Conformational States of the Small G Protein Arf-1 in Complex with the Guanine Nucleotide Exchange Factor ARNO-Sec7*

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Arf1 is a small G protein involved in vesicular trafficking, and although it is only distantly related to Ras, it adopts a similar three-dimensional structure. In the present work, we study Arf1 bound to GDP and GTP and its interactions with one of its guanosine nucleotide exchange factors, ARNO-Sec7. The 31P NMR spectra of Arf1-GDP·Mg2+ and Arf1-GTP·Mg2+ share the general features typical for all small G proteins studied so far. Especially, the β-phosphate resonances of the bound nucleotide are shifted strongly downfield compared with the resonance positions of the free magnesium complexes of GDP and GTP. However, no evidence for an equilibrium between two conformational states of Arf1-GDP·Mg2+ or Arf1-GTP·Mg2+ could be observed as it was described earlier for Ras and Ran. Glu156 of ARNO-Sec7 has been suggested to play as "glutamic acid finger" an important role in the nucleotide exchange mechanism. In the millimolar concentration range used in the NMR experiments, wild type ARNO-Sec7 and ARNO-Sec7(E156D) do weakly interact with Arf1-GDP·Mg2+ but do not form a strong complex with magnesium-free Arf1-GDP. Only wild type ARNO-Sec7 competes weakly with GDP on Arf1-GDP-Mg2+ and leads to a release of GDP when added to the solution. The catalytically inactive mutants ARNO-Sec7(E156A) and ARNO-Sec7(E156K) induce a release of magnesium from Arf1-GDP·Mg2+ but do not promote GDP release. In addition, ARNO-Sec7 does not interact or only very weakly interacts with the GTP-bound form of Arf1, opposite to the observation made earlier for Ran, where the nucleotide exchange factor RCC1 forms a complex with Ran-GTP·Mg2+ and is able to displace the bound GTP.

The members of the Arf (ADP-ribosylation factor) protein family are involved in membrane dynamics and vesicular trafficking (1). Arf1 is a small Ras-like guanine nucleotide-binding protein and adopts a similar fold as all other proteins of the Ras superfamily. However, Arf shows two structural elements that are missing in Ras: an extra β-strand (β2E) and an N-terminal helix (2, 3). The N-terminal helix is amphipathic and myristoylated and is involved in the GTP-dependent interaction of Arf1 with membrane lipids (4, 5). In Arf1 and the corresponding Arl (Arf-like) proteins, the switch I region is located between helix α1 and strand β2a, an interswitch region encompasses strands β2 and β3, and the switch II region contains the loop starting at strand β3 and the helix α2 (6).

Similar to Ras and other small G proteins, Arf1 switches between the GTP-bound "on" state, where it transmits signals to its effectors in the signaling pathway, and the GDP-bound "off" state. A characteristic feature of the nucleoside triphosphate form is the coordination of the γ-phosphate group of GTP with the Mg2+ ion and the amide group of Thr48 from switch I in Arf1 (7). Simultaneously, the hydroxyl group of Thr48 is coordinated to the metal ion. This bond pattern seems to be essential for stabilizing the correct conformation of the effector loop for effector recognition. The molecular details of the GDP-GTP switching mechanism are largely conserved among all small G proteins as well as the general features of the activation-deactivation cycle. GDP is replaced by GTP, which is present in higher concentration in the cytoplasm, by guanine nucleotide exchange factors (GEFs).

The specific GEFs for Arf1, which can be subdivided into four different subfamilies containing the Geo/Gnom/GBF family, the Sec7/BIG family, the ARNO/cytohesin/GRP family, and the EFA6 family (8), share a common domain (Sec7) of about 200 residues, which contains the catalytic activity. The Sec7 domain is also active on a truncated form of Arf1 that lacks the first N-terminal helix of 17 residues ([Δ17]Arf1). It demonstrates that the Sec7 domain of ARNO (ARNO-Sec7) interacts with the core domain of Arf1 and not with the myristate or the N-terminal amphipathic helix (9). Recently, a glutamic finger hypothesis in the catalysis of GDP-GTP exchange by the guanine nucleotide exchange factor ARNO-Sec7 has been formulated (10). Indeed, a specific glutamate residue, Glu156, located in the hydrophilic loop of ARNO-Sec7 helps to destabilize the GDP bound to Arf1 by displacing the Mg2+ and by repulsing the GDP β-phosphate. The conservative mutation E156D as well as the charge reversal mutation E156K reduce the exchange activity of ARNO-Sec7 on Arf1 by several orders of magnitude. In addition, ARNO-Sec7(E156K) forms a complex with the Mg2+-free form of [Δ17]Arf1-GDP without inducing the release of GDP. The published crystal structure of a complex between nucleotide-free [Δ17]Arf1 and the Sec7 domain of Geo2 supports the hypothesis of a glutamic acid finger (7). A shift in the position of the switch 1 region in Arf1 exposes its active site to Glu156 of the Sec7 domain. This results in steric and electrostatic repulsion of the β-phosphate and the Mg2+ ion on Arf1, promoting nucleotide dissociation (7).

