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Multi-probe Relaxation Dispersion Measurements Increase Sensitivity to Protein Dynamics

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Abstract

Carr–Purcell–Meiboom–Gill (CPMG) relaxation dispersion measurements are a valuable tool for the characterization of structural transitions on the micro-millisecond timescale. While the measurement of ^{15}N relaxation dispersion is now routine, the measurements with alternative nuclei remain limited. Here we report ^{15}N as well as ^1H R_2 relaxation dispersion measurements of the N23PP/S148A “dynamic knockout” mutant of dihydrofolate reductase. The ^1H dispersion measurements are complementary to ^{15}N data as many additional residues are observed to have dispersive behavior for the ^1H nucleus. Simultaneous fitting of the dispersion profiles for the two nuclei increases the accuracy of exchange parameters determined for individual residues and clustered groups of residues. The different sensitivity of the two nuclei to changes in backbone torsional angles, ring currents, and hydrogen bonding effects provides important insights into the nature of the structural changes that take place during the exchange process. We observe clear evidence of direct and indirect hydrogen bond effects for the ^{15}N and ^1H chemical shift changes in the active-site, modulation of ring current shielding in the CD-loop and backbone torsional changes in a cluster of residues associated with the C-terminus. This work demonstrates the power of combined ^1H and ^{15}N probes for the study of backbone dynamics on the micro-millisecond timescale through the analysis of chemical shift changes.

Graphical Abstract

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Introduction

The study of biomolecular dynamics by nuclear magnetic resonance spectroscopy has advanced significantly over the past decade with the ability to monitor dynamic processes on the microsecond - millisecond timescale using relaxation dispersion measurements like CPMG and $R_{1\rho}$.¹⁻³ Advances in protein labeling techniques and pulse sequences now allow the sampling of dynamic processes at a multitude of sites within the backbone and the side-chains of proteins. Despite this, the majority of studies utilize only ^{15}N single quantum relaxation dispersion measurements. One reason for this is the difficulty in fitting of multiple probes to global kinetic parameters. This difficulty has now been removed with the introduction of programs that allow flexible analysis of multiple data sets at multiple fields (see Morin et al. and references therein).⁴ Another difficulty is the interpretation of the fitted ω values and how to relate the ω to structural changes in the excited state. In the case of ^{15}N , the changes in ω are often in direct response to structural changes in the backbone of the polypeptide chain.^{5, 6} In contrast, for both ^{13}C and ^1H there are significant long-range interactions that can lead to chemical shift changes of these nuclei without any direct structural change for the probe.⁷ In addition to the increased number of structural probes gained by measuring ^1H dispersion, a further advantage is the increased precision of the fitted parameters for faster exchange processes.⁸ Here we apply CPMG relaxation dispersion measurements to the ternary E:folate:NADP⁺ complex of the N23PP/S148A “dynamic knockout” mutant of E. coli DHFR,⁹ which does not undergo the classic closed-to-occluded conformational transition observed in the wild type enzyme.^{10, 11} We report the fitting of both ^{15}N and ^1H single quantum relaxation dispersion data collected at two magnetic field strengths for the N23PP/S148A mutant DHFR for backbone amide and side-chain indole probes and discuss the sensitivity of the ^1H and ^{15}N probes to different structural changes. In order to calculate structures of the excited states of proteins, it is necessary to determine the sign of ω , since only the absolute value of ω is obtained from CPMG dispersion experiments.^{12, 13} Unfortunately, for DHFR complexes, the sign of ω can be determined only for a small number of residues, especially in the case of ω_{H} .¹⁴ Despite this we demonstrate that detailed structural insight can be gained from the absolute chemical shift changes for multiple nuclei when analyzed on the basis of knowledge of the structure of the ground state. Classical analysis of chemical shifts using chemical shift hypersurfaces to relate chemical shifts deviations from random coil values to variations in structural parameters, like backbone torsion angles, hydrogen bonding, ring currents and side-chain torsions gives insight into the structural changes taking place. We conclude that ^1H dispersion profiles are an important complement to ^{15}N dispersion profiles and that they allow both more precise determination of kinetic parameters as well as deeper insights into the array of dynamic processes and structural changes that can be detected.

