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Optimization and stabilization of Rho small GTPase proteins for solution NMR studies

The case of Rnd1

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Abbreviations: GMPPNP, β: γ-imidoguanosine 5'-triphosphate; NMR, nuclear magnetic resonance; GTP, guanosine triphosphate; GDP, guanosine diphosphate; TEV, tobacco etch virus; LB, lysogeny broth; IPTG, isopropyl β-D-1-thiogalactopyranoside; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CD, circular dichroism; WT, wild type; MW, molecular weight; TFE, trifluoroethanol; TMAO, trimethylamine N-oxide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

Rho GTPases of the Ras superfamily have important roles in regulating the organization of the actin filament system, morphogenesis and migration of cells. Structural details for these proteins are still emerging, and information on their dynamics in solution is much needed to understand the mechanisms underlying their signaling functions. This report reviews conditions for solution NMR studies of Rho GTPases and describes our optimization and stabilization of Rnd1 for such experiments. Rnd1 belongs to the Rnd protein subfamily branch of Rho small GTPases and functions in neurite outgrowth, dendrite development and in axon guidance. However, as we report here, solution NMR studies of this protein are challenging. Multiple methods have been employed to enhance the stability of Rnd1, including by cleavage of an N-terminal His expression tag and by addition of non-hydrolysable GMPPNP (β: γ-imidoguanosine 5'-triphosphate) nucleotide. Further stabilization of Rnd1 against aggregation was achieved through a structure informed point mutation while maintaining its conformation and binding affinity for a partner protein. The NMR spectrum of the optimized protein reveals significant improvement in NMR signal dispersion and intensity. This work paves the way for structural and protein-protein/protein-ligand interaction studies of Rnd1 by solution NMR and also provides a guide for optimization and stabilization of other Rho GTPases.

Introduction

Rho GTPases of the Ras superfamily regulate diverse cellular and developmental events including cell morphology, cell to cell interaction, cell migration and adhesion.[1,2] Abnormalities in Rho GTPase function have vital consequences on the reorganization of actin cytoskeleton, such as an increase in cell migration seen in cell metastasis and tissue invasion that are part of cancer progression.[3,4] Of the 22 vetebrate Rho GTPases, the structures of 12 have been determined by X-ray crystallography, in part due to intensive efforts by the Structure Genomics Consortium. Often structures are available for the inactive (GDP bound) as well as for the active (GTP bound) form, although in the latter the nucleotide is typically exchanged by a non-hydrolysable analog, such as GMPPNP or GTPγS. So far only three Rho GTPases, i.e., Cdc42,[5-12] Rac1,[13-18] RhoA,[19-21] have been extensively studied by solution NMR. A summary of the expression and purification of these proteins, as well as the concentration and buffer condition of the NMR samples, is given in **Table 1** and is further discussed at the end of the paper (section 3.7). All three proteins give high quality spectra under appropriate conditions. It is possible that the GTPases that have been most widely utilized in biological settings are also the ones that are the most well behaved in solution. NMR studies of other Rho GTPases could be more challenging, as this report on the optimization and stabilization of Rnd1 for solution NMR illustrates.

Rnd1 is one of three Rnd proteins, Rnd1, Rnd2 and RhoE/Rnd3, which are a subset of the Rho family. Rnd proteins exhibit unusual properties in that they bind but (with possible exception of Rnd2) do not hydrolyze GTP (guanosine triphosphate) at an

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Table 1. Summary of protein expression, purification, concentrations and buffer condition of Rho GTPases Cdc42, Rac1, RhoA and Rnd1

Rho GTPase[a]	Description/ [Reference]	expression	protein concentraton	temp	buffer
Cdc42	Cdc42-GDP Cdc42-GMPPCP[5,6] Cdc42-GTPγS [7]	1-187, GST tagged (GST cleaved after purification, leaving GSLSLIISA-N-terminal addition)	0.8 mM	25°C	5 mM Na.phosphate buffer (pH 5.5),[b] 25 mM NaCl, 5 mM $MgCl_2$, 1 mM NaN_3
Cdc42 (T35A)	active switch mutant of Cdc42[8,9]	1-187, His_6-tagged (His_6 cleaved after purification)	0.2–0.5 mM	25°C	10 mM Na.phosphate buffer (pH 5.5),[b] 25 mM NaCl, 5 mM $MgCl_2$, and 1 mM NaN_3
Cdc42 (F28L)	Cdc42-GDP [9]	1-187, His_6-tagged (His_6 cleaved after purification)	0.2–0.5mM	25°C	10 mM Na.phosphate buffer (pH 5.5),[b] 25 mM NaCl, 5 mM $MgCl_2$, and 1 mM NaN_3
Cdc42 (Q61L, ACK bound)	Cdc42-GMPPNP [10]	1-184, His_6-tagged (His_6 cleaved after purification)	~1 mM		5 mM Na.phosphate buffer (pH 5.5), 25 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 1mM GMPPNP
Cdc42 (WASP bound)	Cdc42-GMPPCP [11]	1-179, untagged	~1 mM	25°C	25 mM Na.phosphate buffer (pH 5.5), 25 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT
Cdc42 (Q61L, PAK bound)	Cdc42-GMPPNP [10]	1-184, His_6-tagged (His_6 cleaved after purification)	~1 mM	25–30°C	5 mM Na.phosphate buffer (pH 5.5), 25 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT
Cdc42 (SoPE2 bound)	Cdc42-GDP [12]	1-184, His_6-tagged (His_6 cleaved after purification)	1.3 mM		20 mM Na.phosphate buffer (pH 5.5), 50 mM NaCl
Rac1	Rac1-GDP [13]	residues 1-188, C178S, no tag	0.5–0.7 mM	25°C	50 mM Tris maleate buffer (pH 6.8), 50 mM NaCl, 20 mM $MgCl_2$, 10 mM DTT, 0.1 mM GDP, 0.1% sodium azide
	Rac1-GMPPNP [14]	residues 1-184, C178S, no tag	0.8 mM	25°C	"physiological buffer" (pH 6.8), 4 mM DTT, 4 mM $MgCl_2$
Rac1 (Q61L, PRK1 bound)	Rac1-GTP [15]	residues 1-191, GST-tag (cleaved after purification)	1 mM	25°C	20 mM Tris-HCL (pH 7.4) 20mM NaCl,
Rac1 (RhoGDI bound)	Rac1-GDP [16]	Residues 1-191 no tag	0.3 mM	15°C	50 mM Na.phosphate buffer (pH 6.3), 50 mM NaCl, 1 mM DTT, 5mM $MgCl_2$
Rac1 (Q61L Plexin RBD bound)	Rac1-GTP [17,18]	residues 1-184, C178S, no tag or His_6-tag	1 mM and vars. ratios	25°C	50 mM Na.phosphate buffer (pH 6.8), 50 mM NaCl, 1 mM DTT, 5mM $MgCl_2$
RhoA	RhoA-GDP [19] RhoA-GTPγS	residues 1–181, His_6-tag (cleaved after purification)	0.3 mM	20°C	20 mM HEPES buffer (pH7.0), 100 mM NaCl, 5 mM $MgCl_2$, 2 mM TCEP
	RhoA-GDP [20]	residues 1–181, His_6-tagged	0.9 mM	25°C	25 mM Tris-HCl buffer (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT
RhoA (DH-PH bound)	RhoA-nucleotide free [21]	residues 1–181, His_6-tagged	0.3 mM	25°C	200 mM MOPS/Tris buffer (pH 7.5), 1 mM DTT
Rnd1 (plexin RBD bound)	Rnd1-GTP [17,18]	residues 5-200, His_6-tag (cleaved after purification)	1 mM and vars. ratios	25°C	50 mM Na.phosphate buffer (pH 6.8), 50 mM NaCl, 1 mM DTT, 5mM $MgCl_2$
Rnd1	Rnd1-GMPPNP [this report]	residues 5-200, W66L mutant, His_6-tag (cleaved after purification)	0.54 mM	25°C	20 mM Tris-HCl (pH 7.0), 50 mM NaCl, 1.0 mM TCEP, 4 mM $MgCl_2$, 0.01% NaN_3

