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Cu^{2+}-cyclen as Probe to Identify Conformational States in Guanine Nucleotide Binding Proteins

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Supporting Information

ABSTRACT: ^{31}P NMR spectroscopy is a suitable method for identifying conformational states in the active site of guanine nucleotide binding proteins detecting the nucleotide placed there. Because there is no labeling necessary, this method is gaining increasing interest. By ^{31}P NMR spectroscopy two major conformational states, namely state 1(T) and state 2(T), can be detected in active Ras protein characterized by different chemical shifts. Depending on the conformational state Ras shows clearly different physiological properties. Meanwhile analogous conformational equilibria could also be shown for other members of the Ras superfamily. It is often difficult to determine the conformational states of the proteins on the basis of chemical shift alone; therefore, direct detection would be a great advantage. With the use of Cu^{2+}-cyclen which selectively interacts only with one of the major conformational states (state 1) one has a probe to distinguish between the two states, because only proteins existing in conformational state 1 interact with the Cu^{2+}-cyclen at low millimolar concentrations. The suitability was proven using Ras(wt) and Ras mutants, Ras complexed with GTP, GppNHp, or GTPγS, as well as two further members of the Ras superfamily namely Arf1 and Ran.

Guanine nucleotide-binding proteins (GNB proteins) act as molecular switches, being involved in essential cellular signaling pathways regulating cell cycle, transport processes or cell formation (see, e.g., ref 1). It is known, that GNB proteins in the active, GTP-bound form exist in an equilibrium between (at least) two different conformational states. These can be distinguished by different chemical shift values in ^{31}P NMR spectroscopy using, as a probe, the nucleotide which is bound in the active center of the proteins.[2] ^{31}P NMR spectroscopy becomes more and more popular in investigating such conformational equilibria of different members of the Ras-superfamily of GNB proteins such as Ras, Ran, Arf, or Cdc42.[3−5] In Ras proteins this equilibrium is easy to detect in the nucleotide triphosphate bound form (T) because of the separation of the resonances, which correspond to the effector binding state 2(T) and the conformational state 1(T) with low affinity to effectors.[6,7] Recently, we could show by ^{31}P NMR spectroscopy that state 1(T) is primarily recognized by the guanine nucleotide exchange factor, Sos.[8] Crystal structures of nucleotide-free complexes of Ras with Sos[9] of the state 1 mutant Ras(T35S)[5] as well as an NMR-derived structure of state 1 of Ras·GppNHp stabilized by metal-cyclens[10] are available. They all indicate the loss of interaction of the totally conserved threonine in switch I and the γ-phosphate group in the active form.

States 1 and 2 were originally defined on the basis of their ^{31}P chemical shifts with the α- and γ-phosphate resonances of the bound nucleotide shifted downfield in state 1 relative to state 2. In the meantime, the two states are also functionally and structurally defined. However, sometimes it is difficult to conclude from the chemical shifts alone in what state the protein under consideration (another protein of the Ras-superfamily or a mutant protein) actually occurs since chemical shifts can additionally be influenced by mutations or differences in the amino acid composition. It is especially difficult to recognize the conformational state of a Ras-like protein when it exists predominantly in one state. Here, the absolute chemical shift values are not very helpful. What is missing is a fast method for discriminating the two states spectroscopically that we propose in the following.

For Zn^{2+}- and Cu^{2+}-cyclen[10] (Figure 1A) we could show that they bind near the γ-phosphate group in Ras only when it exists in conformational state 1(T).[11] State 1(T) of Ras is also characterized by a more than 100-fold decrease of affinity to effector molecules.[6] It appears reasonable to assume that the paramagnetic Cu^{2+}-cyclen can be used as a general tool to recognize state 1(T) also in other GNB proteins. For a demonstration of this feature we have used the proto-oncogene product Ras, the ADP-ribosylation factor Arf1, and the nuclear Ras-related protein Ran.

