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Solvent Saturation Transfer to Proteins (SSTP) for Structural and Functional Characterization of Proteins

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Abstract

Protein structure determination using NMR is dependent on experimentally acquired distance restraints. Often, however, an insufficient number of these restraints are available for determining a protein's correct fold, much less its detailed three-dimensional structure. In consideration of this problem, we propose a simple means to acquire supplemental structural restraints from protein surface accessibilities using solvent saturation transfer to proteins (SSTP), based on the principles of paramagnetic chemical-exchange saturation transfer (paraCEST). Here, we demonstrate the utility of SSTP in structure calculations of two proteins, TSG101 and Ubiquitin. The observed SSTP was found to be directly proportional to solvent accessibility. Since SSTP does not involve the direct excitation of water, which compromises the analysis of protein protons entangled in the breadth of the water resonance, it has an advantage over conventional water-based magnetization transfers. Inclusion of structural restraints derived from SSTP improved both the precision and accuracy of the final protein structures in comparison to those determined by traditional approaches, when using minimal amounts of additional structural data. Furthermore, we show that SSTP can detect weak protein-protein interactions which are unobservable by chemical shift perturbations (CSPs).

Keywords

Yb-DOTAM; paraCEST; Ubiquitin; TSG101; Structure Refinements; CSP; NMR

Introduction

NMR structure determination of proteins depends on the incorporation of distance restraints acquired from experimental data. Yet, often these calculations are obstructed by an insufficient amount of distance restraints (e.g., due to resonance broadening and/or overlap). Furthermore, in cases where protein instability is a factor and/or when deuteration is required, the acquisition of enough distance restraints becomes even more challenging. To lessen this difficulty, Wang et al. developed a method to convert experimentally observed solvent-protein interactions to structural restraints which can be implemented in NMR structure calculations¹. Although the precision and accuracy of protein structures improved

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following incorporation of the acquired solvent accessibility data, this methodology relies on the analysis of solvent-protein interactions via water nuclear Overhauser effects (NOEs). Since water NOE experiments are based on the direct excitation of water, structural information from amide protons which are spatially close to other protons that resonate in the vicinity of the water resonance (e.g. protein exchangeable groups such as H^N, OH, and non-exchangeable aliphatic H^α) is compromised and unavailable for determining a protein's structure.

To overcome these difficulties of using water excitation-based magnetization transfers as an aid in the determination of protein structures by NMR, we have developed solvent saturation transfer to proteins (SSTP), based on the principles of paramagnetic chemical exchange saturation transfer (paraCEST), as a supplemental means to acquire structural restraints using protein solvent accessibilities. Chemical exchange saturation transfer (CEST) involves (1) selective spin saturation using a weak magnetic field and (2) transfer of this saturated magnetization to a non-saturated spin via chemical exchange. This technique is well-known in magnetic resonance imaging (MRI) in which contrast agents with exchangeable protons are used to produce a marked effect on image contrast^{2,3,4}. However, contrast agents based on diamagnetic compounds whose H^N or OH resonance frequency falls within a few ppm of bulk water, are strongly attenuated by the direct excitation and saturation of water^{3,5}. This off-resonance attenuation, however, is suppressed considerably in paraCEST which uses pseudo contact shift (PCS) compounds as contrast agents to displace their H^N and OH resonances by a few hundred or even higher parts per million from that of water^{2,3,6}. In addition, this method allows the extraction of higher exchange rates while still adhering to the slow to intermediate MR exchange regime⁷. Recently paramagnetic lanthanide (III) (Ln=Eu, Dy, Th, Yb) complexes with DOTA (1,4,7,10 tetraazacyclododecane) have been demonstrated to have two sites of exchangeable protons (amide and coordinated water)². In particular, Yb-DOTA-tetraamide (Yb-DOTAM) shows a substantial CEST effect when its amide protons (~16 ppm upfield from water) are irradiated with a weak magnetic field, resulting in upwards of ~70% water saturation at pH 8.0^{8,9}. Of note, the exchange of protons between amides or coordinated water in lanthanide DOTA-tetraamide chelates with those of bulk water have been well-characterized¹⁰