Full length proteins are Cdc42 (res. 1-191), Rac1 (res. 1-191), RhoA (res. 1-210), Rnd1 (1-223); 1 mM DTT was used in lysis buffer

appreciable rate. Multiple functions have been revealed for Rnd1, such as its role in the regulation of neurite outgrowth, dendrite development, and axon guidance.[22-24] Lately, a crystal structure was solved for Rnd1 (pdb id: 2CLS) and of several of its complexes with an effector of interest, the Rho GTPase binding domain of plexin.[25,26] This provided important structural insights for this protein, but further studies are needed in order to understand the structure-dynamics-function relationship of Rnd1 and how the protein participates in cell signaling mechanisms. Solution NMR spectroscopy has become a versatile and key tool for the characterization of protein structure in solution, protein dynamics and also for the delineation of biomolecular interactions.[27,28] For example, NMR relaxation measurements suggest an allosteric mechanism for cell signaling when plexin is bound to the Rho GTPase, and Rnd1 homolog, Rac1.[14] Compared to Rac1 thermodynamic measurements and molecular dynamics simulations also reveal a likely different model for Rnd1 GTPase binding.[18]

By contrast to the three Rho GTPases studied previously by solution NMR, this is the first direct study of Rnd1 by NMR and we found this protein more challenging to work with. This is due to several problematic features, such as its relative long-term instability and its tendency to aggregate. Similar problems were not reported in the crystallization of the protein or its complexes;[25] however, the crystallization conditions are far from physiological. We utilized multiple approaches to address these problems for solution NMR; specifically, we combined the cleavage of the His tag with the screening of buffer conditions and introduction of point mutations in the protein.[29-32] Previously, we

presented a strategy for the selection of sites to be mutated that only relied on NMR and sequence data.[31] However, here the crystal structure of a dimeric form of Rnd1 is available and guided our approach. After optimization, a well behaved Rnd1 protein was obtained in solution and the NMR spectrum was remarkably improved compared with the spectra before optimization. Importantly, ITC measurements showed that the mutant protein retains binding affinity for one of its binding partners, the plexin-B1 Rho GTPase binding domain. Successful optimization and stabilization of Rnd1 achieved here forms a basis for future detailed structural, dynamical and functional investigations in solution by NMR. It also provides a useful guide for the study of other Rho GTPase proteins.

Materials and Methods

His tagged Rnd1 protein expression, purification. The plasmid encoding the Rnd1 GTPase (residues 5–200) was a gift from Dr. Declan Doyle, formerly at the Structural Genomics Consortium at Oxford. The gene had been PCR-amplified and inserted into the pLIC-SGC1 vector (similar to pet15 of Invitrogen). Mutants were made using the QuikChange Lightning site directed mutagenesis kit (Agilent). Plasmids were transformed into *E. coli* BL21 cells, which were inoculated in 5 ml LB (Lysogeny Broth) medium at 37°C and subsequently grown in 0.8 L LB containing 1mM ampicillin at 37°C. When the cell culture reached an OD_{600} of 0.6–0.8, protein expression was induced with 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) at 25°C overnight. Induced cells were harvested, centrifuged at 4,500 G for 20 min at 4°C. The pellets were re-suspended to 25 ml in Tris buffer (20 mM Tris-HCl, 500 mM NaCl, 1.0 mM TCEP (Tris (2-carboxyethyl)phosphine hydrochloride), 4.0 mM $MgCl_2$, pH 8.0). Just before sonication, standard protease inhibitors were added. Following sonication, lysates were centrifuged at 30,000 G for 30 min at 4°C. The protein was purified from the supernatant by use of 1 ml of Ni-NTA Agarose (Qiagen) beads. Following 2 h of binding at 4°C, the beads were washed twice for 20 min with a buffer containing 20 mM TRIS-HCl, 500 mM NaCl, 30 mM imidazole, 1.0 mM TCEP, 4 mM $MgCl_2$, pH 7.5. Proteins were eluted from beads using 5 mL elution buffer containing 20 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, 1.0 mM TCEP, 4 mM $MgCl_2$, pH 7.5. The purity of proteins was judged by 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). ^{15}N uniformly labeled proteins were expressed in *E. coli* BL21 grown in M9 media.[15,33] NH_4Cl and ^{13}C D_6-glucose was included as the sole nitrogen and carbon source respectively. Partial deuteration of the protein was accomplished by growing the bacteria in medium as above, but where H_2O was replaced by 100% D_2O.