Spectra B and C in Figure 1 are ^{31}P NMR spectra of different human H-Ras variants bound to the commonly used stable GTP analogue GppNHp. For Ras(wt) (Figure 1B, bottom spectra) clearly two sets of resonances are obtained. These can be best seen for the α- and the γ-phosphate signals of the bound nucleotide, corresponding to two conformational states that are in chemical exchange with a correlation time in the millisecond time scale at room temperature.[2] Addition of Cu^{2+}-cyclen leads to the disappearance of the Pγ-resonance which correspond to state 1(T), whereas the one representing state 2(T) is unperturbed. As a control the well-studied mutant Ras(T35A) was used because this mutant exists predominantly in conformational state 1 (Figure 1B, upper spectra). After addition of 5 mM Cu^{2+}-cyclen

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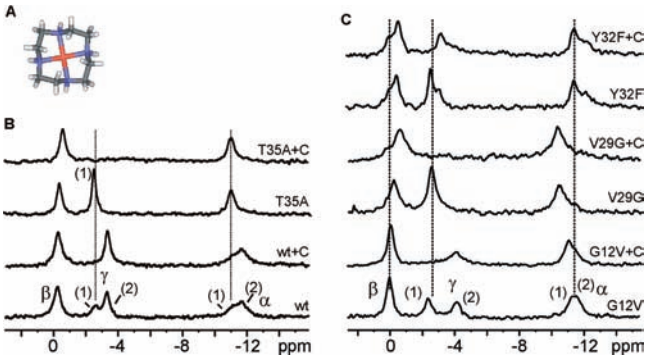

Figure 1. Impact of Cu^{2+}-cyclen binding to Ras·GppNHp. (A) Structure of 1,4,7,10-tetraazacyclododecan (cyclen) copper(II) complex. Chloride was used as a counterion. (B) ^{31}P NMR spectra of 1 mM Ras(wt)·GppNHp in the absence and the presence of 5 mM Cu^{2+}-cyclen. (C) Spectra of selected Ras mutants in the absence and the presence of 5−7 mM Cu^{2+}-cyclen. The phosphorus resonances are assigned to the α-, β-, and γ-phosphate, respectively. The Ras-variant and the presence of Cu^{2+}-cyclen (+C) are indicated.

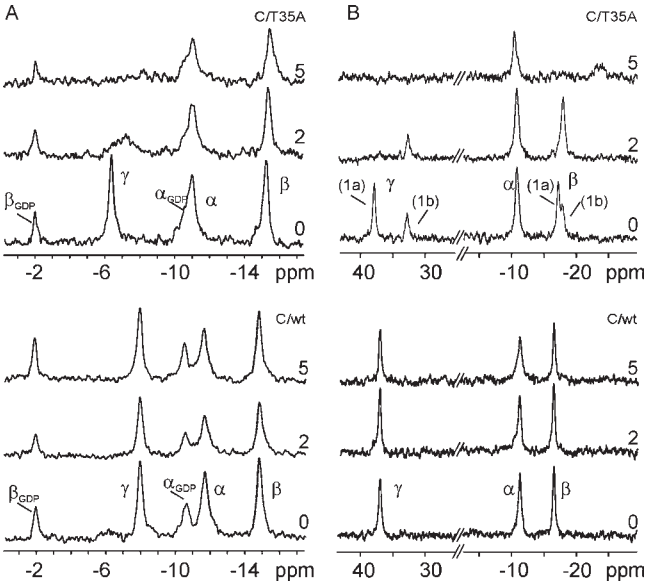

Figure 2. Titration of Cu^{2+}-cyclen to GTP and GTPγS complexes of Ras. ^{31}P NMR spectra of 1 mM Ras(T35A)·GTP (A, top) or Ras(wt)·GTP (A, bottom) and Ras(T35A)·GTPγS (B, top) or Ras(wt)·GTPγS (B, bottom) in the absence and the presence 2 or 5 mM Cu^{2+}-cyclen.

the Pγ-resonance is broadened beyond detection by the paramagnetic relaxation enhancement. In Figure 1C the NMR spectra of exemplary Ras mutants G12V, V29G, and Y32F are shown where the assignment to a conformational state from the chemical shifts is difficult. Mutation of Tyr32 such as in the mutant Ras(Y32F) leads to state-independent chemical shift changes since the ring current shifts due to the tyrosine residue perturbations. By addition of Cu^{2+}-cyclen the assignment to a given state can be unequivocally performed since the Pγ-resonance corresponding to state 1 disappears. This is also true for Ras(G12V) and Ras(V29G).