Method

Sample preparation

Uniformly ^{15}N , ^{13}C labeled and ~68% randomly deuterated samples of E. coli DHFR N23PP/S148A were prepared by expression of a 6His-SUMO fusion protein in M9 media containing ^{13}C glucose, $^{15}\text{NH}_4\text{Cl}$ and $(^{15}\text{NH}_4)_2\text{SO}_4$ with 68% recycled deuterium oxide.

Cells were harvested and lysed with 0.01 mg/mL DNase and 0.25 mg/mL Lysozyme in 50 mL of lysis buffer containing 50 mM Tris pH 8, 500 mM NaCl, 5 mM MgCl₂, 5 mM 2-Mercaptoethanol, 0.2 % v/v Triton X-100, to which EDTA-free protease inhibitor cocktail was added (Pierce). Protein was first affinity purified using Nickel NTA with 50 mM Tris pH 8.0 buffer containing 250 mM NaCl and eluted using an imidazole gradient. The protein was concentrated and exchanged into fresh buffer containing 1M urea and incubated overnight at 25 °C with recombinant 6His-ULP1 protease at a 1:5000 molar ratio to cleave off the affinity tag. The 6His-ULP1, the affinity tag, as well as any uncleaved protein, were removed by a second Nickel NTA affinity purification step. The cleaved E. coli DHFR N23PP/S148A was further purified by size exclusion chromatography in NMR buffer (50 mM phosphate buffer pH 6.8 containing 100 mM KCl, 1 mM EDTA, 1mM DTT, 0.02 % NaN₃). The protein fractions containing DHFR were concentrated and stored at -80 °C. NMR samples of ~1.5 mM N23PP/S148A DHFR were exchanged into fresh NMR buffer containing 20 mM NADP⁺, 12 mM Folic Acid, and 10 % D₂O in amber tubes as previously described.¹⁰

NMR experiments and processing

Standard HNCA, HN(CO)CA, HNCACB¹⁵ and CC(CO)NH¹⁶ triple resonance assignment experiments for deuterated samples of the E:folate:NADP⁺ complex of N23PP/S148A DHFR were used to confirm the backbone amide assignments. The assignment of the indoles was confirmed using the pattern of NOE peaks in a 3D (¹H)-¹⁵N-HSQC-NOESY-¹H-¹⁵N HSQC experiment.¹⁷ All relaxation dispersion experiments were recorded at 301 K and spectrometer frequencies of 500 MHz and 800 MHz. The ¹⁵N and ¹H CPMG relaxation dispersion profiles were recorded using pulse sequences appropriate for protonated samples in an interleaved fashion as previously described by Oyen et al.¹⁴ using Poisson gap nonuniform sampling in the indirect dimension.¹⁸ Briefly, a series of scan interleaved CPMG experiments were recorded as a pseudo-3D experiment with 60 % NUS sampling of the full complex data matrices of size 1024 x 128 with 16 scans per FID and a recycle delay of 3.0 seconds. Values of 1/τ_{cp} between 100 Hz and 2000 Hz were recorded for ¹⁵N CPMG, while values of 1/τ_{cp} between 100 Hz and 3200 Hz were recorded for ¹H. Each pseudo 3D experiment took ~1.5 days to complete. NUS data was reconstructed using compressed sensing and virtual echo with MDDNMR^{19, 20} and all data were processed using NMRPipe.²¹

CPMG data analysis

Peaks intensities were obtained from parabolic fitting using CCPNMR analysis.²² Relaxation dispersion curves were fitted using the program Glove²³ with numerical simulation of the Bloch-McConnell equations.^{23, 24} During individual fits the upper limit on p_B was restrained to be 10 %, while for k_{ex} the upper limit was restrained to 12000 s⁻¹.

Results

Throughout the results and discussion we will refer to residues by the residue numbering of the wild-type protein, to minimize confusion when comparing the wild type and mutant proteins. Using this numbering scheme, position 23 in N23PP/S148A is actually a proline

dipeptide but these prolines are “invisible” in the current NMR experiments since they lack an amide proton.

Initial data inspection

While 100% of the observable backbone amides could be assigned, not all could be used for relaxation dispersion analysis. Ten residues (A29, E48, L110, V119, D127, D132, S135, V136, D142, A145) could not be used due to severe overlap in the spectrum, while two residues (G56, H124) were excluded as their cross peaks were too weak in the CPMG experiments.

