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Combining NMR and Molecular Dynamics Studies for Insights into the Allostery of Small GTPase–Protein Interactions

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Abstract

Combinations of experimentally derived data from nuclear magnetic resonance spectroscopy and analyses of molecular dynamics trajectories increasingly allow us to obtain a detailed description of the molecular mechanisms by which proteins function in signal transduction. This chapter provides an introduction into these two methodologies, illustrated by example of a small GTPase–effector interaction. It is increasingly becoming clear that new insights are provided by the combination of experimental and computational methods. Understanding the structural and protein dynamical contributions to allostery will be useful for the engineering of new binding interfaces and protein functions, as well as for the design/in silico screening of chemical agents that can manipulate the function of small GTPase–protein interactions in diseases such as cancer.

Keywords

Protein–protein interaction; Plexin RhoGTPase binding domain; Rac1; Rnd1; GTPase–effector interaction; Nuclear magnetic resonance relaxation; Lipari–Szabo order parameters; Cross-correlation analysis

1. Introduction

1.1. Allostery Based on Changes in Dynamics

Monitoring protein dynamics and uncovering its role in protein conformation and function is a major challenge in biophysics and structural biology. All three aspects – dynamics, structure, and function – are intimately linked, at least for key regions of proteins. In an allosteric system, the binding of ligand or modification at one site of the protein causes a change in affinity or catalytic efficiency at a spatially distinct and often distant site (1). Although allostery is most often associated with multimeric proteins, long-range communication via structural and/or dynamics changes can also occur in small single-domain proteins (2). However, in the majority of cases the protein features (i.e., conformational and/or dynamical) involved in such “communication conduits” or coupling networks are still elusive. Comparison of NMR relaxation measurements and molecular dynamics simulations of proteins in different bound and unbound states can identify the role of dynamics in these networks and thus provide insight into the mechanistic and molecular basis of the allosteric behavior.

Several models have been developed to describe allosteric mechanisms. Smock and Gierasch (3) discussed the dynamic properties in cellular signaling proteins, suggesting that proteins fluctuate among multiple states on energy landscapes that have been selected during evolution. Upstream signals remodel those landscapes and cause signaling proteins to transmit information to downstream partners. Using the landscape paradigm, fluctuations may already be sufficient in the unbound protein to sample the bound conformation (1). The bound conformation would have higher affinity for a ligand or substrate, and thus association with such a molecule would move the equilibrium to populate the bound state (so-called “equilibrium shift mechanism”). As an alternative mechanism, it has recently become clear that communication between two or more different sites in proteins can be accomplished by changes in protein dynamics alone, with no change in the average protein structure (4, 5). NMR can show, for example, that the average structure remains the same (e.g., by the lack of significant chemical shift changes), whereas the fluctuations are increased in frequency or amplitude. These dynamic changes will still have significant effect on the recognition of binding sites as they alter the reference state for entropy changes that occur upon complex formation. Below, we describe such a case from our studies on a small GTPase–effector protein interaction.

1.2. NMR as a Powerful Tool to Investigate Protein Structure and Dynamics

A number of experimental techniques are available for the detailed characterization of protein structures and structural changes, including optical spectroscopies, X-ray crystallography, and Nuclear Magnetic Resonance (NMR) spectroscopy. Initially, the main objective of studying proteins by NMR was to obtain knowledge of the architectural organization of these molecules, necessary for understanding the links between structural motifs and biological functions. During the last 30 years, theoretical, methodological, and instrumental developments of NMR have concentrated on the structure determination of proteins with increasing molecular weights. Under favorable circumstances, the possibilities offered by the labeling with nitrogen-15, carbon-13, and deuterium, as well as TROSY techniques (6) now allow the NMR structural analysis of soluble proteins with molecular weights up to and greater than 800 kDa.

NMR is unique in that it yields site-specific information on multiple timescales (Fig. 1). As a result, NMR has become a technique of choice for the study of macromolecular dynamics (7, 8). In particular, backbone relaxation measurements (^{15}N spin probes are ideal because of their uniform distribution along the protein backbone) can be used to monitor internal motions on the fast (pico to nanosecond) timescale, to indicate fluctuations on a slower (micro to millisecond) timescale and to assess the overall rotational diffusion of the molecule (nanosecond time-scale). Measurement of protein relaxation has a long history. For example, Wagner and colleagues worked on the relaxation of carbon-13 nuclei at natural abundance in the Bovine Pancreatic Trypsin Inhibitor (9). Since most proteins cannot be concentrated sufficiently to allow a study using carbon-13 (or nitrogen-15) at natural abundance, techniques were then developed to label these molecules completely, partially or selectively with different isotopes. A direct consequence of these labeling techniques has been to greatly facilitate the study of main- and side-chain relaxation in proteins. Seminal investigations were carried out by Kay et al. (10), Clore et al. (11), Palmer et al. (12) and

Wagner et al. (13); Buck et al. (14), first measured Arg/Asn/Gln/Trp side-chain ^{15}N relaxation and methyl ^{13}C - and ^2H relaxation were first monitored by Palmer et al. (15), and Nicholson et al. (16) as well as by Muhandiram et al. (17). Below we illustrate aspects of working with ^{15}N main-chain relaxation measurements, largely on the ps–ns timescale that is comparable to currently achievable molecular dynamics simulations. Other NMR techniques specifically aimed at timescales beyond the ns (global tumbling) regime of proteins are mentioned at the end of the chapter (see Subheading 3).