The molecular pathway of Arf that leads to GDP dissociation most probably involves several steps: docking, conformational change at the switch/Sec7 interface, and Mg/GDP release.

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‡ The abbreviations used are: GEF, guanine nucleotide exchange factor; Gpp(NH)p, guanosine 5′-β,γ-imidodiposphate; DTE, diithothreitol.
These steps can be inferred from biochemical studies (e.g., brefeldin A (11), membrane lipids (9)) but have not been trapped by crystallography. Phosphorus NMR was shown to be an alternative to characterize different steps of GTP-induced conformations in effector recognition (15–17) as well as of GDP dissociation (16) of small G proteins. At a millimolar concentration of protein and nucleotide used, one can detect weak ternary complexes between a small G protein, nucleotide, and exchange factor. These complexes represent critical intermediates in the nucleotide exchange pathways but can hardly be isolated, since they tend to disappear to the advantage of more stable binary complexes during purification. Thus, if one considers a simple scheme for the exchange reaction: ArfGDP + Sec7 ⇌ ArfGDP-Sec7 ⇌ ArfSec7 + GDP, phosphorus NMR is an excellent method to be able to distinguish in principle the phosphorus α and β signals at each step and notably when the nucleotide encounters the catalytic machinery of the exchange factor, here the Glu finger, before being expelled.

We set out to study the interaction of ARNO-Sec7 with [17]Arf1 by phosphorus NMR using the wild type ARNO-Sec7 protein as well as the mutated forms E156D, E156K, and E156A. In all cases, the data suggest that stable ternary complexes of Arf1-GDP-Sec7 exist in solution in both the Mg2⁺-free and the Mg2⁺-bound form, depending on the ion concentration.

MATERIALS AND METHODS

Protein Expression and Purification—[17]Arf1, a truncated form of Arf1, lacking the first 17 N-terminal residues, and ARNO-Sec7 (residues 50–252 of ARNO) were expressed in Escherichia coli and purified as described (5, 10, 12). After purification, [17]Arf1 is complexed at 60–70% to GDP and at 30–40% to GTP as determined by tryptophan fluorescence. To shift the equilibrium toward the GDP-bound form or the GTP-bound form, [17]Arf1 and ARNO-Sec7 protein, usually 1.8 mM concentration typically varied between 0.8 and 1.9 mM. To provide a lock and the Mg2⁺-free solution, the samples contained 10% D2O. In the study of complexes not noted otherwise, phosphorus spectra were recorded at a temperature of 85 °C.

31P NMR Spectra of Wild Type ARNO-Sec7 Complexed with Arf1—31P NMR spectra of [17]Arf1 complexed with guanine nucleotide were recorded in 20 mM Tris-HCl, 1 mM MgCl2, and 1 mM dithiotreitol at pH 8.0 (buffer A). 800 mM MgCl2, and 1 mM dithiotreitol at pH 8.0 (buffer A). 800

800 i.e.

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Preparation of the NMR Samples—31P NMR spectra of [17]Arf1 complexed with guanine nucleotide were recorded in 20 mM Tris-HCl, 1 mM MgCl2, and 1 mM dithiotreitol at pH 8.0 (buffer A). 800 μL of the sample was placed in 8-mm NMR tubes (Shigemi). The protein concentration typically varied between 0.8 and 1.9 mM. To provide a lock signal, the samples contained 10% D2O. In the study of complexes between [17]Arf1 and ARNO-Sec7 protein, usually 1.8 mM [17]Arf1 and different concentrations of ARNO-Sec7 solutions were added (i.e., 1.9 mM [wt], 1.28 mM [E156D], 1.3 mM [E156K], and 0.74 mM [E156A]). The ARNO-Sec7 proteins added were in 50 mM Tris-HCl, 1 mM MgCl2, 100 mM NaCl, and 1 mM dithiotreitol at pH 7.5.

NMR Spectroscopy—31P NMR experiments were performed on a Bruker DRX-500 NMR spectrometer working at a phosphorus resonance frequency of 202 MHz. The 31P NMR spectra were indirectly referenced to sodium 2,2-dimethyl-2-silapentane-5-sulfonate according to Ref. 13. A δ-value of 0.4048073561 was used, which corresponds to 85% external phosphoric acid contained in a spherical bulb. Unless noted otherwise, phosphorus spectra were recorded at a temperature of 278 K with a total spectral width of 40 ppm. For one-dimensional 31P NMR spectra, 1024–12,288 free induction decays were accumulated after excitation with a 65° (90°) pulse using a repetition time of 5.8 s. A total of 32,768 time domain data points were recorded and transformed to 32,768 real data points, corresponding to a digital resolution of 0.25 Hz/point.

