdom) and a gold target. These needles were filled with 1–4 μl of aqueous solution and centrifuged for several seconds using a PicoFuge™ (Tomy Capsule, Tomy Tech Inc, Palo Alto, CA). The needles were mounted on the micromanipulator and were gently brought in contact with a surface near the inlet of the mass spectrometer. Air pressure was applied to the needle until a small droplet appeared on the tip. The needle was then adjusted 2.5 mm opposite the inlet of the heated capillary. A potential of 900 V was applied to the needle, although some air pressure was maintained to initiate the electrospray process. For measurements of aqueous solutions, the relative intensities of the corresponding peaks were maximized when the collision-induced decay at the inlet of the mass spectrometer was minimized. The tuning procedure of the mass spectrometer was applied for this purpose. The original data (*i.e.* abundance of the multiple charged protein populations *versus m/z*) were deconvoluted into mass spectra (relative abundance *versus* mass) by means of the deconvolution software BioExplore. The error of the mass spectrometer was 100 ppm in the normal mass range (150–2000 *m/z*), which was used for all experiments shown here. Proteins exhibited symmetric peaks only at very low ionic strength, in particular when they were sprayed from volatile organic solvents. Asymmetric peaks (with a tail at higher masses) were generally observed when proteins were sprayed from aqueous solution at moderate ionic strength. This lead to some increase of all detected

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[1] The abbreviations used are: GMPK, guanylate kinase; NDK, nucleoside-diphosphate kinase; GST, glutathione *S*-transferase; $Ap_5A$, $P^1$,$P^5$-di(adenosine 5′)-pentaphosphate; $Gp_5A$, $P^1$-(5′-guanosyl)-$P^5$-(5′-adenosyl) pentaphosphate; GDPβS, guanosine 5′-*O*-2-(thio)diphosphate; ADPβS, adenosine 5′-*O*-2-(thio)diphosphate.

masses of the particular protein. The shift was dependent on the salt concentration and on the spraying conditions. For one experiment, it was the same for all complexes of each protein so that the accuracy of difference measurements was not affected. Adducts of salt or water had been observed before (12). Note that $Mg^{2+}$ (24 Da) substitutes for two protons with its two charges and that $Na^{+}$ (23 Da) substitutes for one proton. Therefore, the resulting mass increase of the protein is 22 Da for both $Mg^{2+}$ and $Na^{+}$.

*Protein Samples*—Lysozyme from chicken egg white was obtained from Serva GmbH, Heidelberg. GMPK was prepared as follows: *Escherichia coli* BL21(DE3) transformed with the pGEX-hGMK vector were grown to $A_{600}$ of 0.8 in ampicillin-supplemented (100 mg/l) 2YT medium. Expression of the GST-GMPK fusion protein was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside, and the culture was incubated at 30 °C overnight. Cells were recovered by centrifugation, resuspended in lysis buffer (50 mM Tris/HCl, pH 7.5, 5 mM EDTA, 100 mM KCl, 1 mM phenylmethylsulfonyl fluoride), and lysed with a microfluidyzer. The homogenate was ultracentrifuged (45,000 rpm, 1 h) to remove insoluble material. The supernatant was loaded onto a glutathione-Sepharose 4B (Amersham Pharmacia Biotech) column previously equilibrated with 50 mM Tris/HCl, pH 7.5, 200 mM KCl, 5 mM $MgCl_2$. After extensive washing and cleavage by thrombin on the column overnight, GMPK was collected, concentrated using Vivaspin concentrators with a 10,000 molecular weight cutoff, and loaded onto a gel-filtration column (S-75, Amersham Pharmacia Biotech) equilibrated with 50 mM Tris/HCl, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$. The fractions containing GMPK were pooled, concentrated to 31 mg/ml, aliquoted, and shock-frozen in liquid nitrogen before storage at −70 °C. Its sequence corresponds to Swiss-Prot entry Q16774 with the additional 4 N-terminal amino acids GSHM, which resulted from the thrombic cleavage of the GST moiety.

Purification of truncated $p21^{ras}$ was performed essentially as described previously (13, 14). The final purity of the protein was >95%, as judged from sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Purified $p21^{ras}$ contained 1 mol of guanine nucleotide bound/mol of protein, 85–95% of which could be exchanged against free GDP. The protein concentration was determined with the Bradford assay using bovine serum albumin as standard (15). [8-$^3$H]GDP binding activity was determined by the filter binding assay (13). Standard buffer was 64 mM Tris/HCl, pH 7.6, 1 mM dithioerythritol, 10 mM $MgCl_2$, and 1 mM sodium azide. Exchange of protein-bound nucleotide, usually GDP, was as described (16).

Yeast nucleoside-diphosphate kinase kinase was cloned in the expression vector pJC20 and overproduced in bacteria essentially as described for adenylate kinase (17). The protein was purified from sonicated bacterial extracts using established procedures (18). After centrifugation, the supernatant (equilibrated in 50 mM Tris/HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol) was passed through a Q-Sepharose Fast Flow column (Amersham Pharmacia Biotech), which adsorbed bacterial nucleoside-diphosphate kinase kinase and other contaminants. The flow-through was then loaded onto a Blue-Sepharose CL-4B column (Amersham Pharmacia Biotech). The desired yeast enzyme was eluted with a linear 0–2 M NaCl gradient, concentrated in Centricon (Amicon), and further purified by gel-filtration chromatography on Superose 6 (Amersham Pharmacia Biotech).