1.3. Studies Are Complemented by Use of Molecular Dynamics Simulations

Since the first few ps-length simulations of proteins by molecular dynamics in the late 1970s (18), molecular dynamics have revolutionized our understanding of protein structure and function. Nowadays simulations of tens, if not hundreds of ns are standard and multi-ms simulations are becoming increasingly common (19). This is exciting because many of the motions at correlation times at or faster than the global motion of the protein and that are detected experimentally by NMR relaxation, can be directly compared between calculation and experiment. Simulations can provide atom-level insights that are difficult to obtain from the experimental data. The aim of our project on small GTPase– protein interactions is to correlate the dynamic motions predicted by molecular dynamics simulations with the same timescale motions that are observed experimentally, providing greater insights into the latter.

1.4. Allostery in Small GTPases

The Ras superfamily of small GTPases is nearly ubiquitous in cell signaling, functioning as molecular switches. The structure and dynamics of several so-called switch regions within the protein largely depends on the nucleotide, GDP or GTP, which is bound. The different states (structural and dynamical) are in turn recognized by GTPase regulatory or effector proteins in signaling mechanisms (20). Trivially, the shift that occurs when GDP exchanges for GTP in the neighboring protein switch regions could be regarded as allosteric. However, we believe that the conformational behavior of these switch regions by themselves is rather mundane and that allostery in small GTPases involves longer-range communication networks in the protein. The identity of residues which allow this longer-range coupling and the mechanistic role of these residues in allostery is, therefore, of paramount interest. It has become clear, for instance, that the conformational equilibria in GTPases that are normally associated with the presence or absence of the terminal γ -phosphate of the nucleotide are sensitive to mutations not just in or close to the switch regions (21, 22), but also to sequence changes outside these regions. Specifically, an extensive mutagenesis study found that even though such residues were distant to the nucleotide, they can significantly establish the functional specificity of small GTPases (23). A statistical coupling analysis, followed by a mutagenesis study on G-proteins revealed that the nucleotide binding regions are indeed linked via a network of conserved side chains to other regions of the proteins, some far away from the nucleotide binding site (24). Thus, the GTPases may communicate signals also in an allosteric manner across the protein.

Allosteric communication in GTPases has been corroborated by a number of other studies: experimental and computational work, carried out on Ras-family proteins as they associate with lipid membranes (25) or are posttranslationally modified (26), has suggested longer-

range communication utilizing specific structures outside the switch regions. Importantly, the residues involved in these communication conduits may be GTPase specific. As yet, there are only a few examples of GTPase–protein interactions which involve binding to surfaces other than the switch regions. Yet, it is thought that such regions can provide some of the specificity of the GTPase–protein interactions (27–29). In studies that have suggested allostery, evidence either derives from functional work in vivo (where the exact binding partners and their interactions are typically not identified), from sequence analyses or from investigations of the free, but modified or lipid-bound GTPases. However, the concept of allostery requires characterization also of the ternary complex (30). Although such structures are likely to be central to the function of GTPases, to the knowledge of these authors, no GTPase ternary complex structures have been determined to date.

In this article, we discuss work from our and other laboratories that concerns GTPase–protein binary complexes. The comparisons of unbound and bound states, already provides information on the allosteric nature of GTPase–protein interactions. This became apparent in our study of the plexin-B1 RhoGTPase binding domain (RBD) and its interaction with the small GTPases, Rac1 and Rnd1, which we have studied over a number of years. The binding domain has a ubiquitin-like fold (with insertion of several lengthy loops, see Fig. 2) and is part of the intracellular region of plexin-B1, part of a family of transmembrane receptors that function in axon guidance and cell migration/positional maintenance. Rac1 and Rnd1 are members of the Rho family of small GTPases, which bind specifically to the plexin domain and effect a conformational change that is used to activate the receptor. Our combined NMR and MD studies suggest an allosteric mechanism that is propagated through the different GTPases in the complexes with plexin and is specific to them.

2. Methods of Analysis and Examples of Results and Their Interpretation

2.1. NMR Relaxation Measurements and Analysis

Backbone motions are characterized by measuring heteronuclear relaxation parameters, which are then translated into dynamic parameters (actually a representation to describe the motional amplitudes and timescales) using models of varying complexity. However, the interpretation of relaxation parameters in terms of dynamics models is typically not unique because the number of experimentally measureable parameters is limited and is insufficient to describe physically realistic models. For instance most analyses are essentially based on three parameters (observables possibly measured at several values of the magnetic field): (1) the T_1 or longitudinal relaxation time of ^{15}N is usually measured with the inversion-recovery method, (2) the T_2 or transverse relaxation time is usually measured with the CPMG (Carr–Purcell–Meiboom–Gill) method, and (3) the $\{^1\text{H}-\}^{15}\text{N}$ NOE (Nuclear Overhauser Effect) carried out in an interleaved manner, with and without a proton saturation period applied before the start of the ^1H – ^{15}N correlation experiment (31). For ^{15}N T_1 and ^{15}N T_2 measurements, several spectra are recorded corresponding to different delays to follow the recovery of the magnetization (T_1) or its disappearance in the transverse plane (T_2), see Fig. 3. More elaborate NMR experiments, increasing the number of observables has also been proposed (e.g., ref. 13). Raw data are processed and the relaxation rate constants are obtained by fitting of the peak intensities ($\pm$ sampled uncertainties) to a single exponential

function using the nonlinear least-squares method. Experimental uncertainties are determined from baseline noise and/or duplicate measurements.