Initial inspection of the CPMG data for N23PP/S148A shows that relaxation dispersion is observed for many more ^1H probes than for ^{15}N . In Table 1 we present the counts of residues that have exchange contributions $R_{ex} > 3.0$ Hz at 18.8T, as indicated by differences in R_2 values measured at $1/\tau_{cp} = 100$ Hz and 2000 Hz pulsing frequencies for ^{15}N and between $1/\tau_{cp} = 100$ Hz and 3200 Hz for ^1H . Based on this criterion, there are 14 residues that show ^{15}N dispersion and 23 residues that exhibit ^1H dispersion, with “two-point” $R_{ex} > 3.0$ Hz. The same trend is observed if a cutoff of 5.0 Hz is used for R_{ex} , however the number of dispersing residues in each case is about half. Therefore, regardless of the chosen cutoff, there are approximately twice as many residues dispersing in proton as there are in nitrogen. To estimate the exchange timescale of the relaxation dispersion for each probe we calculate the α' value described by Millet et al which approximates α .²⁵ Fast exchange on the chemical shift timescale restricts α to the range $1 < \alpha < 2$, while for slow exchange α is restricted to the range $0 < \alpha < 1$. For intermediate exchange on the chemical shift timescale ($k_{ex}/\omega = 1$) $\alpha = 1$.²⁵ Values of α' between zero and one are indicative of slow chemical exchange, while values larger than one are usually indicative of fast exchange. Values of around one are consistent with intermediate exchange.²⁵ We observed only one residue (Y128) with $\alpha' < 1$ for the backbone, while 11 have values of $\alpha' < 2$ (see Table 1). Two of the five tryptophan indoles have appreciable ^{15}N dispersion, while three indoles exhibit ^1H dispersion. Only one of the tryptophan (W47) indoles had an α' value < 1 indicative of slow exchange for ^1H dispersion, while none of the indoles were judged to be in slow exchange for ^{15}N .

Fitting of two probe data for two fields

Excluding the residues that have R_{ex} less than 3.0 Hz for both of the nuclei, there are 30 residues that show dispersion in the backbone of N23PP/S148A mutant plus three tryptophan side-chains that exhibit indole NεH dispersion. Of the 30 backbone amides, 7 display only ^{15}N dispersion, 7 have both ^1H and ^{15}N dispersion, while 16 residues exhibit only ^1H dispersion. This indicates that there is complementary information to be gained by combined analysis of these two probes. Fitting the 30 backbone and 3 tryptophan indole dispersion curves for individual ^{15}N - ^1H pairs using the Bloch-McConnell equations,²⁴ we find that the exchange parameters for more than half of the residues are under-determined and the values of the fitted p_B converge to the artificially imposed upper limit of 10 %. These residues have rather featureless dispersion profiles, which increase monotonically and do not exhibit turn over at the slow pulsing limit, from which robust fits of k_{ex} and p_B cannot be obtained; we note that if the upper limit on p_B is increased to 49 %, then the fits for these

residues converge to this higher value. Those residues that individually have values of p_B significantly below the upper limit are the amides of V10, E11, V13, F31, T73, G97, Y128, E129, D131, W133, E134, and R158. In addition the dispersion profiles of the tryptophan indoles of W30, W47, and W133 also appear to be well defined with values of p_B that are below the fitting threshold of 10 %. In figure 1 we show representative dispersion profiles for the E:folate:NADP⁺ complex of N23PP/S148A DHFR that illustrate the high sensitivity of the ¹H probe even in the absence of any discernable ¹⁵N dispersion.

In figure 2 we show the location on the X-ray structure for residues that exhibit relaxation dispersion. The residues with large R_{ex} were observed to cluster at three distinct locations within the protein. These groupings were validated by ensuring that the clustered global reduced χ^2 was no more than two times larger than the individually fitted reduced χ^2 values. The most distinct cluster is composed of residues W47, R52, G67 and T73 in the CD-loop and the dispersion curves were fit to a k_{ex} of 3100 s⁻¹ and p_B of 2.1 %. A second distinct cluster contains residues K109, Y128, E129, D131, W133, E134, L156, E157, and R158. Global fitting of the dispersion curves for this cluster, previously referred to as the C-terminal associated cluster in other DHFR complexes, yielded k_{ex} of 630 s⁻¹ and p_B of 3.3 %.^{10, 26} Finally the active-site cluster, with k_{ex} of 4200 s⁻¹ and p_B 4.2 %, is composed of residues A6, A7, L8, A9, V10, E11, R12, V13, W22, W30, F31, G97, R98, Y111, L112, I115, F125, and C152. The fitted parameters and errors determined by Monte Carlo simulation for all fitted clusters are presented in table 2.