His tag cleavage from the Rnd1 protein. The construct expressing tobacco etch virus protease (TEV) was purchased from ACC (order number MBA-145). Expression and purification of TEV protein followed an established protocol.[34] After purification, both TEV and His tagged Rnd1 were dialyzed into a buffer suitable for cleavage, consisting of 20 mM Tris-HCl, 500 mM NaCl, 10 mM imidazole, 1.0 mM TCEP, 4 mM $MgCl_2$, pH 7.5. Then the TEV protease was mixed with His tagged Rnd1 at a molar ratio of 1:4 (TEV/Rnd1) and 8% (v/v) glycerol was added. After gentle rocking for 14–15 h at 4°C, the solution was loaded to Ni-NTA Agarose (Qiagen) beads. This binding was performed for 30 min to remove TEV and partially undigested Rnd1; then the slurry was briefly centrifuged at 4°C, leaving the untagged Rnd1 in the supernatant. The efficiency of cleavage was monitored using SDS-PAGE to be $> 90\%$.

NMR measurements. NMR experiments were performed at 298K on a Bruker Avance 800 MHz spectrometer equipped with a TXI cryoprobe. Samples were prepared in the buffer at pH 7.0 containing 20 mM Tris-HCl, 1.0 mM TCEP, 50 mM NaCl, 4 mM $MgCl_2$, 0.01% NaN_3 and 10% D_2O. The concentrations of proteins for HN-TROSY experiments were 250 μM. Concentrations of proteins for the 3D HNCO experiment were 540 μM. All data were processed with NMRPipe[35] and analyzed with the program Sparky.[36]

Calorimetric measurements. Calorimetric measurements of binding between Rnd1 and an effector protein, plexin-B1 RBD, were performed using an isothermal titration microcalorimeter (VP-ITC, MicroCal). Both the proteins were exchanged with identical buffer prior to the experiment using dialysis in phosphate buffer, pH 7.0 composed of 50 mM NaCl, 4 mM $MgCl_2$, and 1 mM TCEP. Isothermal titration calorimetry (ITC) measurements were performed at 25°C. Rnd1 was placed into a temperature controlled cell (volume 0.20 mL) and the second protein plexin-B1 was placed into a syringe (capacity 0.04 mL) for injection into the cell. The concentration of Rnd1 was 40 μM and the concentration of plexin-B1 was 400 μM. The data were analyzed using Origin software package supplied by MicroCal. An offset due to a small dilution effect is constant and was subtracted. Based on the concentrations of the titrant and the sample, the software used a nonlinear least-squares algorithm to fit the series of heat flows to an equilibrium binding equation. The best fit was optimized, providing values for the stoichiometry (molar ratio) of the interaction (N), enthalpy of binding (ΔH) and binary equilibrium binding constant K_a [$K_a = 1/K_d$ which is equal to (Rnd1.Plexin) / (GTPase) x (Plexin)]. The free energy of binding (ΔG) is determined from the well-known equation, $\Delta G = -2.303$ RT log K_a and the entropy change (ΔS) upon binding is determined using Gibbs-Helmholtz equation, $\Delta G = \Delta H - T\Delta S$.

Results and Discussion

High yield expression and purification of His tagged Rnd1 protein. Well expressed and highly purified proteins are important for in vitro studies of protein structure and dynamics. Solution NMR usually requires concentrations in the 0.2–1.0 mM range at volumes of 0.2–0.5 mL. Here, we expressed recombinant N-terminally His tagged Rnd1 in *E. coli* bacteria in both LB and M9 medium supplemented with $^{15}NH_4Cl$. A relatively large volume of buffer (5 mL) was used for elution from Ni-NTA beads in order to avoid high concentrations of the protein since the protein was more prone to aggregation with the His tag. In total, 5 ml of unlabeled and 2 ml ^{15}N labeled proteins were produced at 200 μM concentration after purification from

0.8 L of *E. coli*. The purity of protein was greater than 95% as analyzed by SDS-PAGE. Both yield and purity of Rnd1 was adequate and appeared favorable for NMR studies (~12 mg/L for ^{15}N labeled and ~5 mg/L for ^{15}N, ^{13}C and for ^{2}H, ^{15}N, ^{13}C labeled protein).

Optimization of solution conditions. The dispersion of amide signals in the 2D HN-TROSY NMR spectrum of Rnd1-His is as would be expected for a well folded protein (several resonances are seen at 10 ppm, others are seen down to 7 pm, **Fig. 1**). However, an uneven distribution of peak intensities and signal line widths were observed and many resonances appear to be missing; less than half of the expected 208 signals—main chain amides and NH_2 side chains- are seen (the total number of signals is calculated as 196 amides, i.e., 208 residues—13 Prolines—1 N-terminus + 2 Trp side chain NH + 3 Asn NH_2 + 9 Gln NH_2). Some of the strongest peaks are likely to arise from an overlapping of resonances from residues, which have identical chemical environments. Broad lines can arise from intermediate internal protein dynamics, as well as from an exchange between monomers and dimers or higher order oligomers.[37,38] Beyond this transient aggregation, a considerable precipitation (approx. 40% of total protein) was seen at the bottom of NMR tube after 2–3 d at 25°C. Accordingly, repeat spectra recorded after this time showed that NMR signals were significantly diminished. These observations indicated that His tagged Rnd1 was unstable in solution, possibly because of partial unfolding followed by oligomerization and gradually by subsequent aggregation.