NMR parameters were measured for the plexin-RBD and Rac1 GTPase at two fields, separately and in the complex. Since the size of the protein complex is considerable (~37 kDa) and crowding of resonances in 2D spectra would have been severe, the measurements of the complex were done on separate samples. These samples had one protein ^{15}N labeled, while the other was unlabeled, with the unlabeled protein at an excess concentration to ensure that all labeled protein is in the bound state. The most widely used model to extract dynamical information from relaxation parameters is the Lipari–Szabo model (32). In this approach, the considered relaxation vector feels two molecular motions at very different timescales (Fig. 4). This model does not rely on knowledge of the orientation of the relaxation vector regarding the rotational-diffusion tensor but introduces the concept of a local director; this is called the “model-free (MF) approach”. In order to describe the tumbling of the relaxation vector, one uses a so-called correlation function $C(t)$. It represents the correlation between the orientation of the relaxation vector at time 0 and its orientation at time t . MF supposes that the motions can be decomposed into global and internal protein dynamics and that the two are not interdependent; so the correlation function can be decomposed into a series of independent contributions:

$$C(t) = C_o(t) C_i(t) \quad (1)$$

Here, $C_o(t)$ represents the overall rotational motion, it can be written as e^{-t/τ_m} where τ_m is the correlation time characteristic of this motion that is supposed to be isotropic. $C_i(t)$ stands for the effective internal (local) motion, it can be written as $C_i = S^2 + (1 - S^2)e^{-t/\tau_e}$. Because this motion is restricted, the correlation function does not decay to zero but to the value S^2 which is called the order parameter. τ_e is the correlation time for the effective internal motion. As depicted in Fig. 4, the local director D of the MF approach moves according to the overall molecular motion (slow), while local motions (fast and small) are held around the director. S^2 is the order parameter describing the restriction of fast motions around semi-angle θ . The higher the order parameter, S^2 , the more restrained the N–H bond motion is by its environment, i.e., the more tightly the proteins is packed (S^2 value of 1 means there is no internal motion). Other approaches exist, for cases where the assumption of two independent motions cannot be made (e.g., ref. 32).

Fourier transformation of the above equation leads to the spectral density function, $J(\omega_i)$, as

$$J(\omega_i) = S^2 \frac{2\tau_m}{1 + \omega_i^2 \tau_m^2} + (1 - S^2) \frac{2\tau_e}{1 + \omega_i^2 \tau_e^2} \quad (2)$$

This spectral density is sampled at different NMR frequencies ω and allows us to quantify NMR relaxation since relaxation rates (inverse of relaxation times, T_1 , T_2) and heteronuclear NOE are a linear combination of spectral densities with some scaling factors (equations given elsewhere, e.g., ref. 31 and see Fig. 5). In Eq. 2, the first part on the right-hand side represents the overall motion while the second part represents the internal motion. For each

kind of motions in the MF approach, the relaxation time can be calculated by fitting parameters to the correlation function of the internal motions. $\tau^{-1} = \tau_m^{-1} + \tau_e^{-1}$ with the τ_m being the correlation time for global molecular motion and the τ_e for effective internal motion. S^2 can be calculated from the average of plateau value of the correlation functions (Fig. 5). In some cases, the Lipari–Szabo model proved to be insufficient and led to an extended model by introducing two types of internal motions (33). The local motions are then characterized by two correlation times, τ_f and τ_s , and two order parameters S_f^2 and S_s^2 . Other issues such as anisotropy are also omitted here for lack of space, but are extensively presented in the literature (34). Thus, the relaxation parameters can be calculated from the order parameters and global correlation time. In fact, the latter variables in Eq. 2 above are fitted iteratively to minimize the difference between the observed and back-calculated NMR relaxation parameters.

For the MF analysis, ^{15}N experimental relaxation data are first used as input, together with the protein structures (pdb files) to fit the global motion, and then to derive the local motions (34, 35). Specifically, after residues that exhibit either significant conformational exchange (high $1/T_2$), or rapid motion on the fast timescale (low NOE factor) are excluded, the remaining T_1/T_2 ratios are used to determine the rotational diffusion tensors that describe the overall tumbling. It should be noted that the relative orientation of proteins in the complex is not required for the analysis. The structures of the proteins in their free states are used to assess the overall motion of each protein in the complex as well as issues such as anisotropy (not discussed here), assuming that the topology of each protein is not changed dramatically upon complex formation. Results reported for the plexin-B1 RBD–Rac1 system show that both proteins experience a similar axially symmetric global motion when they are part of the complex. The rotational diffusion directors for the global motion are superimposable, indicating the relative position of protein in the complex (36). The average values for the global correlation time and of the anisotropy were then used to perform the Lipari–Szabo analysis to derive values for the generalized order parameter (S^2) and the effective correlation time for internal motions (τ_{oc}) for each residue. The procedure also takes into account possible exchange contributions (R_{ex}), which are associated with fluctuations on the microsecond to millisecond timescale. Specific experiments exist that characterize more extensively motions on this timescale (see Note 1). Similar to our work on the RBD–Rac1 complex (36), other studies have used NMR relaxation to characterize protein–protein (e.g., refs. 37, 38) and protein–ligand (e.g., refs. 39, 40) interactions.