In addition to the GTP-analogue, GppNHp, the conformational state of complexes with other analogues, such as GTPγS or the physiological ligand GTP, should be able to be addressed by

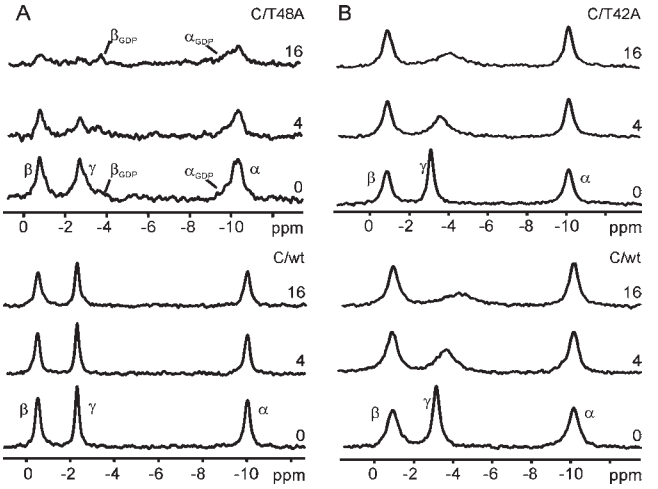

Figure 3. Titration of Cu^{2+}-cyclen to the Ras-related proteins Arf1 and Ran bound to GppNHp. ^{31}P NMR spectra of 1 mM Arf1(wt) and Arf1(T48A)·GppNHp (A) and Ran(wt) or Ran(T42A)·GppNHp (B) in the absence and the presence of 4-fold or 16-fold excess of Cu^{2+}-cyclen. (Top) mutant protein, (bottom) wild-type protein. α_{GDP} and β_{GDP}, signals of bound GDP.

the use of the Cu^{2+}-cyclen method. It was shown that the nature of the nucleotide (analogue) used modulates the conformational equilibria.[2,12,13] Figure 2 presents the obtained spectra for wild-type and (T35A)Ras in complex with these two guanine nucleoside triphosphates. For GTP-bound Ras the expected effects are obtained. Ras(wt)·GTP predominantly exists in the conformational state 2, whereas the alanine mutant is in state 1, which clearly could be demonstrated by the strong line broadening of the Pγ-resonance in the presence of Cu^{2+}-cyclen (Figure 2A).[13] GTPγS bound to wild-type Ras results in only one set of resonances and is assumed to exist predominantly in state 2.[12] In agreement with that, addition of Cu^{2+}-cyclen does not have any effect. GTPγS bound to Ras(T35A) shows two sets of resonance lines, one with chemical shifts close but not identical to those of the wild-type spectrum, and one with clearly different shifts. Functional data suggested that both sets of lines correspond to state 1 and that the line splitting is caused by different possible orientations of the sulfur of the thiophosphate group in the complex.[12] In agreement with this interpretation, addition of Cu^{2+}-cyclen leads to the disappearance of both signals assigned to states 1a and 1b. The signal of the β-phosphate becomes broadened and starts shifting with increasing concentration of Cu^{2+}-cyclen. Interestingly, state 1a is more sensitive than state 1b (Figure 2B). In state 1a the negatively charged sulfur atom can be coordinated by the Cu^{2+}-ion, whereas in the coordination pattern supposedly for state 1b only delocalized electrons are provided for coordination.[12]