*$^{18}$O-Labeling of Phosphate and Sulfate*—The ammonium salt of phosphoric acid-$^{18}O_4$ ($H_3P^{18}O_4$) was obtained as a 500 mM solution by dissolving 104 mg (500 μmol) of phosphorus-pentachloride ($PCl_5$, Aldrich, 95%) in 1 ml of $^{18}$O-water (Cambridge Isotope Laboratories, 97–98%) and adjusting the solution to a pH of 7.0 with aqueous $NH_3$. The ammonium salt of sulfuric acid-$^{18}O_4$ ($H_2S^{18}O_4$) was prepared in a similar manner by adding 32.5 μl of (400 μmol) sulfuryl chloride ($SO_2Cl_2$, Fluka, 99%) to 1 ml of $^{18}$O-water. The percentage of conversion and purity of the phosphate and sulfate was analyzed using mass spectrometry. The ratio of $H_2P^{16}O_4^-$ to $H_2P^{16}O_3{}^{18}O_1^-$ to $H_2P^{16}O_2{}^{18}O_2^-$ to $H_2P^{16}O_1{}^{18}O_3^{2-}$ to $H_2P^{18}O_4^-$ was 27:0:0:73; the ratio of $HS^{16}O_4^-$ to $HS^{16}O_3{}^{18}O_1^-$ to $HS^{16}O_2{}^{18}O_2^-$ to $HS^{16}O_1{}^{18}O_3^-$ to $HS^{18}O_4^-$ was 0:0:2:9:89. The $^{18}$O-modified anion was stable at a pH of 7.0 at room temperature for at least 24 h. Exchange of $^{18}$O against $^{16}$O could only be observed at pH < 1.

## RESULTS

*Characterization of the Proteins*—When proteins are investigated by means of electrospray mass spectrometry, they are usually transferred to a mixture of a volatile organic solvent and water. Employing nanospray (2) mass spectrometry we could use $H_2O$ without organic solvents and thus avoid denaturation in the liquid phase. The resulting mass spectrum consists of the expected series of multiple charged (protonated) molecules depending on the proton concentration (pH). Fig. 1*A* shows the original mass spectrum (relative abundance *versus m/z*) of human GMPK (EC 2.7.4.8) in $H_2O$. Seven peaks with 12–18 protons on each protein population are shown. Next to each main peak, two minor peaks with a slightly different charge distribution were observed. When the spectrum was deconvoluted as described under "Experimental Procedures," the molecular masses of 22,006, 22,104, and 22,202 Da were derived (Fig. 1*B*). The expected molecular mass of GMPK is 22,006 Da as deduced from the amino acid composition. The two main adducts thus have additional masses of 98 Da each. This mass difference may correspond either to the binding of $PO_4^{3-}$) (95 Da), which has to be neutralized by 3 protons, or to $SO_4^{2-}$ (96 Da), which has to be neutralized by 2 protons. In both cases, protons have to compensate for the negative charges so that a shift in the charge distribution (envelope of the original spectrum) is expected and observed (Fig. 1*A*). To discriminate between the binding of $H_3PO_4$ and $H_2SO_4$, we prepared $^{18}$O-labeled ammonium salts of the acids $H_3P^{18}O_4$ and $H_2S^{18}O_4$. These heavy sulfates and phosphates have a mass increased by 8 Da. When a 3-fold excess of $S^{18}O_4^{2-}$ in relation to the protein concentration was added, at least three adducts of $SO_4^{2-}$ to GMPK were observed with masses of 108, 212, and 317 Da. In contrast, the experiments performed with $P^{18}O_4^{3-}$ did not show any isotope exchange. In fact, the presence of phosphates ($P^{18}O_4$ or $P^{16}O_4$) generally reduced the relative abundance of all 98 Da adducts. We conclude that complexes of GMPK with sulfate and not with phosphate are readily detected in the mass spectrometer. Because the addition of phosphate reduces the relative abundance of bound sulfate, there must be competition in solution, although complexes with phosphate cannot be detected in the spectra. It should be noted that sulfate has not been employed at any stage during the purification of the bacterially produced protein.

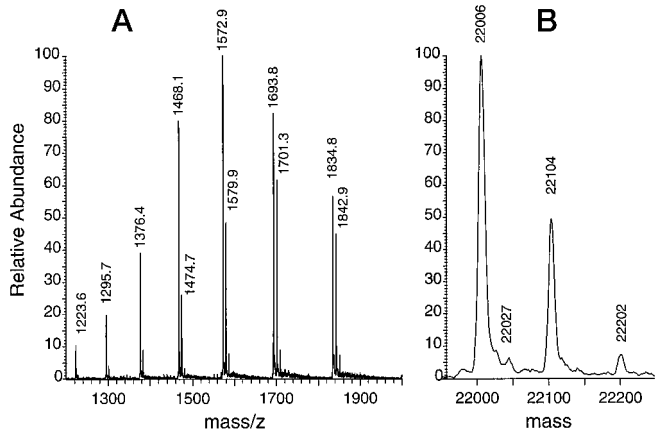


FIG. 1. **Mass spectrum of 10 μM GMPK in $H_2O$.** *A,* original mass spectrum (relative abundance *versus m/z*). Different charge states (12–18 protons) of the molecule appear at different mass/charge ranges. *B,* deconvoluted spectrum of *A* as generated by the deconvolution algorithm BioExplore (relative abundance *versus* mass (Da)). The peaks were assigned to free GMPK (22,006), GMPK·$Na^+$ (22,027), GMPK·$H_2SO_4$ (22,104), and GMPK·2 $H_2SO_4$ (22,202).