2.2. Protein Internal Dynamics Changes Upon Plexin-B1 RBD–Rac1 Complex Formation as Viewed by NMR-Derived Order Parameters

As an example of the analysis and interpretation of the experimental data, we have characterized the backbone dynamics of Rac1 (bound to nonhydrolyzable GTP analog,

¹Classical MD is currently confined to being able to sample motions that occur on the tens, if not hundreds of ns and in some recent cases up to a ms timescale. Thus, to overcome this limitation in sampling, several alternatives, or as above, hybrid computational approaches have been developed. One set of strategies uses biased simulations by applying a force, as seen above. Another derives possible motions from simplified forces between atoms (normal mode, contact or network analysis) (60) and finally coarse-grained models are being used to sample longer timescales (61). Several of these techniques have been used to examine a coupling of residues and motions across protein interfaces, suggesting that coupling networks may connect two proteins across interacting surfaces (61, 62).

GMPPNP) and of plexin-B1 RBD both as free proteins and in the plexin-B1 RBD–Rac1.GMPPNP complex. Molecular correlation times, τ_m , extended Lipari–Szabo model-free order parameters, $S^2 = S_f^2 S_s^2$ and local correlation times, τ_e or τ_{loc} as well as chemical-exchange (R_{ex}) contributions, were derived using ^{15}N relaxation data acquired at two spectrometer frequencies (36). The global and internal dynamics of the two proteins change upon their association in a manner that is wide ranging and complex; specifically, changes in the dynamics are observed far from the site of binding in the Rac1 GTPase and several decreases in the extent of fluctuations appear to be compensated by increase especially on the side of the RBD (see Fig. 6). Several residues at the binding interfaces of both proteins experience conformational exchange on the μs – ms timescale, suggesting that these regions fluctuate locally between alternate contacts in the protein complex. Flexible regions of the plexin-B1 RBD, such as residues in the first long loop, residue 16–26, and those at the N- and C-termini of the protein, are not extensively perturbed by binding and have unchanged S^2 order parameters. These regions are distant from the interface that interacts with the GTPase. Upon complex formation two regions in the RBD show reduced fluctuations in terms of S^2 . The first (res. 77–84) is at the end of β -strand 4 and at the beginning of the dimerization loop, L4. The second region includes the latter part of another long loop, L2 (residue 50–58), with residues 50, 54, 57, and 62 particularly affected. The first of these two regions is close to one of the interfaces with Rac1, while the second is near the beginning of β 3 which is adjacent to β 4, a strand that is rigidified on binding. It is likely that the strand is extended by making additional contacts with β 3, also making that latter slightly more rigid. These findings show that binding is communicated beyond the region of immediate contact. Specifically, we postulate that the binding interface strand b4 is allosterically coupled, via β 3, to sections of loop L2. Support for this notion also comes from cancer mutant, L1815F and L1815P which cause chemical shift perturbations not only locally but also in the start of β 3 and in L2. The functional role of these longer-range changes is not yet clear, although we speculate that they may alter the interactions of the RBD with the adjacent domains in the intracellular region of the plexin receptor (41).

Regions of small GTPases that are involved in protein–protein interactions (switch 1 and switch 2) typically experience decreased mobility in the complex, compared to the unbound state, suggesting that protein flexibility may be required for binding (e.g., ref. 42). The ability to form complexes with Rac1 can be highly specific to the state of the nucleotide cofactor which is bound to it. For example, Rac1 loaded with GTP or GTP analogs, binds to the plexin RBD with a K_d of $\sim 6 \mu\text{M}$ whereas no interaction, even transient, could be detected for the GDP state of Rac1 even at mM concentrations (43). This level of discrimination suggests that several regions which contact the nucleotide – the switch regions 1 and 2 as well as the P-loop (residues 10–17) and parts of two other regions, residues 115–118 and 158–160, are directly or indirectly involved in the interaction with the plexin RBD. Internal dynamics appear overall diminished in Rac1 on binding to plexin (Fig. 6). However, dynamics in the switch 1 and 2 regions of active Rac1 bound to the RBD remain complicated, with chemical exchange precluding an NMR analysis in and around these regions. Nevertheless, a number of observations can be made for other regions. Several residues in the C-terminal half of the protein (e.g., 129) stand out as hinges, with S^2 values lower than the surrounding residues in the free protein (44). In the bound protein