Comparison of multi- and single-probe fitting

We compared the results obtained by fitting the two field data for proton and nitrogen probes individually with those obtained by simultaneously fitting dispersion curves for both nuclei. The fitted parameters and errors estimated from Monte Carlo simulations are shown in table 2. Fitting all of the ¹⁵N data to a global k_{ex} and p_B yields an apparent rate of 910 +/- 20 s⁻¹ with a p_B of 3 %. This value is very much smaller than the global k_{ex} (2970 +/- 80 s⁻¹) fitted to the ¹H dispersion curves, while the fitting of both ¹H and ¹⁵N dispersion curves simultaneously results in an intermediate value of 1850 +/- 30 s⁻¹. Moreover, the average $\chi^2_{\text{clust}}/\chi^2_{\text{indiv}}$ is 2.0 for the ¹⁵N data alone, while for the ¹H data the value is ~ 3. For the two probes fitted together the $\chi^2_{\text{global}}/\chi^2_{\text{individual}}$ is still larger than 2.0, indicating that the fit is not adequate. For residues in the C-terminal associated cluster, it is clear once again that fitting the ¹H and ¹⁵N dispersion curves individually gives rise to different p_B and k_{ex} values. The fitted parameter values for the ¹⁵N data are within error of the value determined when fitting both probes together, however the k_{ex} is higher and the p_B is lower. In figure 3 we show two-dimensional histograms of Monte Carlo error fitting simulations for a single residue, Y128 in the C-terminal associated cluster. The fits to ¹H or ¹⁵N dispersion profiles alone give rise to a broad range of exchange parameters. It is evident that the fitted parameters k_{ex} and p_B are highly correlated over the range of fitted values ($\rho = -0.95$ for ¹⁵N and $\rho = -0.98$ for ¹H). Fitting of both probes simultaneously for Y128 reduces the variability in the exchange parameters, however the degree of correlation between the exchange parameters remains largely unchanged ($\rho = -0.97$ for ¹⁵N and ¹H). When the dispersion profiles for all residues in the C-terminal associated region are fitted together the correlation is slightly weaker ($\rho = -0.89$) and k_{ex} and p_B are better defined. It is interesting to note that

the exchange parameters obtained by fitting the ^1H and ^{15}N data simultaneously for Y128 differ from those obtained by fitting to the dispersion curves for all residues in the C-terminal cluster. This illustrates that even a curve that displays turn over can be fitted to a broad range of exchange parameters.

Figure 4 shows the distribution of p_B and k_{ex} from the Monte Carlo error simulations for the C-terminal associated, active-site, and CD-loop clusters. In the case of the C-terminal associated cluster it is clear that fits to both the ^{15}N and ^1H probes individually yield precise estimates of the exchange parameters p_B and k_{ex} even though the actual values differ. The observed differences between the two probes are similar to the variation observed for the individual fitting of Y128 and the average $\chi^2_{\text{clust}}/\chi^2_{\text{indiv}}$ is improved when the data for the two probes are combined (see Table 2). In contrast to the C-terminal associated region, no ^{15}N dispersion was observed for the CD-loop. However, the observed ^1H dispersion allowed the rate and population of the CD-loop cluster process to be determined. Finally for the active-site cluster, the k_{ex} values obtained by fitting the dispersion curves for ^{15}N and ^1H individually are similar to that obtained when the two probes are fitted simultaneously. However, the resulting values of p_B are quite different, with a very large population ($> 10\%$) predicted by fitting the ^{15}N data alone. A tendency to converge towards unreasonably large values of p_B and/or k_{ex} when fitting dispersion profiles with Glove is symptomatic of an underdetermined system and is often encountered when probes are in fast exchange. The results of the Monte Carlo simulations in figure 4 show that k_{ex} and p_B for this cluster are not reliably defined by the ^{15}N dispersion data. Indeed, for both the CD-loop cluster and the active-site cluster, where values of k_{ex} are $\gg 2000\text{ s}^{-1}$, the inclusion of the ^1H dispersion data is essential for robust determination of k_{ex} and p_B values.