$p21^{ras}$ has a very high affinity for GTP and GDP (19), so that preparations of this GTPase isolated after expression in *E. coli* contain GDP. To obtain the mass spectrum of the free protein without nucleotides, it was fully denatured in 5% formic acid in 50% methanol. The spectra showed molecular masses of 18,851 and 18,720 Da corresponding to the sequence of residues 1–166 and residues 2–166 without N-terminal methionine. When the same sample was sprayed from neutral aqueous solutions, the masses of both peaks were increased by 467 Da, the sum of

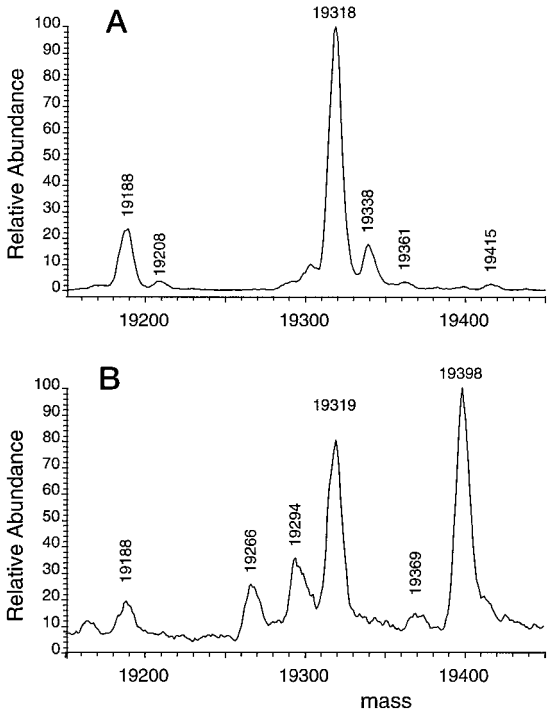


FIG. 2. **Deconvoluted mass spectrum of 10 $\mu$M p21$^{ras}$ in $H_2O$.** *A*, spectrum of recombinantly produced p21$^{ras}$. The peaks were assigned to p21$^{ras}$-(2–166)·GDP·$Mg^{2+}$ (19,188), p21$^{ras}$-(2–166)·GDP·$Mg^{2+}$·$Na^+$ (19,208), p21$^{ras}$-(1–166)·GDP·$Mg^{2+}$ (19,318), p21$^{ras}$·(1–166)·GDP·$Mg^{2+}$·$Na^+$(19,338), p21$^{ras}$-(1–166)·GDP·$Mg^{2+}$·2$Na^+$(19,361), and p21$^{ras}$-(1–166)·GDP·$Mg^{2+}$·$H_2SO_4$ (19,415). *B,* 10 $\mu$M p21-GDP incubated with 100 $\mu$M GTP for 30 min in $H_2O$. The additional peaks were assigned to p21$^{ras}$-(2–166)·GTP·$Mg^{2+}$ (19,266), p21$^{ras}$-(1–166)·GDP (19,294), and p21$^{ras}$-(1–166)·GTP·$Mg^{2+}$ (19,398).

$Mg^{2+}$ and GDP (Fig. 2*A*). The relative abundance of the remaining free protein was only 0.4% of the respective complexes and cannot be shown in a graph. The mass recorded at 19,416 Da (difference of 98 Da to the main peak) again corresponds to bound $H_2SO_4$. An isotope exchange experiment (same as described above for GMPK) confirmed that the adduct was caused by $H_2SO_4$ and not by $H_3PO_4$. The number and relative abundance of $SO_4$ adducts could be increased by adding $(NH_4)_2SO_4$.

To get an estimate for the binding energy, the complex of p21$^{ras}$, GDP, $Mg^{2+}$, and $H_2SO_4$ was submitted to collision-induced decay with helium inside the ion trap. When *m/z* 1766 (corresponding to 11 charges at the quaternary complex) was selected and exposed to 30% of the relative collision energy, approximately 50% of the sulfate dissociated. At 35% relative collision energy less than 0.1% sulfate remained. This energy corresponds to the complete breaking of peptide bonds when peptides are analyzed. At this stage only 22% of the bound magnesium and 11% of bound GDP was dissociated. 50% of GDP was dissociated when the relative collision energy was increased to 45%. The process was accompanied with fragmentation of the protein.

The addition of GTP to Ras-like proteins generally leads to a relatively slow exchange of GTP for GDP (20, 21) in the absence of exchange factors. Fig. 2*B* shows such an exchange experiment studied by means of mass spectrometry. The exchange reaction was time-dependent. In addition to the nucleotide exchange illustrated in Fig. 2*B*, additional binding of at least two GTP molecules to p21$^{ras}$ was recorded. This and similar observations are discussed below in the context of binding studies.

Upon addition of nucleotides to NDK from *S. cerevisiae* no nucleotide·protein complexes could be detected even at nucleotide concentrations of 1 mM GDP or GTP. The enzyme preparation showed multiple adducts of 98 mass units in aqueous