these hinges are tightened up. Similarly, a number of residues no longer experience ps–ns dynamics in the bound protein. Moreover, fluctuations for the majority of residues in the region which includes $\alpha 5$ (residues 117–120), the insert helix $\alpha 6$ (res. 123–130) as well as helices $\alpha 7$ and $\alpha 8$ are reduced on binding, possibly because the hinge residues, mentioned above, are no longer flexible. These results are intriguing because the insert helix, $\alpha 6$, is not part of the region that contacts the plexin RBD. However, subtle conformational changes and the dynamical changes at the hinges seem to allow changes in protein dynamics to be propagated over longer distances, specifically to the back of the protein (i.e., $\alpha 5$ – $\alpha 8$). It is interesting to compare the NMR-derived results for the RBD–Rac1 interaction with the dynamics changes observed in the Rac1 homologous GTPase, Cdc42 on forming a complex with a 46-residue effector peptide from PAK (45). The switch 1 region is in contact with the peptide and experiences diminished fluctuations upon binding, as does the insert helix and helix $\alpha 3$. The apparent correlation of binding with a change in the flexibility of $\alpha 3$, which is adjacent to the insert helix ($\alpha 6$), was noted and makes these findings in part similar to ours on plexin RBD–Rac1 complex formation. Similar to our work, this study suggests that a “network” of amino-acid contacts exists in the proteins, which serves to couple these fluctuations possibly in all Rho family small GTPases.

2.3. Molecular Dynamics Simulations and Analysis

The structure of the plexin-B1 RBD has been determined by NMR spectroscopy (46), and the structure of the RBD–Rac1 complex has been extensively refined in the Buck laboratory employing HADDOCK and XPLOR-NIH, including the use of residual dipolar coupling and relaxation tensor constraints. The structure of the plexin-B1 RBD in complex with a Rac1 homologous GTPase, Rnd1 was determined by X-ray crystallography (47). Classical all-atom MD simulations were carried out on the two RBD–GTPase complexes and on the free proteins. For simplicity of this book chapter, we report data for the low pH/protonated form of RBD histidines throughout. As a first step, the protein complexes were solvated in a rectangular box of explicitly represented water. Counterions were added to neutralize the system and to represent the ionic charge in the experimental sample. After brief minimization, the initial structures were prepared and the NAMD program was used to run constant pressure (1 atm) and temperature (300 K) dynamics for 55 ns (48). The CHARMM27 all-atom potential function (49) was used with CMAP correction and with the standard Particle-Mesh Ewald method for long-range electrostatic interactions under periodic boundary conditions.

Simple analyses were carried out to indicate the equilibration and stability of the trajectories. Figure 7 shows the RMSD (real mean square deviation) from the starting structure and the accessible surface area (ASA) that is buried in the protein–protein complexes (ASA). These are calculated according to the following:

$$Rmsd(t) = \frac{1}{N} \sum_i \sqrt{(r_i(t) - r_i(0))^2}$$

with $r_i(0)$ and $r_i(t)$ as the initial and simulation time evolved coordinates, respectively.

$$\Delta ASA = ASA \text{ (free proteins)} - ASA \text{ (complex)},$$

using a probe radius equivalent to that of water of 1.4 Å. The observation that the simulations reach and stay near a low RMSD plateau value shows that the complexes are stable, as a protein that unfolds or proteins that completely dissociate would have an increase to a large RMSD value. The same is true for the buried surface area, which would dramatically decrease if the proteins dissociated.

Order parameters, S^2 and τ_e for main-chain N–H bonds (for each individual residue) are calculated in CHARMM from autocorrelation functions of $C_2(t)$ over trajectory segments.

$$S^2 = \lim_{t \rightarrow \infty} C_2(t), \quad \text{where} \quad C_2(t) = A \langle P_2[\mu(\tau) \cdot \mu(\tau+t)] \rangle \quad (3)$$

A is a such that $C(0) = 1$. The second Legendre polynomial $P_2(x) = \frac{1}{2}(3x^2 - 1)$. The angle brackets ($\langle \rangle$) represent the trajectory average over a sliding window of 5 ns (close to the global tumbling in the simulations) (see ref. 50). The unit vectors $\mu(\tau)$ and $\mu(\tau+t)$ describe the orientation of the N–H vector at time τ and $(\tau+t)$ in relation to a fixed reference frame. To construct this frame, the global motion of the protein is removed prior to this analysis by C α superposition on a reference structure (e.g., the starting structure), so the correlation function represents the internal (local) motion only. Again, similarly to the analysis of the experimental data, alternative but more complex approaches are available that do not assume that global and internal motions are separable (and uncorrelated) (e.g., ref. 51). S^2 is the plateau value, calculated for the $C_2(t)$ function in the NMR module of CHARMM. τ is estimated by integration of the correlation function up to time T_{conv} , the time when first crosses

$$\tau_e = \frac{1}{1 - S^2} \int_0^{T_{\text{conv}}} (C_2(t) - S^2) \cdot dt. \quad (4)$$

In some cases, while S_s^2 and S_f^2 are not calculated separately, the τ_e estimated from the trajectories corresponds to τ_s , a slower motion. An issue is the converge of the internal motions of individual bond vectors. This is most easily assessed by examination of the correlation function (see Fig. 8). Running multiple simulations with different assigned starting velocities or even structures allows one to check for convergence and estimate an uncertainty in the calculated S^2 and τ_e values. When this uncertainty is too large, nonconvergence is indicated. In this case, longer simulations and/or enhanced sampling protocols such as replica exchange are required (52). Further alternative strategies exist to sample conformational space (see Note 2).