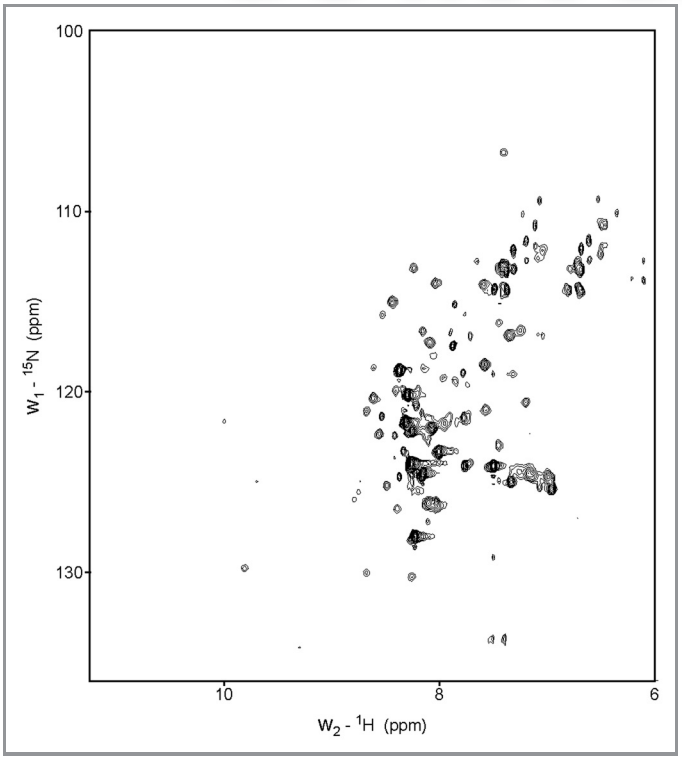

Figure 1. Two dimension HN-TROSY spectrum (24 scans) of N-terminally His-tagged Rnd1 at 250 μM, 298K.

A number of solution NMR parameters may be optimized to give a better spectra: temperature is the most easily changed condition. A higher temperature leads to faster global protein motion and typically improves spectral line widths, but may also increase protein unfolding, and thus, aggregation. Depending on pH (see below), higher temperature may cause line broadening due to the exchange of solvent accessible amide hydrogens with those of the solvent. Lower temperature has the opposite effects. In the case of Rnd1, the protein is large enough so that additional line width, due to lowering the temperature, is problematic. At the same time, higher temperatures lead to increased aggregation and precipitation (data not shown). Sample pH can be varied in principle but amide hydrogen exchange (above pH ~7.5) and proteins unfolding at extremes of pH are often complicating factors. In the case of Rnd1, we wanted to stay near physiological pH, as the purpose of the project was to study the protein's functional interactions with other binding partners. Buffer conditions may be varied, e.g., buffer type and salt concentration. In another study in our lab, on the Rnd1 homologous GTPase Rac1 we found that a physiological buffer[39] offered advantages.[14] However, this was not the case for Rnd1 and the protein gave very similar spectra in three different kinds of buffer: Tris-HCl buffer, phosphate buffer and physiological buffer containing the same sodium chloride concentration (data not shown). A typical phosphate buffer was chosen. Cosolvents, such as structure stabilizing TFE (2, 2, 2-Trifluoroethanol),[40] TMAO (trimethylamine N-oxide)[41] or CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate)[42] may be added in small amounts (< 5–10% v/v). There are some examples where these solvents help to dissociate proteins but the effect of these solvents on protein-protein interactions in general has not yet been thoroughly investigated and these solvents did not improve spectra of Rnd1 (data not shown).

Cleavage of His tag and addition of GMPPNP stabilize Rnd1. Protein aggregation can critically affect protein conformation, function and activity. Usually, His tags are thought to have a negligible effect on protein structure and function. But, several papers reported that His tags do have effects on occasion, including on protein expression level, protein solubility and also on protein binding with target proteins.[43,44] We thus hypothesized that the His tag may promote the oligomerization, aggregation and substantial instability of Rnd1. In our study, the pLIC-SGC1 vector used for Rnd1 expression has a His_6 tag; this 22 residue N-terminal expression tag also contains a TEV (tobacco etch virus) protease cleavage site. Following the protocols described for this protease,[34] the His tag was removed from the protein. The NMR HN-TROSY spectrum was acquired (**Fig. 3A**) and compared with that of the His tagged protein (**Fig. 1**). An increased number of peaks was seen (approximately an additional 90 resonances) upon tag removal. Especially the line width of some of the more dispersed signals was improved. However, gradual precipitation continued to be a serious problem after several days at room temperature. We further hypothesized that the aggregation/precipitation may be due to thermodynamic instability of the protein fold. Small GTPases are known to become unstable under a range of circumstances that principally arise from the oxidation

of thiol containing side chains and the formation of inter and intra molecular disulfides and/or from loss of nucleotide cofactor. The former is prevented by inclusion of reducing agents, such as 1 mM TCEP in our case. The latter is usually prevented by inclusion of 4 mM $MgCl_2$ in all buffers. The remaining precipitation of the protein raises the possibility that Rnd1 slowly hydrolyzes GTP to GDP over a long time at room temperature and that the GDP bound state is aggregation prone (e.g., GDP may be lost more easily than GTP from Rnd1). In order to prevent both scenarios we added a non-hydrolysable analog, GMPPNP at 0.5 mM concentration to the protein in the latter stages of the purification (it was not possible to "load" Rnd1 with nucleotide using a protocol that is typically employed for Rac1, Cdc42 and RhoA—see section 3.7 for details). In structural biology studies, this analog of GTP has become particularly popular since long-term stability of the protein-nucleotide complex is required in order to grow homogeneous crystals or to obtain a single state of the protein.[45,46] Addition of GMPPNP to the solution combats several potential mechanisms and did result in a marked improvement in the long-term stability of Rnd1 samples. Spectra of Rnd1 with GMPPNP added did not change with time, suggesting that the exchange of GMPPNP for GTP does not cause a significant perturbation to chemical shifts.