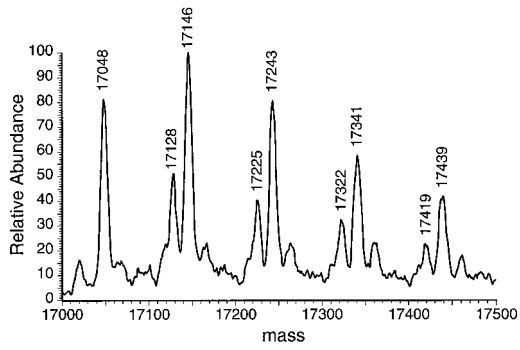


FIG. 3. **Deconvoluted mass spectrum of 15 $\mu$M NDK in $H_2O$ with 100 $\mu$M GTP.** The masses assigned to the peaks by the deconvolution program are indicated. They correspond to NDK (17,048), NDK·$HSO_3$ (17,128), NDK·$H_2SO_4$ (17,146), NDK·$HSO_3$·$H_2SO_4$ (17,225), NDK·2 $H_2SO_4$ (17,243), NDK·$HSO_3$·2$H_2SO_4$ (17,322), NDK·3$H_2SO_4$ (17,341), NDK·$HSO_3$·3$H_2SO_4$ (17,419), and NDK·4$H_2SO_4$ (17,439).

solutions (Fig. 3) and at denaturing conditions (5% HCOOH in 50% methanol). An isotope exchange experiment confirmed that these adducts consisted of $H_2SO_4$. When phosphate was added, the relative intensity of these adducts decreased as observed before with GMPK. The addition of triphosphates (such as GTP shown in Fig. 3) generally leads to the appearance of adducts of 80 mass units corresponding to phosphorylation of the enzyme, *i.e.* the covalent addition of $HPO_3$.

*Enzymatic Activity of GMPK*—Incubation of GDP with guanylate kinase resulted in the production of GMP, GTP, and, after incubation for several hours, of guanosine 5′-tetraphosphate. Fig. 4 shows the mass spectra of 10 $\mu$M GDP incubated with 10 $\mu$M GMPK for 10 min. When data are recorded in the negative ion mode (Fig. 4*A*), the nucleotides and their complexes with $Na^+$ or $Mg^{2+}$ can be detected. The relative abundance of GMP:GDP:GTP was 40:100:24 for the free nucleotides. When a mass spectrum of the same solution is recorded in the positive ion mode, the protonated protein species are recorded. The deconvoluted mass spectrum is shown in Fig. 4*B*, yielding a large number of complexes. None of these nucleotide·GMPK complexes are observed when GMPK is denatured, either with 10% HCOOH or 50% methanol. The relative abundance of the complexes with the nucleotides was 2:28:100 for GMP:GDP:GTP. As generally expected from any enzymatic activity, the relative intensity of the GMPK·nucleotide complexes shown in Fig. 4 was dependent on time and the concentration of GDP. Because of the limited time resolution of approximately 3 min, we could not detect the first steps of the reaction with sufficient accuracy. Also, the temperature of the spraying capillary could not be controlled. Notwithstanding these limitations, we found that at low concentrations (2 $\mu$M GDP, 10 $\mu$M GMPK), no complex with GTP was formed within the first 10 min and complexes of GMPK·GDP (22,448) were almost exclusively detected. The formation of GMPK·GTP (22,528) correlated with the formation of GMPK·$GDP_2$ (22,891).

All these findings can be explained with a simple modification of the commonly employed reaction scheme (22).

$$E + ADP + GDP \Leftrightarrow E + ATP + GMP$$

SCHEME 1

If guanosine phosphates can substitute for adenosine phosphates, this leads to the following.

$$E + GDP + GDP \Leftrightarrow E + GTP + GMP$$

SCHEME 2

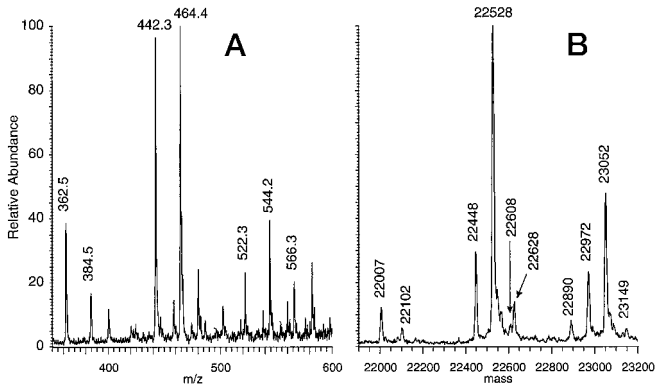


FIG. 4. **The addition of 10 $\mu$M GDP to 10 $\mu$M guanylate kinase in $H_2O$.** *A*, original spectrum (*m/z*) obtained in the negative ion mode after incubation for 10 min. The peaks were assigned to GMP (362.5), GMP·$Na^+$ (384.5), GDP (442.3), GDP·$Na^+$ (464.3), GTP (522.3), GTP·$Na^+$ (544.3), and GTP·$2Na^+$ (566.3). *B,* deconvoluted spectrum (positive ion mode) of the same solution obtained 5 min after the addition of GDP. The peaks were assigned to free GMPK (22,007), GMPK·$H_2SO_4$ (22,105), GMPK·GMP (22,368), GMPK·GDP (22,448), GMPK·GTP (22,528), GMPK·guanosine 5′-tetraphosphate (22,608), GMPK ·GTP·$H_2SO_4$ (22,626), GMPK·2GDP (22,890), GMPK·GDP·GTP (22,971) GMPK·2GTP (23,051).

According to this scheme, 2 molecules of GDP have to interact with the protein as a ternary complex to generate GTP and GMP. Such a ternary complex is highly unlikely at low GDP concentrations. The low abundance of GMP·GMPK complexes in Fig. 4 may be explained with a low affinity of GMPK toward GMP as compared with GDP or GTP. This low affinity would also drive the reaction toward the formation of GTP·GMPK, *i.e.* to the right side of reaction 2. If this reasoning is correct, then the addition of GMP should drive the reaction toward the left side and lead to a decrease of the GTP·GMPK complex. Indeed, when 10 $\mu$MGMP were added to a mixture of 10$\mu$MGMPK and 10 $\mu$MGDP, the relative abundance of the peak corresponding to GTP·GMPK decreased from 35 (before) to 7% after the addition of GMP. Noncovalent binding of ligands to proteins is caused by an array of weak interactions. Of these, only electrostatic interactions are maintained (indeed enhanced) in the vacuum of a mass spectrometer. Our finding low affinity for GMP in relation to GDP or GTP may be an artifact of the method (see "Discussion").