In this work, we used the exchangeable amide protons of Yb-DOTAM and the basic principles of paraCEST to indirectly transfer, using water chemical exchange, saturated magnetization to proteins. Thus, this methodology, which we have termed solvent saturation transfer to proteins (SSTP) is essentially a two-step process. One, the selective saturation of the Yb-DOTAM amide protons undergoing chemical exchange transfer with water and, two, the transfer of this indirect water saturated magnetization to the amide protons of proteins via their chemical exchange transfer with water. In contrast to water-based magnetization transfer methods such as water NOE, SSTP does not involve the direct excitation of water. As such, resonances with frequencies close to the resonance of water are unaffected. Here we demonstrate the utility of SSTP to acquire structural and functional insights into proteins. First, we show that SSTP can be used to procure structural restraints for protein structure calculations of Ubiquitin and TSG101. We, next, show that inclusion of SSTP restraints with minimal amounts of NMR experimental data (chemical shifts, hydrogen bonds, and H^N and/or H^Y NOEs), provides an improvement in precision and accuracy of the resulting

protein structures, in comparison to those determined without SSTP restraints. Finally, we show that SSTP can be used to probe protein-protein interactions between TSG101 and Ubiquitin. SSTP can resolve site-specific residue interactions, similar to those identified using chemical shift perturbations (CSPs), and is a very sensitive probe of weaker interactions which are, otherwise, difficult to observe with CSPs.

Materials and Methods

Protein Expression and Purification

The cDNA of the human TSG101 UEV domain (1–144) with a TEV cleavable N-terminal Histidine Tag and Ubiquitin were incorporated into pET28b and pET13b vectors, respectively, and transformed into *Escherichia coli* Rosetta2 (DE3) pLysS cells for TSG101 or BL21(DE3) cells for Ubiquitin. For ^{15}N isotope labeling, cells were grown in minimal media with ^{15}N labeled ammonium chloride ($^{15}\text{NH}_4\text{Cl}$) as the sole source of nitrogen at 37 °C until the OD ~ 0.8 at which time protein expression was induced by the addition of ~1mM IPTG (Isopropyl β -D-1-thiogalactopyranoside) and continued overnight at 18 °C. Cells were then harvested with centrifugation at 6000 rpm for 30 minutes. Immediately after harvesting, cell pellets were stored at –80 °C.

Ubiquitin purification—Frozen cell pellets for Ubiquitin were thawed and re-suspended in 50 mM Tris-HCl pH 7.5, containing Roche protease inhibitor. The cell suspension was then passed twice through an EmulsiFlex-C3 (AVISTIN) and centrifuged at ~20000 rpm for 1 hr at 4 °C. The supernatant was then titrated, while stirring on ice, with glacial acetic acid until pH ~ 4 and further stirred for ~2 hours at 4 °C. The resulting mixture was, then, centrifuged at 20000 rpm for 1 hour. The supernatant was collected and dialyzed against 50 mM sodium acetate, pH 4.5 (Buffer A) in Thermo Scientific Dialysis Cassettes 2 kDa MWCO overnight with at least one buffer change and, subsequently, centrifuged at 20000 rpm for 1 hour at 4 °C. The resulting supernatant was next passed over a High Performance (HP) SP sepharose column and washed with 15 column volumes of Buffer A. Ubiquitin was then eluted at ~30% of a linear salt gradient using buffer B (50 mM sodium acetate and 1 M NaCl pH=4.5). Ubiquitin containing fractions were collected, concentrated, and further purified using a Superdex 16/60 G75 Size Exclusion Column equilibrated with 50 mM sodium phosphate, pH 7.

TSG101 purification—For purification of TSG101, frozen cell pellets were thawed and re-suspended in buffer A (20 mM Tris-HCl 100 mM NaCl pH 7.0) containing Roche protease inhibitor. This suspension was then passed twice through an emulsifier EmulsiFlex-C3 (AVISTIN). Cell lysate was centrifuged at ~20000 rpm for 1 hr at 4 °C. The supernatant was then passed over a HP Ni-NTA column pre-equilibrated with Buffer A and washed with 15 column volumes of buffer A. TSG101 was eluted with a linear gradient of imidazole using Buffer B (100 mM Tris-HCl pH 7.5 100 mM NaCl 1 M imidazole). Cleavage of the histidine tag was performed using TEV protease (100:1, protein:TEV ratio) overnight at room temperature. The sample was then passed through a G75 size exclusion column equilibrated with buffer B (100 mM Tris, 100 mM NaCl, pH 7.5, 1 mM EDTA). Purified

TSG101 was buffer exchanged into NMR buffer (20 mM potassium phosphate 50 mM NaCl 0.5 mM EDTA pH 5.8) using Amicon Ultra centrifugal filters (Millipore).