All data were processed on a Silicon graphics O2 work station using the software package UXNMR (Bruker, Karlsruhe, Germany) for data processing. Phosphorus NMR spectra for peak integration and for peak fitting were filtered by an exponential window function causing no significant line broadening.

Data Analysis—Quantitative analysis of the NMR spectra was carried out with a fitting and peak integration method, using the program Origin 6.0 from Microlab Software (Northampton, MA). First, all spectra were equally processed and scaled up or down depending on the number of scans, and a base line was determined by fitting the peak free areas with a constant. From the nonlinear fit of the more simple spectra with Lorentzian lines, the chemical shifts of the Arf1-GDP and Arf1-GTP phosphate groups were determined as accurately as possible under different free Mg2⁺ ion concentrations (see Table I). These values were then used as constants in the analysis of the more complicated spectra (complexes with ARNO-Sec7), where a severe overlap of lines occurred. When fitting these kinds of spectra with a multi-Lorentzian model, we tried to reproduce the measured spectrum as closely as possible and making sure at the same time that consistent line widths were used for the single components. As an example, with a growing fraction of [17]Arf1-GDP bound to ARNO-Sec7, the line width increases because of a higher total correlation time of the larger complex. This approach led to satisfying results in most cases. The biggest errors resulting from this method were caused by the slight deviation of the measured signals from a strict Lorentzian line shape (in the case of separated signals, a simple integration was carried out as well in order to test the results and to reduce the error) and especially by the bad signal to noise ratio, a problem that was not negligible even with data acquisition times of several hours.

RESULTS

31P NMR Spectra of the Arf1-Nucleotide Complexes—Fig. 1, a and b, shows the phosphorus NMR spectra of [17]Arf1-GTP-Mg2⁺ and [17]Arf1-GDP-Mg2⁺, respectively. The two spectra were recorded in the presence of Mg2⁺ in millimolar concentrations (i.e. under conditions where the nucleotide should be completely coordinated with the metal ion). The [17]Arf1-GDP spectrum (Fig. 1b) contains two strong lines at −9.66 and −3.66 ppm corresponding to the α- and β-phosphate group of bound GDP (Table I). The spectrum of Arf1-GTP-Mg2⁺ (Fig. 1a) shows the expected three phosphorus lines of bound GTP at −9.58, −15.23, and −4.91 ppm. For both [17]Arf1-GTP and [17]Arf1-GDP, additional peaks were observed. By their size and position, these peaks could be assigned easily to free phosphate and contaminating [17]ArfGDP or [17]Arf-GTP.

Usually proteins with a single high affinity site for guanine nucleotides show only one resonance line for each phosphorus atom of a phosphate group if they occur in only one conformational state (Table I). Because only one set of resonance lines is observable for [17]Arf1-GTP and [17]Arf1-GDP, we concluded that Arf1-GDP-Mg2⁺ and Arf1-GTP-Mg2⁺ exist predominantly in a single (NMR-distinguishable) conformational state, in contrast to other small G proteins such as Ran or Ras. This is true for the whole temperature range from 277 to 303 K studied, where no indication for a second conformational state could be obtained from NMR spectroscopy.

Sec7 domains are believed to expel the bound GDP from Arf1 in part by destabilizing the bound Mg2⁺. Therefore, it was important to record the phosphorus NMR spectrum of isolated [17]Arf1-GDP in the absence of Mg2⁺. Fig. 1c shows the phosphorus NMR spectrum of [17]Arf1-GDP after complexation of Mg2⁺ with 2 mM EDTA (leaving −1 μM free Mg2⁺ in solution). Two new resonances appear at −10.56 and −4.54 ppm corresponding to the α- and β-phosphate group of bound GDP in Mg2⁺-free [17]Arf1-GDP (Fig. 1c). The removal of Mg2⁺ is not perfect, since the resonance lines assigned previously to [17]Arf1-GDP-Mg2⁺ are weakened but still visible. Note that the signal of the β-phosphate resonance of [17]Arf1-GTP-Mg2⁺ (which is present as a contaminating species here) is not influenced much by the addition of EDTA, suggesting that the magnesium ion is not removed from the complex as to be expected from the higher affinity of Mg2⁺ for Arf1-GTP than for Arf1-GDP.