At much longer incubation times (exceeding 12 h for the experiment shown in Fig. 4), the peak at 22,608 Da in Fig. 4*B* corresponding to an adduct with guanosine 5′-tetraphosphate became the most prominent of all complexes. This reaction may well be described by scheme 3.

$$E + GTP + GDP \Leftrightarrow E + \text{guanosine 5′-tetraphosphate} + GMP$$

SCHEME 3

When ADP was used instead of GDP, a similar pattern of complexes could be detected,but the complex AMP·GMPK was even less abundant than the complex GMP·GMPK in Fig. 4. After 1 h of incubation of 10 $\mu$MGMPK with 10 $\mu$MADP the ratio of AMP to ADP to ATP was 51:100:30 for the free nucleotides as determined in the negative ion mode in solution. When the complexes with GMPK were monitored in the positive ion mode, the ratio, as determined from the deconvoluted mass spectrum, was 1:100:96.

Adenosine tetraphosphate was detected neither as a complex with GMPK nor as a separate entity in the negative ion mode. The formation of the complexes was also dependent on time and concentration of ADP, in accordance with reaction scheme 4.

$$E + ADP + ADP \Leftrightarrow E + ATP + AMP$$

SCHEME 4

Magnesium ions are required for the enzymatic activity of guanylate kinase. When we added 1 mM $MgCl_2$ and 20$\mu$M ATP to 10$\mu$M guanylate kinase, we indeed found $Mg_n$·GMPK complexes with $n$ = 1, 2, and 3. However, the magnesium-free protein gave the most abundant peak. The ratio of magnesium-bound to non-magnesium-bound enzyme was slightly increased when 1 additional ATP molecule was bound. This indicates that magnesium enhances ATP binding but this nucleotide can also bind in its absence. A similar enhancement was not observed with magnesium and GTP.

In a separate experiment, we monitored binding of the bisubstrate analogue $Gp_5$ A to GMPK. The high affinity of this ligand was reflected in the mass spectrum of 10$\mu$M GMPK and 12$\mu$M $Gp_5$A, where less than 1% of free enzyme was detected. The ternary complex of GMPK with $Mg^{2+}$ and $Gp_5$A became the dominating peak. The binary complex of GMPK and $Gp_5$A without magnesium was detected with a relative intensity of 95% as compared with the ternary one. The relative intensity of $Mg^{2+}$ binding is thus increased from 20 in the presence of GDP to 105% in the presence of $Gp_5$A.

This indicated that the presumably functional complex has a higher affinity toward $Mg^{2+}$ than the nucleotide complex alone. Electrospray mass spectrometry had also been used to study adenylate kinase and its corresponding bisubstrate analogue $Ap_5$A (4). That experiment also had indicated tight binding, but is difficult to compare with our studies, because denaturing conditions (20% acetonitrile) were employed.

*Binding Studies*—To perform systematic binding studies, we added nucleotides that could not be modified by the enzyme. For example, when the GDP analogue GDP$\beta$S was added to GMPK, complexes corresponding to GMPK·$(GDP\beta S)_n$ could be monitored without phosphorylation or dephosphorylation. Straight binding also was observed when ADP$\beta$S, and at higher concentrations, GMP, AMP, UDP, or CDP were used as ligands. In all of these cases, complexes of one or more ligands with GMPK were detected in the mass spectrometer. An additional peak 98 Da higher, which was assigned to the complex with $SO_4^{2-}$, accompanied the corresponding peaks. The relative abundance of this ternary $SO_4^{2-}$ complex was not smaller than the relative abundance of the (binary) $SO_4^{2-}$ complex with the protein alone, whereas a decrease would be expected if there was competition. When we tried to measure saturation of binding, we found (as expected) that the relative concentration of the free enzyme decreased and that the concentration of the nucleotide-containing complexes increased with the nucleotide concentration. But, in contrast to our expectations, the number of nucleotides/enzyme also increased. Fig. 5 shows the binding of 5 GMP molecules/guanylate kinase when 1 mM GMP was added to 10 $\mu$M GMPK. This unexpected finding cannot be rationalized with "unspecific binding" as in conventional binding studies (23), because only true complexes are monitored in mass spectrometry. When the relative intensity of the free enzyme and the different complexes GMPK·GMP, GMPK·2GMP, GMPK·3GMP, and GMPK·4GMP was plotted *versus* the GMP-concentration, the resulting binding curves (Fig. 6) conformed to commonly used models (24). The population of free enzyme decreased monotonically. The population of the monoliganded complex increased transiently in a hyperbolic fashion. The concentrations of all other complexes increased in a sigmoid fashion. In fact, all these curves could be fitted simultaneously with one simple model, whereby all GMP molecules bind with identical affinities to GMPK. However, this apparent simplicity is misleading.