The purity of the TSG101 and Ubiquitin protein samples was checked at each stage of the purification using SDS-PAGE and LC-MS (Agilent 6224 spectrometer equipped with an electrospray ionization (ESI) source and time-of-flight (TOF) analyzer).

Synthesis of Yb-DOTAM-tetraamide complex

Yb (III)-DOTA-tetraamide (Yb-DOTAM) complex was prepared by mixing 1 mol of DOTA-tetraamide (MACROCYCLICS) with 1.1 mol of YbCl₃ at room temperature in 10 ml of water. Since the product of the reaction is acidic, frequent additions of NaOH were required to maintain the optimal reaction pH of 5.5. Excess Yb was then precipitated by increasing the pH to ~7.5. The particulate in solution was pelleted by centrifugation at 14000 rpm for 10 minutes, and then the supernatant was collected and subsequently lyophilized and stored at 4 °C. LC-MS and one-dimensional ¹H NMR were used to confirm the identity of the Yb-DOTAM complex.

NMR Spectroscopy

All NMR measurements were performed with a Bruker Avance-III spectrometer using a triple resonance z-axis gradient cryogenic probe tuned to the proton (¹H) resonance frequency of 600.33 MHz. Prior to SSTP experiments, Yb-DOTAM was added to the protein sample at the desired concentration (see below). The pulse scheme for SSTP is a modified HSQC pulse scheme¹¹, where a selective EBURP¹² excitation pulse of 2.5 ms (0.06W), at the larmor frequency, in the middle of the Yb-DOTAM amide protons at –10.5 ppm, is applied repeatedly, *n* number of times, for optimal saturation of these protons before the first 90° hard pulse on ¹H. This saturation pulse period was shifted “on/off” resonance relative to the Yb-DOTAM amide protons in an interleaved manner via adjustment of the shape pulse offset to a region of the spectrum displaying no peaks at 63 ppm while the saturation pulse power was turned “on” to provide two ¹H, ¹⁵N HSQC’s. Spectra were acquired with 1024 and 128 complex data points. Before and after the saturating pulse the ¹H carrier frequency was set at the water frequency, while that of ¹⁵N was set to 118 ppm for the entire experiment.

Residue specific SSTP was calculated using

$$\text{SSTP} = (1 - I_{\text{on}}/I_{\text{off}}) \quad (1)$$

where *I*_{on} and *I*_{off} are the cross-peak intensities recorded with the saturating pulse “on” and “off” resonance, respectively. To characterize saturation buildup the SSTP was measured at saturation times of 0.25 s, 0.5 s, 0.75 s, 1.00 s, 1.5 s, 2.0 s, 2.5 s and 3.0 s.

For the SSTP TSG101-Ubiquitin interaction experiment, equimolar amounts (1 mM) of TSG101 and Ubiquitin as well as 20 mM Yb-DOTAM were mixed in 50 mM sodium phosphate buffer at pH 7.5. Each sample was placed in a Shigemi NMR tube prior to

experiments. The experiments were performed at 303 K. Data was processed and analyzed using NMRPipe¹³ and SPARKY^{14,15}.

Results

Optimization of Yb-DOTAM concentration in Proteins

Figure 1 shows the ¹H NMR spectrum of Yb-DOTAM dissolved in 50mM sodium phosphate buffer (303 K, pH 5.5). Due to pseudo contact shifts from the Yb paramagnetic center, the DOTA amides resonate at -11 ppm and -14 ppm, at least 16 ppm up field from the bulk water resonance, while the coordinated water of this complex is observed at 97 ppm downfield¹⁶. Enhancements by chemical exchange depend on the concentration of Yb-DOTAM and the rate of exchange. The Yb-DOTAM pH and concentration dependence of Yb-DOTAM on the magnitude of CEST between the DOTA amide and bulk water protons are shown in Supplementary Figure S1. Since, the observed rate of exchange was highest at pH 7.5, all experiments were performed using 50 mM sodium phosphate buffer at this pH.