2.4. Analysis of Motions in Free Proteins and RBD–Rac1/Rnd1 Complexes as Seen in MD

Figure 7 shows two simple analyses carried out on the MD trajectories. Trajectories were calculated for the unbound proteins and complexes. The plexin-B1 RBD binds two homologous GTPases, Rac1 (see experimental results above) and Rnd1 (for which NMR

relaxation experiments are in progress). The real mean square deviation (RMSD) shows that the trajectories, with exception of bound Rac1, are equilibrated within 10 ns in terms of the average amplitude of the majority of the internal motions, as the displacement from the starting structure reaches a plateau value. The RMSD for the complexes is larger than for the free proteins, as in the former case superposition is made on both proteins, a process that is very sensitive to global changes of the proteins relative to one another. The results are similar for the RBD–Rnd1 and RBD–Rac1 simulations (Fig. 7a, b respectively). Calculating the surface area that is buried by the interface of the complex also gives an indication of the global stability and dynamics of the protein–protein association (Fig. 7c, d). The RBD–Rac1 complex shows more interface fluctuations than the RBD–Rnd1 complex.

2.5. Comparison of Order Parameters Derived from NMR Experiments and from MD Simulations

Figure 9, as an example for the results and their interpretation of an NMR/MD comparison, illustrates that a good correspondence is possible between experimental and simulated order parameters, suggesting that the simulations are overall accurate. A linear correlation coefficient of 0.74 for S^2 and 0.63 for τ_e , averaging the results from four independent 55 ns simulations of the RBD. To date very few papers have compared experimental and MD derived values for the effective correlation time, τ_e . This parameter is difficult to estimate and is not further discussed here. In the bound state, the RBD N–H bond vector motions remain highly flexible in loop1, 4 and at the N- and C-termini. In the case of the RBD–Rac1 complex, RBD's L3, the interface β 4 strand as well as α 2 exhibit greater fluctuations than in the RBD–Rnd1 bound state (data not shown). The comparison of order parameters between NMR and MD for unbound Rac1 is not as good, mainly because the majority of S^2 values for the GTPase is high, near 0.9–1.0, and small variations are difficult to reproduce in the simulations. Many of the observations made from the experimentally derived order parameters upon Rac1 binding, described above, are, however, confirmed by the simulations (Fig. 10). Specifically, the MD derived S^2 show that upon RBD binding the end of Rac1 helices α -4 and -6, also switch 2, become more rigid, whereas flexibility in switch 1 increases. In case of binding to Rnd1, an increase in flexibility is observed in switch 2 (switch 1 is already flexible on the ps–ns timescale in the free protein), at the end of the α -4 turn, and the beginning of β -5. Although, no NMR relaxation data is yet available for Rnd1, this observation is intriguing as it confirms a difference in the entropy change seen upon binding of the RBDs to the two GTPases (53). However, this correspondence is necessarily extrapolated as no experimental relaxation data is yet available for side chains. Order parameters are a local probe and are very sensitive to changes in hydrogen bonding, side-chain packing and solvation. NMR measurements of side-chain dynamics and the analysis of side-chain motions from MD trajectories are in progress for both RBD–GTPase complexes.

²NMR also has a timescale problem. NMR relaxation measurements can be made on the ps–ns and μ s–ms timescale, but this method has until recently been blind to motions that are slower than the overall correlation time of the protein but fast enough not to cause exchange broadening. The analyses of extensive RDC (residual dipolar coupling) datasets have been used to extract μ s–ms timescale motions. However, measurements have to date only been done on individual domains (63). These are intensive experiments in terms of time and effort and have not been carried out for μ s–ms dynamics in protein complexes. A popular alternative are relaxation dispersion experiments (e.g., see ref. 64 for a paper on Ras) that can monitor a similar timescale of motions and an analysis that can also extract the extent of conformational change (as indicated by chemical shift change) and the population of the different states involved. The interested reader is referred to a number of excellent articles and reviews in this area (65, 66).

For the detection of a possible coupling between fluctuations, the analyses of cross-correlations are particularly useful, as these represent longer-range dynamics effects.

2.6. Cross-Correlation Analysis to Detect Coincidence of Motions

In order to analyze the atomic fluctuations in proteins, the cross-correlations are obtained from the simulation trajectories for the C α –C α pairs in the plexin RBD and in the GTPases. The covariance of the spatial atom displacements for selected atom pairs is calculated based on cross-correlation coefficient calculation using following equation:

$$C_{ij} = \frac{\langle \Delta r_i \Delta r_j \rangle}{\sqrt{\langle \Delta r_i^2 \rangle \langle \Delta r_j^2 \rangle}} \quad (5)$$

Here, r_i is the displacement of the mean position of the i th atom (here C α) determined from all configurations in the dynamic trajectory segment (5–55 ns) being analyzed. The numerator in above equation is the covariance for the displacement vectors for atom i and j respectively.

For completely correlated motions, that have the same phase as well as period, $C_{ij} = 1$; and $C_{ij} = -1$ for completely anticorrelated motions (have the same period but counter phase). Deviations from 1 or -1 imply the motions of two residues (i and j) are less correlated (or anticorrelated), or mean they deviate from motion along a straight line. When two displacements are in a direction perpendicular to one another, the dot product of the displacement vectors becomes zero, thus the cross-correlation function equals zero, presenting a limitation to this analysis (54). Alternative approaches that do not have these limitations include analysis of correlation between dihedral angle fluctuations or between interresidue energies (e.g., ref. 55) as well as novel computational approaches that probe specific coupling networks (see Note 3).