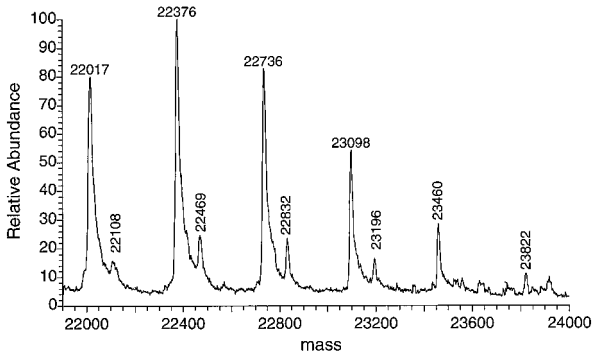


FIG. 5. **Deconvoluted mass spectrum of 10 $\mu$M guanylate kinase incubated with 1 mM GMP in $H_2O$ for 30 min.** The peaks were assigned to GMPK (22,017), GMPK·sulfate (22,108), GMPK·GMP (22,376), GMPK·GMP·sulfate (22,469), GMPK·2GMP (22,736), GMPK·2GMP·sulfate (22,832), GMPK·3GMP (23,098), GMPK·3GMP·sulfate (23,196), GMPK·4GMP (23460), and GMPK·5GMP (23,822).

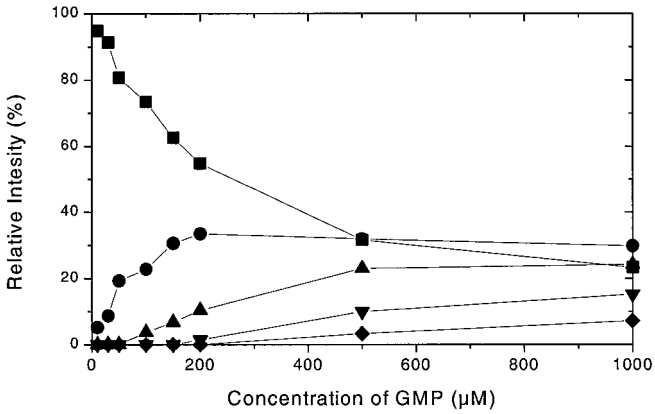


FIG. 6. **Equilibrium binding of GMP to 10 $\mu$M GMPK.** The relative intensity (relative abundance of a particular mass divided by the sum of the relative abundance of all masses multiplied by 100) is plotted *versus* the concentration of GMP. The symbols denote: ■, free GMPK; ●, GMPK-GMP; ▲, GMPK-2GMP; ▼, GMPK-3GMP; ♦, GMPK-4GMP.

In view of the known structure (8) and several binding studies performed with this class of enzymes, the high number of at least five specific binding sites appears to be unlikely. One possibility to reconcile our results with established data is the assumption of nucleotide clusters binding to the protein. A mass spectrum of a nucleotide cluster bound to one site cannot be distinguished from a mass spectrum of nucleotides bound to individual sites. To solve this question, mass spectra of nucleotides in the absence of GMPK were recorded. Indeed, at a concentration of 100 $\mu$M, clusters of up to 4 GDP molecules and cations ($Mg^{2+}$ or $Na^{+}$) were observed in the mass spectrometer. These clusters were detected in the positive ion mode, *i.e.* under the same conditions, which were used for the detection of proteins. When lysozyme, a small basic protein, was incubated with 1 mM GDP, complexes of nucleotides and the enzyme could be observed as well (Fig. 7). Lysozyme is not known to bind nucleotides in solution, although binding to oligonucleotides had been reported (25). Upon close inspection, the mass spectra of the complexes with lysozyme differed from those of nucleotide-binding proteins. They did not only consist of protein and GDP (443 Da) but of protein, GDP, and several cations of 22 Da, which may correspond to either $Mg^{2+}$ or $Na^{+}$ (see "Experimental Procedures"). Accordingly, the relative abundance of all complexes decreased when lysozyme was desalted extensively before GDP was added. We conclude that lysozyme binds ionic clusters containing nucleotides and cations.

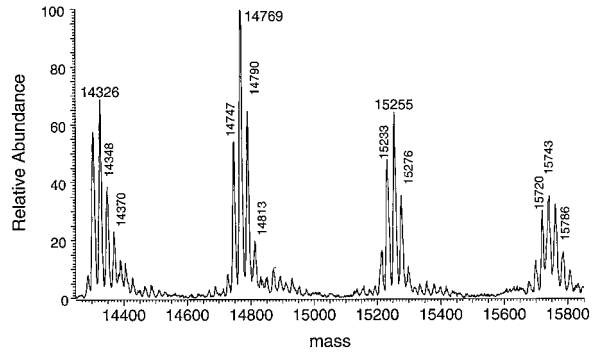


FIG. 7. **Deconvoluted mass spectrum of 20 $\mu$M lysozyme incubated with 1 mM GDP for 15 min in $H_2O$.**

## DISCUSSION

We have shown that mass spectrometry can indeed successfully be applied to noncovalent binding studies of proteins. Whereas we assume that most commercial instruments (triple quadrupole or ion trap) will provide an adequate platform for the detection of all complexes, we would like to point out that the spraying process itself is critical. Nondenatured proteins require aqueous solutions without the addition of volatile organic solvents. Formation of sufficiently small droplets from these solutions would be difficult to archive with standard electrospray techniques. Nano-electrospray is the method of choice.