To optimize the Yb-DOTAM concentration for maximum saturation transfer to proteins, the concentration of Yb-DOTAM was varied from 10 mM to 20 mM in 5 mM increments. The maximum saturation was observed with 20 mM Yb-DOTAM for both Ubiquitin and TSG101, as displayed in Figures 2A and 2B, respectively. Representative ¹H-¹⁵N HSQC spectra of TSG101 with Yb-DOTAM, “on” and “off” resonance of the DOTA amide protons, are shown in Supplementary Figure S2. Residues in the C- and N-terminal as well as loops of the proteins, display high SSTP which most likely results from their participation in fast exchange dynamics in these highly flexible regions. Residue specific saturation is displayed in Figure 2C and 2D at different concentrations of Yb-DOTAM. The trend is not linear and reaches a maximum at 20 mM Yb-DOTAM.

SSTP build up and determination of its rate constant

A series of SSTP's observed at different saturation times were used to produce build-up curves for TSG101 and Ubiquitin. From the fits of these curves to a single exponential, the rate constant which is related to the kinetics of the exchange process was extracted for each residue. The SSTP build-up-curves for Ubiquitin and TSG101 are shown in Figure 3A and 3C, while residue specific rates are shown in Figure 3B and 3D. Residues in exposed and solvent accessible regions of the proteins exhibit transfer efficiencies that are higher than those which are buried. The plateau of saturation was observed at 1.5 s and 2.5 s for Ubiquitin and TSG101, respectively. This differential saturation time of Ubiquitin and TSG101 is expected, since the nuclear longitudinal relaxation time constants (T_1 's) for large molecules (e.g., proteins) increase with molecular weight and the time required for magnetization saturation is proportional to T_1 .

SSTP correlation with solvent accessibility

The solvent accessibility is calculated using an empirical function derived by Wang et al.¹, which is based on the principle that exposed residues tend to have fewer surrounding heavy atoms than those that are buried within a desired cutoff. Specifically, solvent accessibility is

defined as being inversely proportional to the sum of squares of the distances between a specified nuclei and heavy atom,

$$S_{acc}^c = \left[\sum_{i=1}^N \frac{1}{r_i^2} \right]^{-1} \quad (2)$$

where, in our study, r_i is the distance between an amide proton and heavy atom. Solvent accessibilities were restricted to a cut-off distance of 20 Å as described previously¹. To investigate the relationship between solvent accessibility and SSTP, residue-specific values of each were compared using a correlation plot for Ubiquitin and TSG101 shown in Figure 4A and 4C, respectively. The linear regression fit of the experimentally observed SSTP versus calculated solvent accessibility, using the lowest energy NMR structure of Ubiquitin and TSG101^{17,18}, provided the slope and intercept which was used for structure calculations in an iterative manner, as described below, using the following relationship

$$SSTP_{TSG101} = -0.068 + 1.57 S_{acc}^m \quad (3)$$

$$SSTP_{Ubi} = -0.184 + 1.00 S_{acc}^m \quad (4)$$

These initial slope and intercept values were used in the Xplor-NIH potential energy term and further optimized during structure calculations, as described previously¹.

TSG101 and Ubiquitin structure refinement

To test SSTP as a structural restraint in protein NMR structure determinations, it was used for structure refinements of TSG101 and Ubiquitin. Thus, the experimentally observed SSTP data from these proteins (56 and 98 SSTPs for Ubiquitin and TSG101, respectively) were incorporated in simulated annealing calculations using the solvent accessibility potential of XPLOR-NIH¹⁹. This potential is represented by

$$E_{acc} = k (S_{acc}^c - S_{acc}^m)^2 \quad (5)$$

where S_{acc}^c is the calculated solvent accessibility, given by equation (2), and k is the force constant which is varied during structure calculations for the determination of convergence efficiency. All calculations were performed using an extended random coil as the starting structure. During the calculation, temperature was varied from an initial temperature of 3500 K to a final temperature of 25 K in order to reach the global minimum. SSTP restraints were used to determine the correct fold of the proteins using a minimal number of NMR datasets (chemical shifts, H^N and H^γ NOEs, H-bonds).