31P NMR Spectra of Wild Type ARNO-Sec7 Complexed with Arf1-GDP-Mg2⁺ or Arf1-GDP—Fig. 2 shows the 31P-NMR spectra of wild type ARNO-Sec7 titrated with increasing concentrations of Arf1-GDP-Mg2⁺. As was to be expected, ARNO-Sec7(wt) was not detected by 31P NMR, and the 31P signals of the α- and β-phosphate grew in proportion to the increasing fraction of Arf1-GDP-Mg2⁺ added. No larger shifts of the resonance lines of bound GDP are observed in the presence of ARNO-Sec7 (Table I), which would allow the unequivocal as-
The assignment of the resonances of the putative Arf1/GDP-Mg\(^{2+}\) complex. With the molecular mass of 22,413 Da of ARNO-Sec7, the \(T_2\) relaxation rate of bound GDP should increase in the rigid body approximation by a factor of 2.1, which represents a rather small difference in line width for distinguishing between free Arf1/GDP-Mg\(^{2+}\) and Arf1-GDP-Mg\(^{2+}\) complexed with ARNO-Sec7. The total line width at half height of the \(\delta\) lines increased from 60 to 86 Hz, respectively, when the Arf1 to ARNO-Sec7 concentration was 0.84 (Fig. 2d). This means that there is an interaction between the two proteins under our conditions. The detailed analysis of the spectra reveals that at least the \(\beta\)-phosphate line is not homogeneous but consists of a broader, slightly downfield shifted component and a component with virtually the same line width as free Arf1/GDP-Mg\(^{2+}\). The analysis of the \(\alpha\)-phosphate line is difficult because it is superposed by the \(\alpha\)-phosphate line of Arf1-GTP-Mg\(^{2+}\). When fitting the \(\beta\)-phosphate resonance with this assumption, one obtains a transverse relaxation time of 1.27 ms for complex of Arf1/GDP-Mg\(^{2+}\) with ARNO-Sec7 and of 5.25 ms for free Arf1/GDP-Mg\(^{2+}\). This means that in the presence of ARNO-Sec7 exchange broadening occurs (in the absence of ARNO-Sec7, \(T_2\) is about 4 times larger; see above).

At a ratio of Arf1-GDP-Mg\(^{2+}\) to ARNO-Sec7 of 0.84 the rela-

**Fig. 1.**\(^{31}\)P NMR spectra of [\(\Delta 17\)]Arf1 complexed with different nucleotides. The samples were contained in 20 mM Tris-HCl, pH 8.0, 1 mM MgCl\(_2\), 1 mM DTE in 90% H\(_2\)O, 10% D\(_2\)O (buffer A). a, [\(\Delta 17\)]Arf1-GTP-Mg\(^{2+}\) in buffer A, Arf1 concentration 0.83 mM. b, [\(\Delta 17\)]Arf1-GDP-Mg\(^{2+}\) in buffer A, Arf1 concentration 0.88 mM. c, same sample as b after the addition of 2 mM EDTA. Spectra were recorded at 278 K at 202.4 MHz, total recording time 12 h. Note that the [\(\Delta 17\)]Arf1-GDP-Mg\(^{2+}\) sample (b and c) contains ~15% [\(\Delta 17\)]Arf1-GTP-Mg\(^{2+}\) and that after the addition of EDTA (c) ~5% of [\(\Delta 17\)]Arf1-GDP still has an Mg\(^{2+}\) ion bound. \(\alpha\)-Mg\(_{\text{GDP}_{\text{Arf1}}}\) and \(\beta\)-Mg\(_{\text{GDP}_{\text{Arf1}}}\), resonances of the \(\alpha\)- and \(\beta\)-phosphate groups of GDP bound to Arf1; \(\alpha\)-GDP\(_{\text{Arf1}}\) and \(\beta\)-GDP\(_{\text{Arf1}}\), resonances of the \(\alpha\)- and \(\beta\)-phosphate groups of GDP bound to Arf1; \(\alpha\)-Mg\(_{\text{GTP}_{\text{Arf1}}}\), \(\beta\)-Mg\(_{\text{GTP}_{\text{Arf1}}}\), and \(\gamma\)-Mg\(_{\text{GTP}_{\text{Arf1}}}\), resonances of the \(\alpha\)-, \(\beta\)-, and \(\gamma\)-phosphate groups of Mg\(_{\text{GTP}}\) bound to Arf1.
tive population of the free Arf1-GDP-Mg$^{2+}$ to the complexed one would be 0.43 (i.e. the association constant would be on the order of 0.1 mM).

At a ratio of 0.84 and 1.7 of Arf1-GDP-Mg$^{2+}$ to ARNO-Sec7, the signal of the β-phosphate resonance of a small amount of free GDP (partly complexed with Mg$^{2+}$) becomes visible at −5.78 ppm. In addition, the concentration of free inorganic phosphate increases in the sample. This is only seen in the case of the wild-type ARNO-Sec7 and with none of the mutants. A number of explanations seem to be reasonable to account for the free Pi. 1) A contaminating phosphatase is present in the sample (Arf or Sec7 domain), which would hydrolyze GDP as soon as it dissociates from Arf1 upon the action of the nucleotide exchange factor. This would in fact imply the increase of GTP as a separate phosphorus line in our NMR spectra that cannot be seen. 2) Arf1-GDP-Mg$^{2+}$ exists partly as Arf1-GDP-P, Mg$^{2+}$ in solution, and only the catalytically active Sec7-wt domain can expel the Mg$^{2+}$, the P$_i$ and the GDP. Again, even here we would expect a separate additional phosphorus signal for protein-bound P$_i$, which could be severely exchange-broadened beyond detection. A quantitative analysis of the data leads to the conclusion that GDP and P$_i$ are released from Arf1 by ARNO-Sec7, since the increase of its signal is not completely explained by the free P$_i$ and GDP contained in the Arf1 samples added.