Point mutations to solubilize the protein and NMR characterization of mutants. Although His tag cleavage and addition of GMPPNP improved Rnd1 stability, nevertheless, the HN-TROSY spectrum still showed crowded regions with broad resonances, especially in the center area (**Fig. 3A**). The broad resonances are most likely due to transient protein association. Protein-protein association interfaces are dominated by certain residue types (Trp, Tyr and Arg).[47] Previously, we had proposed a strategy for the selection of sites that when mutated may disrupt the association.[31] However, this approach was devised for cases where only NMR data and sequence information was available. Recently, the crystal structure of Rnd1 was solved (pdb id: 2CLS), showing that the protein is a dimer in the crystal lattice and we could, therefore, use a structure based approach (see refs.48 and 49 for other examples). The dimerization interface is apparent in the asymmetric unit of the crystal cell and is composed of both non-polar and polar contacts. These mainly involve residues V46, F47, E48, residues Y73, Y74, D75, N76, V77 and also aromatic residue W66 (**Fig. 2A and B**). Residues V46, F47, E48 are located at the C-terminus of the GTPase switch I region and Y73, Y74, D75, N76, V77 are located in the center of the switch II region. Both switch I and II regions are crucial for interactions and functions of most small GTPases. Thus, dimer formation, if indeed physiological, would oppose the biological function of Rnd1. In order to better study Rnd1 in solution, we wanted to design point mutations that would disrupt Rnd1 dimer formation.

Three representative mutants are discussed here: W66L, Y74S and N76R. W66 is situated at the side of the Rnd1 dimer interface, Y74 is located at the central part of the dimer interface, and N76 is placed at one end of the dimer interface. W66 contributes to the hydrophobic interaction by stacking with the aromatic ring of residue F47. It also forms Van der Waals interactions with the side chain of nonpolar residue V77. Residues

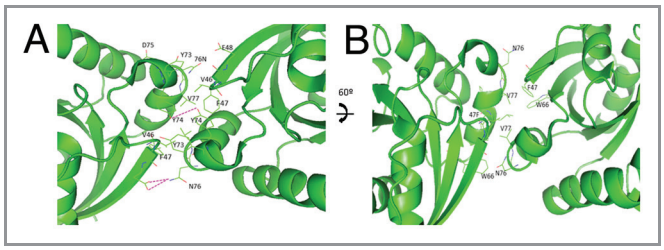

Figure 2. Dimerization interface of Rnd1 from the crystal structure [pdb id: 2CLS]. Left panel, ribbon diagram of the structure shows the binding interface, with major residues involved shown as sticks. Magenta dashed lines indicate hydrogen bonds. Right panel, structure of left panel was rotated 60° around the X axis to show more clearly the interaction of W66 and the hydrophobic cavity.

W66, F47, V77 in one chain, and the same residues in the other counterpart chain, together form a hydrophobic cavity (**Fig. 2B**). Mutation of W66 to L will greatly decrease this hydrophobic interaction. According to the crystal structure, the hydroxyl group of Y74 forms a hydrogen bond with the hydroxyl group of Y74 in the counterpart Rnd1. It also contributes to hydrophobic interaction with its side chain being packed in a cluster of aromatic rings: Y73, F47 in the same chain of the dimer and also F47, Y73 in the other chain (**Fig. 2A**). Mutation of Y74 to S will diminish the hydrophobic interaction due to loss of the aromatic ring. It may also cause a loss of a hydrogen bond to the side chain hydroxyl due to the significant change in side chain length. The second residue, N76 forms multiple hydrogen bonds with the F47 main chain carbonyl oxygen and the side chain oxygens of E48 (**Fig. 2A**). When N76 is mutated to R, the residue can in principle form a salt bridge and also more hydrogen bonds, but in practice Arg has a longer side chain than Asn and this is likely to lead to steric clashes.

In the future, we want to study the change in protein stability and dynamics upon complex formation between Rnd1 and binding partners, such as plexin Rho GTPase binding domains. Thus, the mutations should not strongly interfere with the binding of this effector. However, since much of the interface is shared between Rnd1 dimerization and plexin Rho GTPase binding, the structure of Rnd1 in complex with the Rho GTPase binding domain of plexin-B1 (pdb id: 2REX) also guided our choice of mutants. Specifically, in this structure W66 makes a weak (distant) interaction across the interface with plexin-B1 RBD residue H1804 of RBD. Y74 makes no interaction with RBD, while the N76 side chain NH_2 group forms an H-bond with main chain carbonyl of plexin-B1 RBD residue H1838. This is, in a sense, a negative control as this interaction is likely to be disrupted upon mutation to Arg.

Efficient expression and purification of mutants, and also TEV digestion of the N-terminal His-tag, was possible to a similar level as for the wild-type protein. HN-TROSY spectra were acquired and compared with those of the wild-type Rnd1. Both mutants W66L and Y74S, which were supposed to decrease the interaction between two monomers in the dimer, showed better spectra than wild-type Rnd1. A slight improvement of the crowded areas in the wild-type spectrum was observed (**Fig. 3B and C**). A close-up