The inter-proton NOEs were back-calculated using the published NMR structure of TSG101 (1KPP)¹⁷ with a cutoff of 5 Å. Only H^N-H^N and inter-methyl (H^γ) NOEs were extracted, a total of 230, and used for structure refinement of TSG101. Backbone ϕ and ψ dihedral angles and H-bonds restraints were also included in the refinement. In the case of Ubiquitin (1D3Z)¹⁸, a randomly chosen 10% subset (204) of the reported experimental NOEs were used in addition to the backbone ϕ and ψ dihedral angle restraints and H-bonds. The structure refinements were performed with and without SSTP. A total of 100 structures were generated, from which the 5 lowest energy structures were used as a representative ensemble. Accuracy of these generated structures was checked by comparison with the lowest energy NMR structure of TSG101 and Ubiquitin. The force constant was optimized to have maximum convergence and accuracy. To achieve maximum convergence, a coarse grain grid search of the slope and offset was performed in which the slope was varied from 1.37 Å⁻² to 1.97 Å⁻² while keeping the offset constant. Once the slope was optimized, the offset was varied from 0.04 to 0.14 while keeping the optimized slope constant. This process was performed in an iterative manner until acquisition of the lowest energy structures. The optimized slope and offset for TSG101 were 1.77 Å⁻² and 0.12, while those for Ubiquitin were 1.8 Å⁻² and 0.24, respectively. The statistics of the TSG101 structure calculation with and without incorporation of SSTP are given in Table 1. As the structure convergence improved, the correlation between observed SSTP and predicted solvent accessibility increased from a correlation coefficient R of 0.76 to 0.85 shown in Figure 4B and 0.60 to 0.85 shown in Figure 4D for Ubiquitin and TSG101, respectively. This improvement resulted in an increase in precision of structures. The backbone RMSD decreased from 5.17 Å to 2.73 Å and 1.90 Å to 1.76 Å for TSG101 and Ubiquitin, respectively, following SSTP incorporation. In addition, the backbone pair-wise RMSD to the target structure (PDB ID 1KPP) lowered from 4.12 Å to 3.38 Å for the TSG101 structure determined without and with SSTP as shown in Figure 4E and 4F, respectively.

TSG101-Ubiquitin interactions probed by SSTP

The TSG101 UEV domain (1–144) is important for HIV-1 budding and vacuolar protein sorting pathways (VPS). In the VPS pathway, TSG101 binds to ubiquitylated proteins as they are sorted into vesicles which later bud into late endosomal compartments known as multivesicular bodies (MVBs). TSG101-Ubiquitin binding is required for delivering substrates and hydrolytic enzymes to lysosomes which receive proteins by its fusion to MVBs. The crystal structure of TSG101 with Ubiquitin has been solved to ~2 Å resolution where it was shown that TSG101 binds to Ubiquitin with its highly-solvated interface and that mutations in this interface region inhibit MVBs sorting²⁰.

The direct excitation and transfer of bulk water magnetization has, previously, been used by Dalvit et al. to probe weak protein-ligand interactions at submicromolar affinities²¹. Although water is excited indirectly in SSTP, the mechanism by which magnetization is transferred from water to protein should be analogous to that following the direct excitation of water. Also, residues at the interfacial region of a protein-protein (or -ligand) complex are less accessible to solvent and would be expected to display lower solvent accessibilities and, correspondingly, SSTPs. Therefore, considering the above, we hypothesized that SSTP could be used to detect protein-protein interactions at residue-specific levels. To test the

capability of SSTP to probe intermolecular protein-protein interactions, we performed SSTP experiments on TSG101 in the presence and absence of Ubiquitin. SSTP perturbations were measured as the difference of SSTP observed with and without Ubiquitin. We calculated the square of these SSTP perturbations and plotted them against residue number for comparison with CSPs. Using the approach suggested by Williamson, M. P.²² for acquisition of the appropriate cutoff for assigning perturbation significance, TSG101 residues with CSPs or SSTP perturbations greater than 3 times the standard deviation were excluded, followed by recalculation of the standard deviation for the remaining residues. This process was iterated three times to determine the cutoff of TSG101 CSPs and SSTP perturbations which was 0.023 and 0.0005, respectively. TSG101 residues showing significant SSTP perturbations and CSPs are well-matched as noted by grey shades in Figure 5A. The perturbed residues above the cutoffs were mapped onto the TSG101 structure for CSP and SSTP as displayed in Figure 5B and Figure 5C. The SSTP perturbation plot identified a second binding site between Ubiquitin and TSG101 (dark gray shade) which was validated by measuring TSG101 CSP values at 12 folds increased concentration of Ubiquitin (supplementary Figure S3).