Although the mass spectra recorded with this technique give a new insight into the nature of substrates and products bound to proteins, the question arises how relevant mass spectra are when binding of ligands in solution is in the focus of interest. All ligands whose complexes were identified in our mass spectrometer were either cations ($Mg^{2+}$ or $Na^{+}$) or anions ($SO_4^{2-}$ and all nucleotides or their analogues). Electrostatic interactions are maintained or even enhanced in the vacuum of a mass spectrometer. In contrast, hydrophobic interactions obviously cannot be maintained in the vacuum without water. Van der Waals and similar short range interactions may be lost after the molecules enter the mass spectrometer and collide with the remaining gas in the capillary skimmer region of the mass spectrometer. A similar reasoning applies when weak electrostatic interactions are concerned. If the interactions are too weak, the complexes may dissociate because of collision-induced decay. All this reasoning is reflected in our results: 1) High affinity binding of ions, such as the binding of GDP to $p21^{ras}$ or the binding of $Gp_5A$ to GMPK leads to stable complexes, which are readily detected in the mass spectrometer. 2) Binding of nonionic ligands, such as water molecules, did not lead to complexes detected in our mass spectrometer. $H_2O$ has been clearly localized in the structure of $p21^{ras}$ (10) and GMPK (8) and thus would be expected to bind these proteins with relatively high affinity. 3) The relative abundance of complexes between proteins and nucleotides detected in the mass spectrometer was increased with the number of phosphate groups on the nucleotides. 4) The binding of $PO_4^{3-}$ could not be detected as complexes in the mass spectra, although it could be inferred from their competition with $SO_4^{2-}$ ions. This finding is explained when one assumes that the binding energy of $PO_4^{3-}$ and proteins is weaker than the collision energy in the skimmer region and thus cannot be observed. 5) Relatively large complexes of nucleotides, cations, and proteins were detected in mass spectra, although their abundance in solution may be relatively low. This finding may be explained with an array of electrostatic interactions, which are enhanced in the vacuum. It should be noted that the ligand concentration in solution is increased transiently when droplets generated in the spraying process are evaporated and transferred into the vacuum. This

considerable concentration increase may lead to complexes, which cannot be observed in solution, if and only if these complexes are rapidly formed. Unspecific electrostatic interactions are known to be relatively fast. Therefore, complexes of ions detected by means of the mass spectrometry may not be abundant in solution.

Taken together, our results clearly show the limits of the method in regard to binding studies. Only electrostatic interactions were visible in the mass spectrometer. Of these, unspecific and rapidly formed complexes such as ionic clusters were enhanced by the method. Some electrostatic interactions, such as the binding of $PO_4^{3-}$ to specific sites of the proteins, did not lead to stable complexes in the mass spectrometer and could only be inferred indirectly from competition experiments. Therefore, no dissociation constant relevant to ligand binding in solution can be derived from such studies. Moreover, even the number of bound molecules detected in our mass spectrometer need not necessarily correspond to the number of binding sites. This general feature of electrospray mass spectra is particularly misleading, because information on binding stoichiometry appears to be straightforward (7). Notwithstanding these general limitations of binding studies, we want to point out some results prevalent to the understanding of the nucleotide-binding proteins investigated.

$Mg^{2+}$ was found to bind only weakly to either $p21^{ras}$ or GMPK in the absence of nucleotides. Only upon the formation of the functional complex (with GDP or $Gp_5A$, respectively) did the magnesium-bound moiety become the most abundant population. This finding corresponds to the known x-ray structure of $p21^{ras}$ (10), where 1 magnesium ion was located at the active site in the presence of substrate.

$SO_4^{2-}$ had been identified as forming a complex with all nucleotide-binding proteins in our study. Its exchange rate must be low, because sulfates had not been used in the preparation of GMPK, so that the complexes with $SO_4^{2-}$ must have been preserved during the purification of the protein. The physiological concentrations of both phosphate (26) and sulfate (27) ions are in the millimolar range. The protein concentration of our stock solutions also was in the same concentration range, so that a slow sulfate exchange during storage cannot be ruled out. The relative collision energy of 35% required for sulfate dissociation is similar to the energy required for the breaking of peptide bonds. Therefore, sulfate rather than phosphate complexes must be formed whenever equilibrium is reached. Bound $SO_4^{2-}$ had been observed before in x-ray studies, where these ions had been found localized in the binding site in crystals of GMPK from yeast (8) and $p21^{ras}$ (28). In an independent study, ammonium sulfate had been found to stabilize the nucleotide-free protein and to facilitate the dissociation of GDP from $p21^{ras}$ (29). Our studies indicate that there is no competition between the binding of sulfates and nucleotides, rather the binding of nucleotides facilitates the binding of sulfates or vice versa (*e.g.* Fig. 6). All these findings, namely the stabilization of the nucleotide-free conformation, the facilitated dissociation, and the mutual binding of sulfates and nucleotides may be explained with the same general feature of nucleotide-binding proteins. It may involve a positively charged binding pocket or a string of positively charged and possibly overlapping sites, which may be occupied by negatively charged nucleotides or sulfates. The protein structure would seriously be destabilized when none of these locations are occupied by negative ions. This explains the observed high affinity for $SO_4^{2-}$. Sulfates would then serve as a place-reserving entity ("Platzhalter"), which by themselves need not have any functional properties. They could only be exchanged with other anions such as nucleotides by a mechanism, which may be similar to the facilitated dissociation observed for other high affinity noncovalent interactions (30). Dissociation in the vacuum of the mass spectrometer would be unlikely. Such a mechanism requires the formation of ternary or higher complexes in solution, as is indeed shown here.