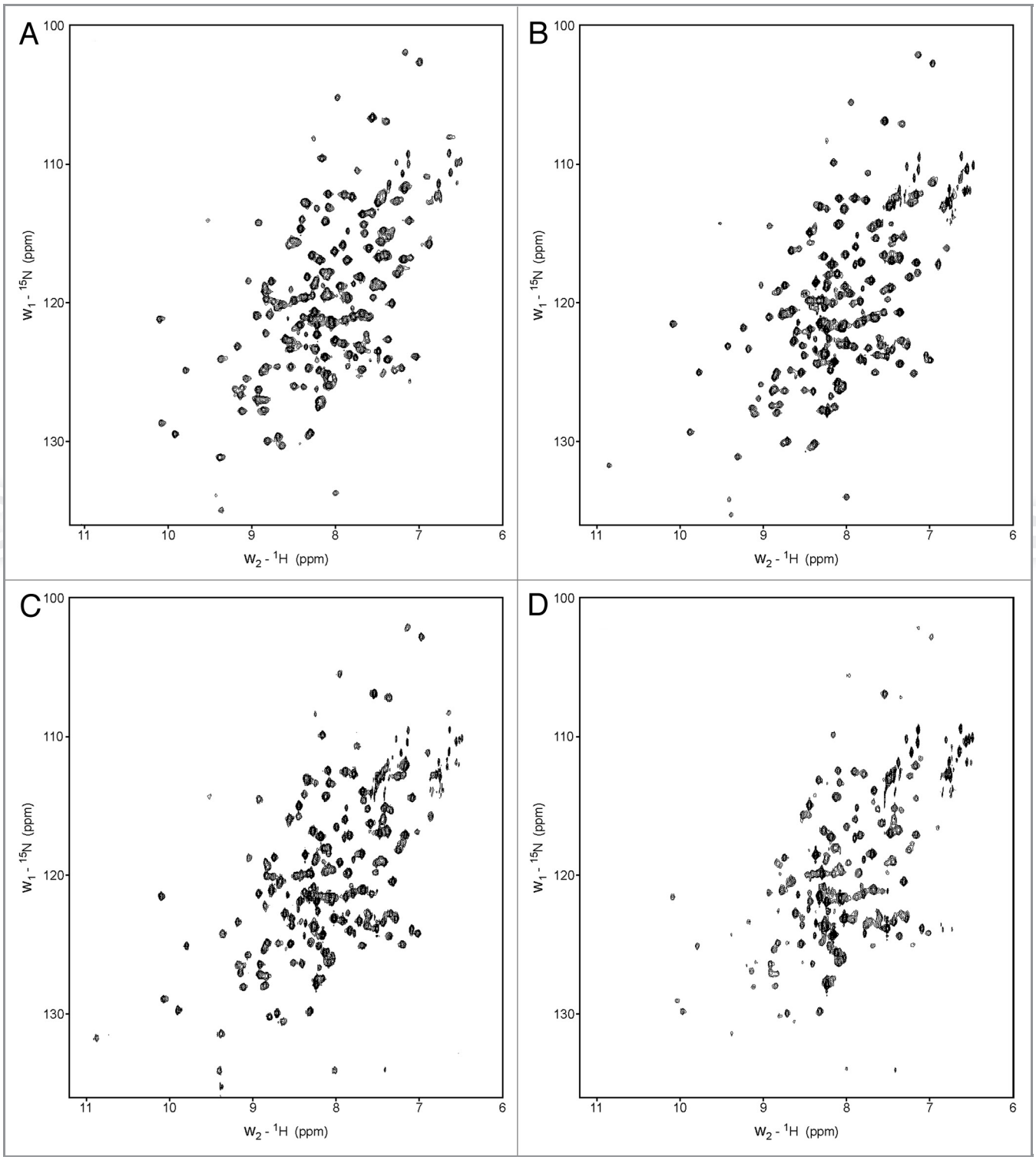

Figure 3. NMR HN-TROSY spectra of Rnd1 wild type and mutants, protein concentration 250 μM run for 24 scans at 298K. (A) Rnd1 wild type, (B) W66L, (C) Y74S and (D) N76R.

view revealed that the spectrum of W66L is better than that of Y74S. Compared with Y74S, the W66L mutant is also a better choice since it more severely changes the non-polar character of the interface, which appears to be more critical for Rnd1 homodimerization than for binding to plexin-B1 RBD. Mutant N76R, gave worse spectra than wild type (**Fig. 3D**). Thus, the Rnd1 mutant W66L was selected for further studies. His-tag cleavage, addition of non-hydrolysable GTP analog, and the

W66L residue change did not result in an appreciable change in protein secondary structure or stability at low protein concentration (20 μM), as monitored by far UV Circular Dichroism (data not shown). However, the combination of these measures nearly completely abolished Rnd1 aggregation/precipitation at NMR concentrations over long periods of time.

Binding between W66L Rnd1 and the plexin-B1 RBD. The overall dispersion and position of the majority of resonances is not affected by the W66L mutation, indicating that the mutation does not appreciably perturb the structure of the protein (**Fig. 3A and B**). However, at the same time we need to ensure that the mutation does not adversely affect the function of the protein. One function of interest is the binding of Rnd1 to its effector, the RhoGTPase Binding Domain (RBD) of plexin-B1 in this case, which is easily studied by ITC measurements (as described in Materials and Methods). ITC showed binding with a stoichiometric ratio N of 0.98. Ka was calculated as 2.09×10^5 M^{-1} (fitting error: 1.98×10^4), and K_d as 4.8 ± 0.5 μM (**Fig. 4**). This dissociation constant is comparable with binding between plexin-B1 RBD and wild type Rnd1, with a K_d of 5.5 ± 0.4 μM.[18] However, the binding enthalpy and entropy contributions are altered by the mutation. The interaction between Rnd1mt W66L and plexin-B1 RBD showed an enthalpy change (ΔH) of -3.9 ± 0.1 kcal/mol and an entropy change*temperature (TΔS) of 3.4 ± 0.2 kcal/mol. This compares with a ΔH of -6.7 ± 0.3 kcal/mol and TΔS of 0.5 ± 0.2 kcal/mol for the interaction between wild-type Rnd1 and the plexin-B1 RBD. Thus, the W66L mutation decreases the favorable enthalpy contribution to binding (L66 is likely to form fewer contacts in the complex) but increases the favorable entropy to binding, possibly because hydrophobic surface area, previously sequestered in a dimeric form, now becomes buried on a transition from monomer to bound Rnd1. Binding of Rnd1 mutant W66L and plexin-B1 RBD was also studied by NMR techniques. A comparison of HN-TROSY spectra of the free and bound Rnd1 protein is shown in **Figure 5**. A shift and/or intensity change of the resonance indicates that the residues are affected by binding of the Plexin effector domain.