Discussion

Yb-DOTAM is a pH responsive probe and can be used over a broad pH range (pH 5.5–8.0). However, a caveat to its use is that DOTA has a propensity to nonspecifically bind with proteins, depending on the solution conditions and protein characteristics, which can result in cross relaxation²³. It has been suggested that modifications to the DOTA moiety and/or an increase in solution ionic strength (e.g., addition of NaCl) are useful approaches to prevent DOTA-protein nonspecific interactions. However, the non-specific binding of Yb-DOTAM to proteins was not observed with the moderate concentrations (10–20 mM) used in our study. The ¹H-¹⁵N HSQC spectra in the presence and absence of Yb-DOTAM were identical. The highest SSTP enhancements occurred at pH 7.5 and 20 mM Yb-DOTAM. Based on the SSTP build-up curves (as shown in Figure 3), the dominant mechanism during the initial stage (< 1.0 s) of SSTP arises from Yb-DOTAM amide exchange with water and negligible SSTP transfer to proteins. At later stages (> 1.0 s) the process is governed by water magnetization transfer to proteins. This SSTP mechanism differs from water-based magnetization transfer methods such as water NOE/ROE where protein amide, H^α, or hydroxyl protons resonating near the water frequency can be saturated from the direct excitation/inversion of water^{24,25}. In the case of SSTP, the amide protons of Yb-DOTAM exchange very efficiently with bulk water and resonate far away (–16 ppm) from the water resonance (–4.7 ppm), making them excellent mediators for the indirect saturation of water without attenuation of nearby exchangeable protein resonances. Moreover, magnetization transfer between water and protein protons occurs, solely, through chemical exchange in SSTP, while in water NOE/ROE it is driven by both chemical exchange and dipolar interactions which can complicate the extraction of protein structural information.

SSTP transfer efficiencies are linearly correlated to solvent accessibilities in proteins. Residues that are in exposed regions of the protein display higher chemical exchange rates than those which are buried. Therefore, SSTP can be used as a structural restraint in protein NMR structure calculations. Considering this, SSTP may find the most utility at the start of

these calculations when insufficient structural restraints are available to acquire the initial fold of a protein. To test this postulate, we performed SSTP experiments and used their resulting SSTP-based structural restraints for structure refinements of TSG101 and Ubiquitin, while using a minimal amount of additional restraints, namely, chemical shifts, H^N/H^Y NOEs and hydrogen bonds. Precision and accuracy of the TSG101 and Ubiquitin structures improved significantly upon incorporation of SSTP restraints. The backbone RMSD of TSG101 and Ubiquitin reduced from 5.17 Å to 2.73 Å and 1.90 Å to 1.76 Å, respectively. Additionally, we have shown that this method is quite useful at identifying residue-specific interactions in protein binding studies. Similar to chemical shift perturbations (CSPs), SSTP perturbations highlighted the residues involved in the TSG101-Ubiquitin interaction. In contrast to CSPs, however, SSTP perturbations were observed at a second and weak binding site between TSG101 and Ubiquitin. The first binding site for TSG101-Ubiquitin has an estimated $K_d \sim 350 \mu M$ ²⁶. Based on its unobservable CSPs at an equimolar ratio of TSG101 and Ubiquitin, the second binding site appears to have a binding affinity that is lower than the protein-ligand binding affinity threshold ($< 1 \mu M$) suggested by Dalvit et al.²¹ using direct water magnetization transfer. Hence, it is quite surprising that SSTP was able to detect this second TSG101-Ubiquitin binding site, which is estimated to have a factor of two lower affinity than the first binding site (unpublished result). The indirect saturation transfer of bulk water magnetization to proteins in SSTP allowed detection of this weak binding site between TSG101 and Ubiquitin, thus demonstrating the high sensitivity of this method for detecting very weak interactions which are undetectable by chemical shift perturbations.

In summary, we have developed a method based on paramagnetic CEST which can be used as a structural restraint in protein structure determinations and provide residue-specific information pertaining to protein-protein interactions. SSTP is more advantageous than water-based NOEs/ROEs which prohibit analysis of protein resonances near that of water. Due to the ease of implementation and data analysis, SSTP finds significant utility during the initial stages of protein structure determination when a minimal number of NMR restraints are available for obtaining the correct fold of a protein. In addition, analogous to CSPs, SSTP perturbations can identify residues involved in protein-protein interactions, even, when they are too weak to be probed by CSPs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. ^1H -NMR spectra of Yb-DOTAM (600 MHz ^1H frequency, 298K) in 50 mM sodium phosphate buffer pH 5.5. The Yb-DOTAM structure is shown in the inset.