The enzymatic mechanism of GMPK appears to be of a more general nature than usually postulated (Scheme 1). In particular, adenosine nucleotides could substitute for guanosine and vice versa, so that the transfer of $HPO_3$ between nucleotides could take place whenever a ternary complex between the enzyme and two nucleotides was formed. Guanosine 5′-tetraphosphate was generated after prolonged incubation of GMPK with either GDP or GTP. In this respect, GMPK is similar to muscle adenylate kinases from rabbit and porcine sources, which catalyze the formation of adenosine 5′-tetraphosphate from ATP and ADP (31). The production of GMP, GTP, and guanosine 5′-tetraphosphate from GDP could also be observed directly (in the negative ion mode) at higher salt concentration and thus was not an artifact of the relatively low ionic strength required for the study of proteins in aqueous solution.

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REFERENCES

1. Mann, M., and Wilm, M. (1995) *Trends Biochem. Sci.* **20,** 219–224
2. Wilm, M., and Mann, M. (1996) *Anal. Chem.* **68,** 1–8
3. Potier, N., Barth, P., Tritsch, D., Biellmann, J. F., and Van Dorsselaer, A. (1997) *Eur. J. Biochem.* **243,** 274–282
4. Briand, G., Perrier, V., Kouach, M., Takahashi, M., Gilles, A. M., and Barzu, O. (1997) *Arch. Biochem. Biophys.* **339,** 291–297
5. Ganguly, A. K., Pramanik, B. N., Huang, E. C., Liberles, S., Heimark, L., Liu, Y. H., Tsarbopoulos, A., Doll, R. J., Taveras, A. G., Remiszewski, S., Snow, M. E., Wang, Y. S., Vibulbhan, B., Cesarz, D., Brown, J. E., del Rosario, J., James, L., Kirschmeier, P., and Girijavallabhan, V. (1997) *Bioorg. Med. Chem.* **5,** 817–820
6. Jorgensen, T. J. D., Roepstorff, P., and Heck, A. J. R. (1998) *Anal. Chem.* **70,** 4427–4432
7. Cheng, X. H., Harms, A. C., Goudreau, P. N., Terwilliger, T. C., and Smith, R. D. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 7022–7027
8. Stehle, T., and Schulz, G. E. (1992) *J. Mol. Biol.* **224,** 1127–1141
9. Wittinghofer, A., and Nassar, N. (1996) *Trends Biochem. Sci.* **21,** 488–491
10. Pai, E. F., Krengel, U., Petsko, G. A., Goody, R. S., Kabsch, W., and Wittinghofer, A. (1990) *EMBO J.* **9,** 2351–2359
11. Schaertl, S., Konrad, M., and Geeves, M. A. (1998) *J. Biol. Chem.* **273,** 5662–5669
12. Witkowska, H. E., Green, B. N., Carlquist, M., and Shackleton, C. H. L. (1996) *Steroids* **61,** 433–438
13. Tucker, J., Sczakiel, G., Feuerstein, J., John, J., Goody, R. S., and Wittinghofer, A. (1986) *EMBO J.* **5,** 1351–1358
14. John, J., Frech, M., and Wittinghofer, A. (1988) *J. Biol. Chem.* **263,** 11792–11799
15. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
16. John, J., Sohmen, R., Feuerstein, J., Linke, R., Wittinghofer, A., and Goody, R. S. (1990) *Biochemistry* **29,** 6058–6065
17. Konrad, M. (1993) *J. Biol. Chem.* **268,** 11326–11334
18. Dumas, C., Lascu, I., Morera, S., Glaser, P., Fourme, R., Wallet, V., Lacombe, M. L., Veron, M., and Janin, J. (1992) *EMBO J.* **11,** 3203–3208
19. Goody, R. S., Frech, M., and Wittinghofer, A. (1991) *Trends Biochem. Sci.* **16,** 327–328
20. McCormick, F., and Wittinghofer, A. (1996) *Curr. Opin. Biotechnol.* **7,** 449–456
21. Klebe, C., Prinz, H., Wittinghofer, A., and Goody, R. S. (1995) *Biochemistry* **34,** 12543–12552
22. Li, Y., Zhang, Y. L., and Yan, H. G. (1996) *J. Biol. Chem.* **271,** 28038–28044
23. Prinz, H. (1992) in *Receptor-Ligand Interactions. A Practical Approach* (Hulme, E. C., ed) pp. 265–288, Oxford University Press, Oxford
24. Prinz, H. (1983) *J. Recept. Res.* **3,** 239–248
25. Laktionov, P. P., Rykova, E. Y., Krepkii, D. V., Bryksin, A. V., and Vlassov, V. V. (1997) *Biochemistry* **62,** 613–618
26. Murer, H., and Biber, J. (1998) *Schweiz. Med. Wochenschr.* **128,** 1247–1252
27. Fernandes, I., Hampson, G., Cahours, X., Morin, P., Coureau, C., Couette, S., Prie, D., Biber, J., Murer, H., Friedlander, G., and Silve, C. (1997) *J. Clin. Invest.* **100,** 2196–2203
28. Scheffzek, K., Kabsch, W., Schlichting, I., Pai, E. F., Lautwein, A., Frech, M., Wittinghofer, A., and Goody, R. S. (1999) *Acta Crystallogr. Sec. D* **50,** 521–526
29. Mistou, M. Y., Cool, R. H., and Parmeggiani, A. (1992) *Eur. J. Biochem.* **204,** 179–185
30. Prinz, H., and Striessnig, J. (1993) *J. Biol. Chem.* **268,** 18580–18585
31. Kupriyanov, V. V., Ferretti, J. A., and Balaban, R. S. (1986) *Biochim. Biophys. Acta* **869,** 107–111


ENZYMOLOGY:

# Binding of Nucleotides to Guanylate Kinase, p21 $^{ras}$, and Nucleoside-diphosphate Kinase Studied by Nano-electrospray Mass Spectrometry

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