Fig. 3a shows a spectrum of Arf1-GDP-Mg$^{2+}$ in complex with ARNO-Sec7. After complexing of Mg$^{2+}$ ions by the addition of EDTA, the signals originating from Arf1-GDP-Mg$^{2+}$ disappear with ARNO-Sec7 disappear and are replaced by new signals at −10.60 and −4.54 ppm at positions close to those of magnesium-free Arf1-GDP (Fig. 4a). The resonance positions do not deviate significantly from those observed for Arf1-GDP in the absence of the exchange factor (Table I). However, the resonance lines are significantly broadened, which is indicative for an interaction of Arf1-GDP with ARNO-Sec7. A simulation of the data leads to a relaxation time of 2.61 ms for the β-phosphate resonance (i.e. the relaxation time decreases by a factor of 1.7 in the presence of ARNO-Sec7).

$^{31}$P NMR Spectra of Arf1-GDP-Mg$^{2+}$ Complexed with Mutants of ARNO-Sec7—A similar behavior is observed for ARNO-Sec7(E156D), which still contains a negatively charged side chain at the position of the glutamate finger but shortened by one methylene unit (Fig. 3b). Again, the NMR signals are indicative for a superposition of resonance lines of free [17]Arf1-GDP-Mg$^{2+}$ and [17]Arf1-GDP-Mg$^{2+}$-ARNO-Sec7(E156D). The resonances of bound Mg$^{2+}$-GDP do not shift much but become broadened and clearly inhomogeneous. A broader component of the ARNO-Sec7 complex is especially well visible for the β-phosphate resonance, which shows a clear downfield shifted shoulder at −3.16 ppm. As discussed above, the main difference between wild type ARNO-Sec7 and the E156D mutant is actually at the level of free GDP and P$_i$, which suggests that the mutant does not dissociate GDP from Arf1.

Replacing the glutamate in ARNO-Sec7 by the uncharged amino acid alanine leads to more dramatic changes in the spectrum; a pair of new lines is created at positions similar but not identical to those of magnesium-free Arf1-GDP, which indicates that in the complex with ARNO-Sec7(E156A), part of the bound Mg$^{2+}$ ions are released (Fig. 3c). The addition of ARNO-Sec7(E156A) does not result in an inhomogeneity of β-phosphate of line [17]Arf1-GDP-Mg$^{2+}$, which was identified as the complex of [17]Arf1-GDP-Mg$^{2+}$ with ARNO-Sec7 in the case of the wild-type Arf1. However, the lines corresponding to the [17]Arf1-GDP can only arise from its complex with the ARNO-Sec7 mutant, since they can only be observed in the presence of this mutant and are significantly shifted and broadened ($T_2$ of the α-phosphate and β-phosphate resonances increases to 2.71 ms for both). Qualitatively, the line broadening effect can be seen when comparing the virtually unperturbed lines of [17]Arf1-GTP-Mg$^{2+}$ with the lines of bound GDP in the presence of ARNO-Sec7. As a consequence of the E156A mutation, we can hypothesize that residue 156 can expel the Mg$^{2+}$ through its first CH$_2$ group of the side chain.

An interesting mutation at position 156 can be created by substituting the negatively charged glutamate by a positively charged lysine. As in the case of the alanine mutant, a complex with magnesium-free Arf1-GDP can be detected. In addition, strong signals of free GDP (partly complexed with Mg$^{2+}$) at −5.67 and −10.35 ppm are visible in the spectrum. However, these signals originate from free GDP already present in the [17]Arf1-GDP-Mg$^{2+}$ sample before adding the exchange factor. As seen in the Ala case, additional signals appear compared with wild type ARNO-Sec7, which can be assigned to the magnesium-free complex of [17]Arf1-GDP-ARNO-Sec7(E156K) (Fig. 3d). The positively charged side chain lysine seems to weaken the Mg$^{2+}$ ion bound to the Arf protein by electrostatic interaction leading to magnesium-free complex.