Figure 2.

Titration of 1 mM Ubiquitin (A) and 1 mM TSG101 (B) with variable concentrations (5–20 mM) of Yb-DOTAM in 50 mM sodium phosphate buffer pH 7.5. The SSTD was calculated using Equation 1. For illustration, 10 mM and 20 mM are shown in the top and bottom panel, respectively. Residue-specific SSTD is plotted as a function of Yb-DOTAM concentrations for Ubiquitin (C), (Red-Q49, Green-N60, Blue-K63) and TSG101 (D), (Red-S48, Green-S49, Blue-E51). Error bars are the calculated standard deviation between two independent experiments.

Figure 3.

(A) SSTP build-up curves for Ubiquitin (Red-G10, Green-I30, Blue-L43, Black-Q2) and TSG101 (Red-L8, Green-Y15, Blue-T25, Black-S4) is shown for select residues. Saturation time is varied from 0.25s to 2s and 0.25s to 3s for Ubiquitin and TSG101, respectively. (B) SSTP rates plotted against residue numbers for TSG101. Error bars are the calculated standard deviation between two independent experiments.

Figure 4.

The correlation plot of calculated solvent accessibility using equation (2) for the five lowest energy structures of Ubiquitin (A and B) and TSG101 (C and D) with and without SSTP, respectively. The correlation coefficient (R) improved from 0.76 to 0.85 and 0.60 to 0.85 for Ubiquitin and TSG101, respectively, following SSTP restraint incorporation in the structure calculation. Overlay of five lowest energy structures of TSG101 calculated using H^N and H^γ NOEs, dihedral and hydrogen bond restraints in the absence (E) and presence (F) of SSTP. Structure convergence improved and the precision increased from 5.17 to 2.75 Å. Error bars are the calculated standard deviation between two independent experiments.

Figure 5.

(A) CSP (top panel) and SSTP (bottom panel) perturbation plots of the interaction between TSG101 and Ubiquitin. The CSP values were calculated using the formula

$$\delta_{HN} = \sqrt{(\delta H_{TSG} - \delta H_{TSG+UBQ})^2 + 0.1(\delta N_{TSG} - \delta N_{TSG+UBQ})^2}$$
, where δH and δN are the proton and nitrogen chemical shift, respectively. Perturbed residues are highlighted with shades of grey in the plot. The light grey shows the perturbation similarity between CSP and SSTP corresponding to the first Ubiquitin binding site of TSG101. Dark grey denotes the second binding site, not observed by CSP. The calculated cut-off for CSP and SSTP perturbations was 0.023 and 0.0005, respectively. Perturbed residues in the CSP and SSTP plots above the cut-off are mapped onto the TSG101 structure with blue (B) and red color (C), respectively. Perturbed residues were calculated by squaring the difference of SSTP with and without Ubiquitin ($= \text{SSTP}_{\text{TSG101}} - \text{SSTP}_{\text{TSG101:Ubi}}$). Two different views rotated

by 180° are shown for each structure map of TSG101-Ubiquitin. Error bars are the calculated standard deviation between two independent experiments.

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Table 1

Structure statistics of calculations performed with a partial set of restraints in the absence and presence of SSTP for TSG101

Structure Calculation	Restraints Set ^a	Backbone RMSD (Å) ^b	Backbone Pair-Wise RMSD to 1KPP (Å) ^c	Correlation Coefficient (R) ^d
Without E _{acc}	230 NOEs	5.17	4.12	0.60
With E _{acc}	230 NOEs	2.73	3.38	0.85

^aThe “230 NOEs” restraint set corresponds to 225 H^N-H^N and 5 inter methyl H^γ NOEs, 282 dihedral and 79 hydrogen bonds restraints. The total number of SSTP restraints used was 98.

^bThe backbone RMSD was calculated using backbone atoms of residues 1–144 in the five lowest energy structures.

^cThe backbone pair-wise RMSD was calculated from the fit of the average of the five lowest energy structures to the lowest energy structure of TSG101(PDB ID:1KPP).

^dThe correlation coefficient R is the linear correlation between the calculated solvent accessibilities using equation (2) and the measured SSTP values.