Deuteration improved ^{13}C resonance intensity and cross-peaks. The development of heteronuclear triple resonance NMR experiments, in conjunction with the enrichment of proteins with NMR active ^{13}C and ^{15}N isotopes, has dramatically increased the scope of the NMR method for structural studies of biological macromolecules.[50] However, compared with proteins of a molecular weight less than approximately 20 kDa, NMR of larger proteins is still more challenging. This arises from multiple aspects: crowded spectra due to a larger number of resonances and broad lines/low resonance signal-intensity due to faster relaxation. An attractive approach is the replacement of protons by deuterium in order to decrease the efficiency of ^{13}C relaxation due to their attached ^{1}H. The application of deuteration for protein assignment and structure determination has been explored in several

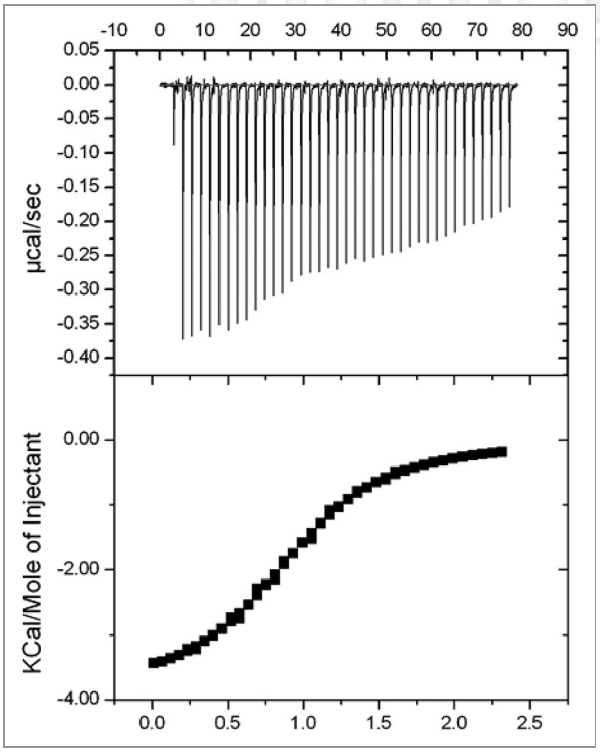

Figure 4. Binding between Rnd1 mutant W66L and Plexin-B1 RBD. (A) Binding isotherm and (B) fitted data for the association from isothermal titration calorimetry (ITC) in phosphate buffer pH 7.0 with 50 mM NaCl, 4 mM $MgCl_2$, and 1 mM TCEP at 25°C.

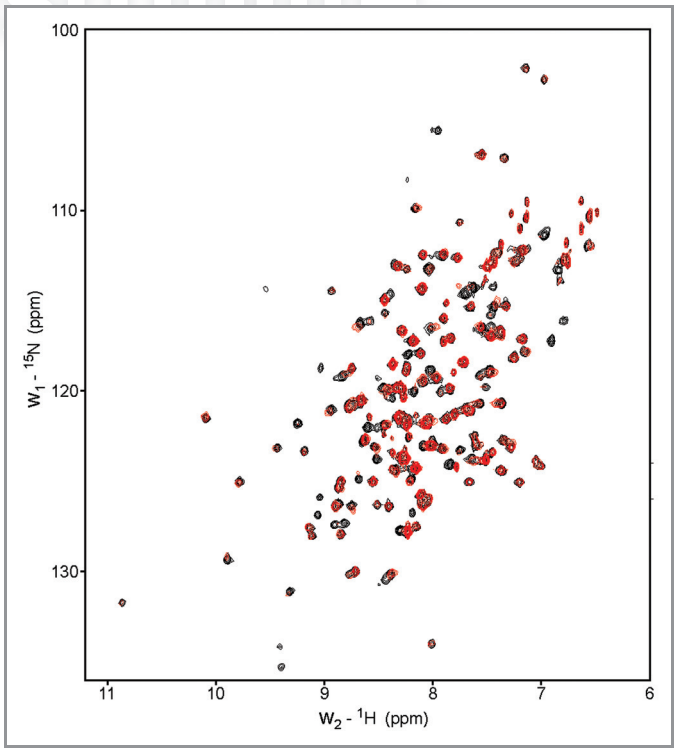

Figure 5. Superimposed NMR HN-TROSY spectra of Rnd1 mutant W66L and its Plexin-B1 RBD bound state. Free form of Rnd1 mutant W66L (black) and bound to Plexin-B1 RBD (red) in phosphate buffer pH 7.0 with 50 mM NaCl, 4 mM $MgCl_2$ and 1 mM TCEP at 25°C.

publications.[50-54] Results proposed that, while 100% deuteration gives maximum sensitivity for backbone assignment experiments HNCOCA and HNCA,[53,54] maximum sensitivity for side chain assignment experiments were achieved with 50% deuteration.[52] For NOESY experiments, deuteration affects HN-HN, HN-HC and HC-HC cross peaks in different ways,[55] but 50% is a useful compromise. So 50% deuteration is an appropriate and useful way for backbone and side chain assignments of proteins up to 30 kDa, while maintaining good signal intensity for $^{1}H/^{1}H$ NOEs.

In this study, first a 3D HNCO experiment was performed with the ^{15}N, ^{13}C labeled protein. The signal to noise ratio was poor and a large number of peaks were missing. We then switched to the triple labeled protein. Labeling is accomplished by growing the protein in 100% D_2O with ^{1}H-glucose. After growing in D_2O medium, amides that are accessible will be back-exchanged to ^{1}H when the protein is purified in normal aqueous solution. For some highly stable proteins, unfolding and refolding protocols need to be employed to allow back-exchange and growing protein with ^{2}H-glucose is more preferable. For Rnd1 this is not an issue since the protein is relatively unstable and undergoes local and possibly transient global unfolding. In this way, many of the normal NH-based experiments can be performed on triple-labeled proteins. The incorporation percentage of deuterium was estimated by mass spectroscopy, confirming that ~50% percent of protons were deuterated. HNCO spectra were acquired for both double labeled and triple labeled Rnd1 mutant W66L (**Fig. 6**). Significant improvement was observed after partial deuteration. This result builds a robust basis for future studies of the Rnd1 GTPase by solution NMR.

Comparison with other solution NMR studies of Rho GTPases and general guidelines. Only 3 of 22 Rho GTPases have been extensively studied by NMR to date, suggesting the possibility that study of the rarer and functionally refined GTPases may be difficult in solution, as their stability could be associated with particular environments. It is interesting to note, that although the GTPases are thought to primarily function at the plasma membrane (to which they are anchored by their modified C-termini),[56] study of truncated forms has been relatively straightforward for the near ubiquitous family members, Cdc42, Rac1 and RhoA. These proteins can also exist in the cytoplasm, where their lipidated tail requires protection by RhoGDI proteins.[16,57] In the case of Rnd1, and likely many other GTPases, protein association and precipitation in aqueous environments is a major challenge, as we have illustrated. Aggregation/precipitation is less of an issue for crystallography (crystallization conditions are far from NMR solution conditions and aggregation may in fact favor crystal formation), but for NMR is a considerable problem since the experiments require both high protein concentration and extended time periods for data recording. For Rnd1, we were fortunate to have information about the surfaces that appear to originate the aggregation and our study could utilize a strategic mutation, W66L, to solubilize the protein. Although this was the key change that makes further study possible, it was combined with several other optimization steps that appear to be general for Rho GTPases.