Since the interaction with ARNO-Sec7 influences the Mg$^{2+}$ binding to [17]Arf1-GDP, a set of measurements was performed in the presence of 2 mM EDTA that should establish a concentration of free magnesium of −1 μM (Fig. 4). As to be expected at low magnesium concentration, the equilibrium between Arf1-GDP and Arf1-GDP-Mg$^{2+}$ is shifted to the magne-
The resonances of $\gamma$-phosphate groups of bound, magnesium-free GDP are observable at 10.60 and 4.54 ppm. The complexes with wild type and ARNO-Sec7 mutants are characterized by small shift changes (Table I) and a clear line broadening. Note that the used concentrations of EDTA are not sufficient to remove significant amounts of magnesium from Arf1-GTP-Mg$^{2+}$, since its signals are not perturbed. In the spectrum of Arf1-GDP in the presence of ARNO-Sec7(E156K), strong signals of free GDP are visible at 5.67 and 10.35 ppm. However, this free GDP was already present as impurity in the Arf1 solution used in this case (see above).

High free magnesium shifts the equilibrium between Arf1-GDP and Arf1-GDP-Mg$^{2+}$ also in the presence of wild type...
Fig. 3. The complex of wild type and mutant ARNO-Sec7 with [Δ17]Arf1-GDP in the presence of magnesium ions. NMR spectra of 1.18 (a), 1.07 (b), 1.75 (c), and 1.08 mM (d) [Δ17]Arf1-GDP in 20 mM Tris-HCl, pH 8.0, 1 mM MgCl₂, 1 mM DTE, 90% H₂O, 10% D₂O in the presence of 1.41 mM wild type ARNO-Sec7 (a), 1.28 mM ARNO-Sec7(E156D) (b), 2.12 mM ARNO-Sec7(E156A) (c), and 1.33 mM ARNO-Sec7(E156K) (d). Thus, the molar ratio of Arf1 to ARNO-Sec7 was held constant at 1.0/1.2. Note that in spectrum d, the Arf1 solution had initially a rather high concentration of free GDP (~0.5 mM). Fractional numbers in the left half of the spectra indicate the relative exchange activity of a certain ARNO-Sec7 mutant with respect to the wild type form (20). The various side chains at position 156 of ARNO-Sec7 are depicted in the right half of the plot. All spectra were recorded at 278 K.

ARNO-Sec7 and the ARNO mutants E156D and E156A completely to the magnesium-bound population (data not shown). An exception is the mutant E156K, where even the presence of 9 mM free magnesium is not sufficient to shift the equilibrium of the Arf1-ARNO complex completely to the magnesium-containing state.

31P NMR Spectra of Arf1-GTP-Mg²⁺ in the Presence of ARNO-Sec7—Arf1-Mg²⁺-GTP exhibits the resonances of the bound
GTP at −9.58, −15.23, and −4.91 ppm (Fig. 1). In the presence of wild type ARNO-Sec7, no shift changes or line broadenings are observable, indicating that under the concentrations used, the population of the complex with ARNO-Sec7 is very low. Consistently, no GTP is released and observable as free GTP in the spectra. Further evidence is provided by a detailed examination of the corresponding 1H NMR spectra, which can be deconvoluted into the single component 1H NMR spectra of Arf1-GTP-Mg²⁺ and ARNO-Sec7(wt) (data not shown) without showing a sign of the formation of the ternary complex.
The Arf1-Nucleotide Complexes—$^{31}$P NMR spectroscopy of the protein-bound nucleotides can give information about the conformational changes and conformational equilibria involving the phosphate environment. As shown earlier by $^{31}$P NMR spectroscopy, Ras-GDP-Mg$^{2+}$ exists in two conformational states in solution whose relative population can be changed by mutations in the effect loop (Thr$^{28}$) or in the phosphate binding pocket (Gly$^{125}$) (19). There is no evidence that such a conformational equilibrium also exists in the Arf1-GDP-Mg$^{2+}$. However, the resonance shifts corresponding to the two states in Ras are rather small (0.05 ppm for the $\beta$-phosphate resonance of bound GDP). With a total line width of 64 Hz of the $\beta$-phosphate resonance of Arf1-GDP-Mg$^{2+}$, a small splitting of 10 Hz would not be directly detectable even if a second conformational state would exist in Arf1-GDP-Mg$^{2+}$.

For the small G proteins Ran (16), Ras (16, 17), and EF-Tu (14, 18), a strong downfield shift of the $\beta$-phosphate resonance and a moderate downfield shift of the $\alpha$-phosphate resonance of GDP after binding to the protein is typical. Besides shifts caused by the anisotropy of the magnetic susceptibility of nearby residues (e.g. ring current shifts) phosphorus chemical shifts in solution are strongly influenced by torsional strain and polarization effects. This can be seen when the positively charged Mg$^{2+}$ ion, which is bound in a monodentate complex to the $\beta$-phosphate group in the small GTPases, is removed. However, only part of the downfield shift (~25%) is contributed from interaction with the metal ion, since the removal of the ion leads to an upfield shift of the $\beta$-phosphate resonance of only ~0.88 ppm (Table I). If electric polarization effects are mainly responsible for these shifts, one would expect other positively charged groups in contact with the $\beta$-phosphate group. From the x-ray structure, possible candidates can be derived; the main candidate is Lys$^{30}$ in Arf1, which is conserved in small GTPases. Lys$^{30}$ corresponds to Lys$^{30}$ in Ras, Lys$^{29}$ in Ran, and Lys$^{24}$ in EF-Tu.