2.7. Analysis of the Results in the Case of the RBD–Rac1 and RBD–Rnd1 Complexes

The correlation maps were calculated for both the unbound proteins and for the protein complexes (see Fig. 11).

2.7.1. Unbound Proteins—For the unbound RBD, moderate to strong correlations are seen for the flexible N- and C-termini and all of the loops which are modestly anticorrelated

³Once identified, allosteric coupling networks need to be validated by computational modeling and if possible by experiments. A priori, it is not clear what functional consequences the plexin-induced dynamics changes in GTPases have for the signaling mechanism. A surface of the GTPases opposite to the binding site for the RBD is rigidified but a binding partner that would specifically recognize/bind with higher affinity to the reduced entropy of this region remains to be identified. In its absence (and since the binding partner will be present in vivo), we are now disrupting the coupling network by mutagenesis, and anticipate to get a functional readout utilizing cellular assays for plexin function. The challenge is similar to those associated with the interpretation of protein dynamics: Are the particular dynamics simply a consequence of the underlying protein structural features that are wiggling and jiggling in a stochastic solvent environment? Or can the dynamics be defined, say, as part of coupling networks that are important for protein function? Assuming that computer simulations are accurate enough, both in terms of sampling and in the potential functions used, molecular modeling of mutants and recent novel computational methods could provide the details required to answer this question. Simulations have the advantages that conditions can be set up that are difficult, if not impossible to realize in the laboratory. Specifically, Agard and colleagues have recently developed a novel computational method to probe coupling networks by perturbing specific residues within them by individual thermal agitation in conjunction with otherwise classical molecular dynamics (67). By forcing dynamics events to take place in one area of the protein, the stochastic nature of the fluctuations is partly overcome.

with protein core motions. Correlations are mostly observed between elements of secondary structure that are in contact in the protein (e.g., $\beta 1$ with $\beta 5$ and $\beta 2$). Much more modest correlations are seen between β -strands $\beta 1$ and $\beta 3$ as well as between $\beta 4$ and $\beta 5$ which have one strand $\beta 5$ and $\beta 3$, respectively to bridge between them. In the case of unbound Rac1 and Rnd1, GTPases structural elements that are spatially close to each other also predominate correlations. There are a few longer-range correlated motions, again through a bridging strand such as $\beta 1$ with $\beta 5$ and $\beta 4$ with $\beta 6$ in both proteins. In the case of Rnd1, an additional correlation is seen between the $\beta 2$ region and the end of $\beta 1$, two regions that are not in direct contact but again bridged. There is also a strong correlation between the switch1 region and $\beta 5$ -strand, two regions that are separated by switch 2, which does not show appreciable correlations with either of them. The region leading to the only remarkable anticorrelation with most of the protein is the insert helix in both GTPases, although here a difference also becomes apparent between the two GTPases. In Rac1 there is considerable correlation between the insert helix motion and the $\beta 3$, although these elements are not close in the structure. Similarly a correlation between the beginning of the insert helix and $\alpha 4/a$ hinge region is only seen in Rac1. In Rnd1 the correlation of the insert helix ($\alpha 6$) region is shifted to $\alpha 2$ /the beginning of switch $\alpha 2$. Again, these structures are not close in space. Thus, there are long range correlated motions in the GTPases, and the several of these as well as some of the next neighbor fluctuations appear to be GTPase specific. These findings are in broad agreement with other MD studies on different members and mutants of the Ras family of small GTPases (53, 56–58).

2.7.2. Intra-protein Motions in the Bound Proteins—In order to visualize the change in correlations for the protein internal motions in the complex, we superimpose the complex either on the bound plexin RBD or GTPase starting structures. In this way the correlations are not affected by fluctuations of the two proteins relative to one another in the complex. For the RBD domain, remarkably the internal motions are changed considerably in certain regions and in a manner that differs between the two complexes. For example, in the RBD bound to Rnd1 next neighbor correlations between $\beta 1$ – $\beta 2$, $\beta 1$ – $\alpha 1$, $\beta 1$ – $\beta 5$, are all increased, as are some of the bridged correlations, $\beta 2$ – $\beta 5$ and $\beta 1$ – $\beta 3$, whereas surprisingly these correlations are all diminished in the Rac1 bound state. A major difference in the RBD structures is that the RBD–Rnd1 started from a crystal structure that has loop1 bound across the back of the RBD structure to interact with $\beta 5$, $\beta 3$ and $\beta 4$. Even over the course of the 55 ns simulation this loop structure largely persists. Intriguingly, in the RBD–Rac1 structure the loop also moves toward the GTPase, even though our NMR relaxation data suggests it is just as flexible in solution as in the unbound state. Similar to the correlations between β -strand motions, correlations involving the two RBD α -helices also respond differentially to binding of the two GTPases. In the Rnd1 bound RBD new correlations are seen between $\alpha 1$ and strands $\beta 3/\beta 5$ and the end of GTPase binding strand $\beta 4$. In the Rac1 bound RBD the latter correlations also exist but $\beta 3$ and $\beta 5$, surprisingly move in a manner that is anticorrelated with $\alpha 1$. Instead, the end of $\beta 4$ and the region that returns from the dimerization loop appears to be correlated with loop2 and the beginning of $\beta 3$. This is consistent with our experimental finding that mutagenesis of Leu1815 to Pro or Phe in $\beta 4$ causes chemical shift perturbations at the beginning of $\beta 3$ and loop2 (46).