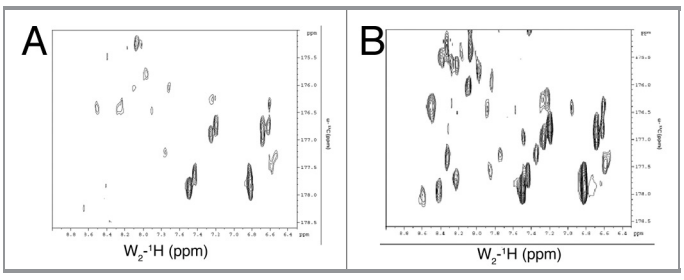

Figure 6. Comparison is shown between a representative region of the 2D projection of HNCO experiments of the double labeled and triple labeled proteins. (A) ^{15}N and ^{13}C double labeled Rnd1 W66L mutant; (B) ^{15}N, ^{13}C, 50% deuterated Rnd1 W66L mutant.

The patterns that emerge from **Table 1** provide several guidelines. (1) In almost all cases, GTPase fragments were used, as the full length proteins are often less soluble. The flexible termini also create strong and highly overlapping signals in the spectra, which may interfere with the characterization of other parts of the proteins. (2) The GTPases need to be kept under conditions that stabilize their nucleotide bound state. Several factors contributed. Temperature: even modestly elevated temperatures can lead to transient/partial unfolding and once the nucleotide is released the unfolding, at least for several Rho GTPases appears to be irreversible.[58] Lower temperature broadens NMR signals for proteins >15 kDa sufficiently to make studies prohibitive. All temperatures in **Table 1** are between 15–30°C. pH and salt concentration: both need to be optimized—in part this is related to protein association, as even transient contacts can lead to partial unfolding and loss of the nucleotide. The overall stability of the protein is also a function of pH (and electrostatic screening of unfavorable interactions). In general, the solution conditions are close to the GTPases isoelectric points (pIs are 5.76 for Cdc42, 8.78 for Rac1, 5.83 for RhoA and 7.96 for Rnd1). Reducing agents: except for low pH studies (i.e., for Cdc42), where disulphide bond formation is inhibited, DTT or TCEP (effective at a lower concentration and more stable with time) needs to be added in order to avoid covalent cross-linking. Especially when formed between proteins, thiol groups that are easily accessible in the structure, can lead to aggregation/protein precipitation. Stabilization by Mg^{2+} and by nucleotide loading/availability: nucleotide free Rho GTPases are generally prone to aggregation/precipitation. It is known from decades of study of Ras and Rho GTPases that the nucleotide bound state is stabilized by Mg^{2+}. Thus, its inclusion is essential for GTPase stability. Cdc42, Rac1 and RhoA GTPases were simply prepared in GDP form by the addition of excess GDP to the lysis-buffer, elution or final buffer. The intrinsic hydrolysis activity of the wild-type GTPase is typically sufficient, in that by the time the protein is extracted and purified from *E. coli*, hydrolysis of all GTP to GDP has occurred. In order to obtain proteins in the active form, the bound GDP was exchanged for a non-hydrolyzable GTP analog (GMPPNP, GMPPCP or GTPγS) following a general protocol[59] with small variations. The Cdc42, Rac1 and RhoA GDP bound proteins are placed at relatively low concentration (~100 μM) in a

buffer containing the non-hydrolysable GTP analog at 10-fold excess in the presence of 10 mM EDTA. Often a stabilizing agent, such as 0.3 M ammonium sulfate, is added for this step to solubilize the apo state of the GTPase. Alkaline phosphatase can also be added for specific degradation of GDP before $MgCl_2$ is added to excess (say 50 mM) over the EDTA that is present. This stops the reaction and secures the bound state. Unbound nucleotides and unfolded/aggregated proteins are typically removed by gel filtration, though it is generally advantageous to keep not only good amounts of $MgCl_2$, but also additional nucleotides in solution. (3) Bound GTPases can be stabilized (or destabilized) by their binding partners. The challenges of studying isolated GTPases by solution NMR do not necessarily transfer to their complexes with effector or regulatory proteins. A range of behaviors has been observed. For example, in the case of the Rac1 constitutively active mutant. Q61L the protein slowly precipitates at room temperature in phosphate buffer, whereas the complex with the Rho GTPase binding domain of plexin-B1 is stable over weeks at a protein molar ratio of 1:1. An extreme example of GTPase stabilization is the nucleotide free state of RhoA, which is unstable by itself but can be characterized when the protein is bound to an exchange factor, the DH-PH module of PDZRhoGEF.[21]

Summary

The Rnd1 GTPase plays important roles in the regulation of neurite outgrowth, dendrite development, and axon guidance. But, so far the solution structure and dynamics of the protein has not been determined. Obtaining a sufficient amount of protein, and a well behaved protein, is the first requirement for solution NMR studies. Rnd1, when expressed with an N-terminal His_6-tag could be purified efficiently with Ni-NTA beads to yield an adequate amount of pure protein. However, Rnd1 was found to be unstable due to aggregation and subsequent precipitation at room temperature. Combined approaches, including His-tag cleavage and addition of non-hydrolysable GMPPNP nucleotide, increased the stability, and abolished protein aggregation and precipitation. In order to prevent aggregation of the protein, a point mutation W66L was made in the putative association interface based on the dimeric crystal structure. This markedly improved the NMR spectra. Further spectral improvement of ^{13}C resonances was possible by random fractional deuteration, in particular this made 3D NMR experiments that involve carbon nuclei suitable for further studies. The strategies described here are likely to be useful in overcoming problems in other poorly behaving proteins, such as the many other GTPases of the Rho family that have not been studied by NMR to date.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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