For Ras proteins the reliability of Cu^{2+}-cyclen binding to the γ-phosphate of the bound nucleotide as a second marker for state 1 (in addition to chemical shifts) has been confirmed here and in many other examples not shown here. Can one use this probe for other members of the Ras superfamily, too? Having a closer look at the two other proteins tested, a similar behavior can be obtained for Arf1 proteins. Wild-type Arf1 most probably exists mainly in the conformation corresponding to state 2(T) when GTP is bound since it does not interact with Arno-Sec7.[4a] As shown in Figure 3A this is also true when GppNHp is bound because there are no significant effects with the addition

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of Cu^{2+}-cyclen on the resonance of the γ-phosphate. This indicates that the threonine residue of switch I is coordinated to the γ-phosphate group as it is found in the crystal structure.[14] In contrast to the Ras protein it exists completely in conformational state 2 also in complex with the analogue GppNHp. Fast exchange conditions between both states can be excluded, which would also result in a single line in the NMR spectrum. In contrast the Arf1(T48A) mutant (corresponding to T35A in Ras) again shows strong line broadening effects after binding Cu^{2+}-cyclen; thus, at higher concentration the γ- and β-resonances are not detected any more at the signal-to-noise level obtained (Figure 3A). Titration of Cu^{2+}-cyclen to Ran proteins leads to the same effects for wild-type Ran as for the mutant. At the Cu^{2+}-cyclen concentration of 4 mM the resonances of the γ-phosphates are strongly broadened (Figure 3B). The affinity of the M^{2+}-cyclen for Ran seems to be somewhat lower compared to that for Ras. The data would indicate that wild-type Ran and Ran(T42A) exist predominantly in state 1 when complexed to GppNHp. In contrast, when GTP is bound, wild-type Ran exists in a mixture between states 1 and 2.[3]

GNB proteins in the active, nucleoside triphosphate-bound form (T) show an equilibrium of two main conformations. States 1(T) and 2(T) were originally defined solely by their phosphorus chemical shifts when Ras was complexed with $Mg^{2+}\cdot$GppNHp.[2a] Only in later experiments the states were connected with additional structural and functional features: State 1(T) is also found predominantly in the partial loss-of-function mutants Ras(T35S) and Ras(T35A) when $Mg^{2+}\cdot$GppNHp[2] or $Mg^{2+}\cdot$GTP[13] is bound. State 1(T) is recognized by exchange factors and state 2(T) by effectors.[8] This functional definition is, in our opinion, the most important one; the phosphorus chemical shifts are now only surrogate markers for these states. Although chemical shifts contain structural information, they are sometimes difficult to interpret, since the observed chemical shifts are the sum of all effects influencing the local magnetic field at the position of the nuclear spins under consideration. Especially, long-range ring current shifts from aromatic rings may sometimes lead to large state-dependent shift changes. Since in many cases it is practically not feasible to perform the binding experiments with effectors and exchange factors, we propose here an additional, state-dependent surrogate parameter, the interaction of Cu^{2+}-cyclen with the γ-phosphate group of the nucleotide in state 1(T) but not in state 2(T). In the cases where the assignment of the states is known, it works perfectly; in the other cases presented here, it is consistent with the general observation in all cases studied by us. The accessibility of the γ-phosphate group for metal-cyclens in state 1(T) is a structural feature (the loss of the interaction between switch I and/or switch II to the γ-phosphate group) connected with our preferred functional definition but is not verified experimentally for all G proteins existing. It could be imagined that specific properties of the protein environment in state 1(T) would reduce the affinity for the marker substantially, but the probability is not high that both phosphorus chemical shifts and cyclen binding would fail simultaneously.

Thus, the addition of Cu^{2+}-cyclen in low millimolar concentrations of about 2−5 mM is a simple and powerful additional method to distinguish between the two main conformational states observed in different activated GNB proteins by ^{31}P NMR spectroscopy where no further labeling is necessary. The method is independent from mutations, or the nature of the GTP-analogue or GTP used. The equilibrium between the two conformations with clearly different structural and functional properties seems to fulfill regulatory properties and possibly can be used as a target for drug development also for GNB proteins others than Ras.

■ ASSOCIATED CONTENT

Supporting Information. Materials and methods section. This material is available free of charge via the Internet at http://pubs.acs.org.

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