In Ras as well as in Ran, the complexes with nucleoside triphosphates occur in two conformational states as has been shown by $^{31}$P NMR spectroscopy (15, 16). In Ran, the two states are easy to observe with GTP, where two sets of signals for the bound GTP are observed (16). Especially the $\gamma$-phosphate signals of the two states are well separated by 1.68 ppm. The equilibrium constant between the two states is strongly temperature-dependent; at 278 K, the equilibrium constant $K$ is 0.67, with the state corresponding to the upfield shifted resonance dominating. Such a behavior cannot be observed in the Arf1-GTP-Mg$^{2+}$ complex, where no indication of a second set of resonances corresponding to a second conformational state can be observed. In this respect, it seems to be more similar to Ras, where in Ras-GTP-Mg$^{2+}$ the second conformational state (if existing) is only weakly populated ($K < 0.1$). It is much easier to observe when GTP is replaced by the GTP analog Gpp(NH)p, where $K$ is 1.06. Since this experiment was not important in the present context, which is focused on the interaction with ARNO-Sec7, these data are not available yet for Arf1.

Interaction of Arf1-GDP-Mg$^{2+}$ with ARNO-Sec7—The acceleration of the nucleotide exchange by exchange factors requires a direct interaction of the exchange factor with the G protein. In a general scheme, one must assume that the exchange factor Sec7 has to interact first with Arf1-GDP; nucleotide-free Arf1 is a very minor species, and the exchange factor does not wait for spontaneous GDP release.

In the Ran system, the interaction of the exchange factor RCC1 in the presence of GDP and magnesium (10 mM) was studied in detail (16). At equimolar concentrations of Ran-GDP-Mg$^{2+}$ and RCC1, about 30% of the bound GDP was released from the complex, and a new GDP-containing complex was observed (40% of the total GDP present initially in Ran-GDP-Mg$^{2+}$). Since GDP was released, about 30% of Ran had to be free from nucleotide and had to be distributed between the four different forms, free Ran, Ran-Mg$^{2+}$, Ran-RCC1, and Ran-Mg$^{2+}$-RCC1, coexisting in the chemical equilibrium.

Under similar experimental conditions, we only detect a small release of GDP from Arf1 when wild type ARNO-Sec7 is added in saturating concentrations. About 7% of the bound GDP is released. In contrast, no GDP release could be observed for the glutamic finger mutants of ARNO-Sec7 studied here.

In the Ran system, a new GDP-containing protein complex (that must correspond to Ran-GDP-Mg$^{2+}$ or Ran-GDP complex with RCC1) becomes visible in the presence of the exchange factor with the $\alpha$-phosphate resonance shifted upfield by ~2.17 ppm from the original position and the $\beta$-phosphate resonance shifted upfield by ~0.50 ppm (Table I). Such a large spectral change cannot be observed in the Arf1 system in the presence of ARNO-Sec7(wt) and ARNO-Sec7(E156D), although the complex of Arf1-GDP-Mg$^{2+}$ with ARNO-Sec7 could be identified (see below). However, at intermediate concentrations of Mg$^{2+}$ in the presence of ARNO-Sec7(E156A) and ARNO-Sec7(E156K), a new set of resonance lines becomes visible, which have to be assigned to a complex of Arf1 with the exchange factor. The $\alpha$-phosphate resonance shifted upfield by ~1.3 ppm from the original position in the Arf1-Mg$^{2+}$-GDP complex, and the $\beta$-phosphate resonance is shifted upfield by ~1.24 ppm (E156A) and ~1.38 ppm (E156K) (Table I), respectively. Since the positions of these line are not influenced by the removal of the protein-bound Mg$^{2+}$ by EDTA, they must represent the metal-free complex of Arf1-GDP with ARNO. In analogy, it is likely that the complex observed earlier in the Ran system also represents the magnesium-free Ran-GDP-RCC1 complex, although in the published study the corresponding experiments were not performed.