A similarly complex picture emerges on the side of the GTPases upon binding. Several changes are common to both GTPases upon RBD binding. Helix turn $\alpha 3/\beta 4$ is also more correlated with the insert helix in both. Motions of a few residues at the center of $\beta 2$ becomes decorrelated in both GTPases, beginning at switch 2, which becomes less correlated in Rnd1 compared to the unbound state. Correlations between $\beta 1-\beta 2$ and $\alpha 1-\alpha 3$ are extended in bound Rnd1 more than in Rac1. Other differences concern the anticorrelation of residue 95 and insert helix ($\alpha 6$) with switch 2 which changes to a correlated motion in Rnd1, whereas in Rac1 there is a new correlation of $\alpha 3$ with the $\beta 2-\beta 3$ turn. There is also less anticorrelation and more correlated movement of other regions with the insert helix, suggesting that this latter structure has tightened up, an observation made for both GTPases upon RBD binding and is also evident from NH order parameters. In summary, the pattern of correlated motions and the changes to this pattern in the GTPases upon RBD binding is complex. The analysis by tools such as the cross-correlation matrix reports on changes as observed for the main chain. However, to rationalize and further understand the changes, we need to consider differences in side-chain packing between the two GTPases and also between the bound and free states. These studies are still ongoing and use a different set of analysis tools, as described in a future publication.

2.7.3. Correlated Motions Between Proteins—Motions across the interface are difficult to interpret, since alignment on neither protein in the complex alone presents an unbiased picture. Alignment on the entire complex structure is one option, but the fluctuations of the proteins relative to one another are considerable; this in itself will lead to spurious long-distance correlations. We are currently exploring a number of approaches, including those that are not based on absolute coordinate but instead on relative coordinate or dihedral angle space. These results are reported elsewhere. It is remarkable, however, that the regions of both proteins, RBD and GTPase, that are in contact with one another are also involved in medium range correlated motions. This is consistent with a proposal from statistical coupling analysis (59) that interfaces are not only correlated during protein evolution but that they also connect to protein allosteric networks 1–2 layers beyond the interface into each protein.

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Fig. 1.
NMR and MD timescales.

Fig. 2. Main-chain fold of ubiquitin in comparison with the ubiquitin-like RhoGTPase binding domain (RBD) of plexin-B1. The longer loops in the plexin domain are labeled, L1-4. Shown below is the RBD binding partner, Rac1, with GTP bound. Regions of interest, switch 1 and 2, the P loop, the insert helix, and helix α -7 are labeled.

Fig. 3.

Following ^{15}N relaxation by three different parameters, T_1 , T_2 , and Heteronuclear NOE.

Heteronuclear NOE (not represented) is the intensity change for ^{15}N when its attached ^1H is saturated.

Fig. 4. Model-free representation. The local director D has the same motion as the global molecule (slow motion characterized with τ_m), while local motions occur around this director (fast motions characterized with τ_e , and restricted according to the angle θ).

Fig. 5.

Relationship between correlation function (a), spectral density (b) and NMR relaxation measurements (c). In the analysis of simulation data, a correlation function is Fourier Transformed to give the spectral density which then predicts NMR relaxation rates (sampling the spectral density function at certain frequencies). For the analysis of the NMR measurements, the process is the reverse. Comparisons between all three sets of parameters (a–c) test the models and self-consistency. Plot (d) shows the standard MF correlation function and (e) shows the more complicated model, incorporating two internal motions, sometimes used to fit experimental data.

Fig. 6. Changes in ^{15}N order parameter, S^2 , in plexin-B1 RBD (*left*) and Rac1 (*right*) upon complex formation mapped onto their respective structures. The plexin domain shows increased as well as decreased main-chain dynamics near the binding interface (*shaded*), while Rac1 becomes more rigid in parts of the protein, some of these regions are opposite to the binding site (*black arrows*). Results adapted from our recent paper (36).

Fig. 7.

(a, b) Plot Cα RMSD of Rac1–RBD and Rnd1–RBD complexes versus their starting structures (superimposed on complex and on single protein in complex). (c, d) Surface area covered in protein–protein interface (same simulations).

Fig. 8.

Selected correlation function decays to illustrate convergence issues. $C_2(t)$ correlation functions are shown for the N–H bond vector of residues 13, 39, and 53 of the RBD. Residue 13, located in loop1, is not fully converged in this simulation.

Fig. 9. Order parameters from MD versus experiment (open and closed squares) showing (a) S^2 and (b) τ_e , comparison for the unbound RBD.

Fig. 10.

GTPase order parameters from MD, comparing S^2 unbound (open circles) and bound (closed circles) for (a) *Rac1* and RBD-*Rac1*; (b) *Rnd1* and RBD-*Rnd1*.

Fig. 11.

Correlation analysis of free and RBD bound GTPases. Correlation analysis for free GTPases are shown above the diagonal and the difference maps (bound-free) are shown below the diagonal. Circled areas show correlation/changes in correlation common to both structures, in the case the free GTPases these are long range correlations. Areas indicated by squares indicate different correlations between the two GTPases.