In the presence of ARNO-Sec7(wt) and ARNO-Sec7(E156D), the lines of bound Mg$^{2+}$-GDP do not shift significantly (Table I) but contain a second component with a line width approximately more than 4 times as large as that of free Arf1-Mg$^{2+}$-GDP; this represents the complex of Arf1-Mg$^{2+}$-GDP with the exchange factor ARNO-Sec7. The affinity determined from the data is in the 0.1 mM range. The line widths in the complex are larger than expected from the molecular mass of the complex in a rigid body approximation and probably reflect an additional exchange broadening. For the two other mutants, such an additional broader component cannot be observed, but the lines of bound GDP are broadened and slightly shifted. This indicates that these mutants also form a complex with Arf1-GDP-Mg$^{2+}$ in concentrations detectable by NMR spectroscopy. Whether the observed lines represent exclusively the complex of Arf1-GDP-Mg$^{2+}$ with ARNO-Sec7 or a mixture of this complex in fast exchange with free Arf1-GDP-Mg$^{2+}$ cannot be decided from the data.

In the presence of EDTA, the lines corresponding to the metal-free Arf1-GDP complex are clearly broadened when ARNO-Sec7 is present, indicating that under these conditions the ternary complex is also formed for ARNO-Sec7(wt) and its mutants.

Our observations are in line with the previously published finding that ARNO-Sec7(E156K) forms abortive ternary complex with Arf1 where GDP remains bound (10). It was described as a first example of an abortive ternary complex between a small G protein, a bound nucleotide (GDP), and a
nucleotide exchange factor. It was suggested to mimic the ternary complex that precedes the dissociation of GDP and the formation of the stable and binary nucleotide-free complex (10). The phosphorus NMR data show that this abortive complex is indeed magnesium-free and that it forms also with the ARNO-Sec7(E156A). In contrast, the wild-type and the mutant protein (E156D) do not form this complex at higher levels of magnesium; however, phosphorus NMR indicates a weak but NMR-detectable interaction with Arf1-GDP-Mg$^{2+}$.

Interaction of Arf1-GTP-Mg$^{2+}$ with ARNO-Sec7—Under similar experimental conditions where in the Ran system the interaction of Ran-GTP-Mg$^{2+}$ with RCC1 exhibited a substantial amount of the bound GTP released from the protein (16), we could not detect a release of GTP from Arf1-GTP-Mg$^{2+}$ by ARNO-Sec7. In addition, we see no effect on the phosphorus line width of Arf1-GTP-Mg$^{2+}$ upon the addition of ARNO-Sec7(wt). In line with these observations, the $^1$H spectrum of a solution containing Arf1-GTP-Mg$^{2+}$ and ARNO-Sec7(wt) can simply be reconstructed as the sum of the $^1$H spectra of the isolated proteins. This indicates again that no specific interaction between the two components is detectable even at the high protein concentrations used (i.e. the affinity for ARNO-sect is very low).

Conclusions—The phosphorus NMR data show that the negative charge of the glutamate residue at position 156 is very important for the interaction of ARNO-Sec7 with Arf1-GDP-Mg$^{2+}$ complexes. At intermediate magnesium concentrations, ARNO-Sec7(wt) and ARNO-Sec7(E156D) interact weakly with magnesium-free Arf1-GDP. After replacing the negative charge at position 156 by introduction of an uncharged (Ala) or positively charged residue (Lys), ARNO-Sec7 shifts the equilibrium to the magnesium-free state and forms a stable complex with Arf1-GDP without releasing GDP. To our surprise, the alanine mutant ARNO-Sec7(E156A) was even more effective in displacing the magnesium ion from the catalytic site than the lysine mutant ARNO-Sec7(E156K) with its long, positive charged side chain. The proposal that Glu$^{156}$ acts as a “glutamic finger” (10) interacting with the Mg$^{2+}$ ion and the $\beta$-phosphate group of bound GDP is supported by our phosphorus NMR data as well as the published crystal structure of the complex (7). ARNO-Sec7 preferentially binds to Arf1-GDP-Mg$^{2+}$ and directly displaces GDP (or GDP-Mg$^{2+}$) but has a low affinity to the metal-free Arf1 complex, since the negative charge of Glu$^{156}$ is here not shielded by the Mg$^{2+}$ ion. Replacing Glu$^{156}$ by Asp reduces the replacement of GDP by the complex formation, since the negative charged side chain is further away from the negatively charged $\beta$-phosphate group of bound GDP. Replacing Glu$^{156}$ by a nonpolar residue (Ala) or a positively charged residue (Lys) reduces the affinity to Arf1-GDP-Mg$^{2+}$ and thereby leads to a preferential binding to Arf1-GDP.

In conclusion, we can hypothesize a dual role for the Glu residue in Sec7. As a first step, the Mg$^{2+}$ ion has to be removed. This can be achieved through a CH$_3$ group and is mimicked by the Ala mutation. An even larger stabilization of the Mg$^{2+}$-free complex can be reached by a substitution through the positively charged amino acid Lys. As a second step, the GDP is removed by the carboxylate ion of Glu$^{156}$.

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Conformational States of the Small G Protein Arf-1 in Complex with the Guanine Nucleotide Exchange Factor ARNO-Sec7

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