Chemical shift changes

The relative magnitudes of ω_N and ω_H derived by fitting the dispersion profiles provide insights into the nature of the structural changes between the ground and excited states in each cluster. In figure 5A we map residues of the C-terminal associated cluster that have large values of $|\omega|$ onto the X-ray structure of the folate: NADP $^+$ complex of N23PPS148A.²⁷ The residues that disperse are colored by the magnitude of the change in chemical shift (ω) between the ground and excited states. Both ω_N and ω_H are large for the backbone amides of residues in the loop between 128 and 133. In addition, the indole $\text{N}\epsilon$ of W133 also has an appreciable ω_N , as does R158.

In contrast, the majority of residues in the active-site cluster show only weak or no ^{15}N dispersion yet have prominent ^1H dispersion. Indeed, the widespread nature of the conformational fluctuations in the active-site region could not be recognized from measurements of ^{15}N dispersion alone⁹ but only becomes evident when ^1H dispersion profiles are recorded. The ω_N and ω_H values for residues in the β -sheet adjacent to the cofactor-binding site are summarized in figure 5B. The patterns of the chemical shift changes between ground and excited states provides important insights into fluctuations in the hydrogen bonding network of the β -sheet (see Discussion).

The CD-loop cluster is also different to the C-terminal associated region in that essentially no backbone ^{15}N dispersion is observed. ^1H dispersion for residues W47, R52, G67 and T73

as well as a small $|\omega_N|$ of ~ 1 ppm from the indole nitrogen of W47 are the only indications that a structural transition is taking place. In figure 6 we show this region of the structure, close to the CD-loop where two tryptophan side-chains are located. Interestingly, two of the amides in this cluster, G67 and T73, lie above and below the aromatic ring of W74, while the R52 amide is close to (within 5 Å) the aromatic ring of W47.

Discussion

Here we have reported the measurement of relaxation dispersion for both nitrogen and proton nuclei that report on the backbone amide groups in N23PP/S148A E. coli DHFR as well as the indole side-chains of tryptophan residues. We have observed that twice as many residues exhibit significant R_2 relaxation dispersion ($R_{ex} > 5$ Hz) for ^1H compared to the more frequently measured ^{15}N probe. The ^1H nucleus is observed to be a very sensitive probe of conformational exchange, often with a larger number of curves that turn over at small values of $1/\tau_{cp}$. To obtain accurate and robust measures of kinetic parameters from fits of CPMG dispersion profiles, it is critical to have curves that “turn over” i.e. in slow exchange and for which values of ω (in Hz) are greater than the lowest pulsing frequency used in the experiment. ^1H dispersion measurements are a valuable complement to ^{15}N dispersion as the exchange contributions (R_{ex} values) are often larger than for ^{15}N , and ^1H dispersion is often observed in cases where ^{15}N dispersion profiles are flat. Indeed, less than 1/3 of all dispersing residues in the N23PP/S148A DHFR complex studies here had R_{ex} greater than 3 Hz for both probes. This leads to a larger number of residues that can be analyzed when considering the two probes together. One prominent example of this is the CD-loop cluster, where no dispersion was observed for ^{15}N probes and the exchange parameters p_B and k_{ex} could only be determined using the ^1H dispersion data. This is not to say that ^1H is a substitute for ^{15}N dispersion; as we have shown here, when ^1H and ^{15}N are used in combination they increase the number of residues that can be accurately analyzed. This is shown clearly by comparing the number of residues in slow exchange for the two probes considered separately and when combined. Only 7 residues in each of the separate backbone ^1H and ^{15}N dispersion sets had $\alpha' < 2$, while the combined datasets contained 11 different residues in slow exchange.

The combined analysis of both ^{15}N and ^1H dispersion profiles at two fields improves parameter estimates for clustered processes. This is especially true of the fast processes that are observed for the active-site cluster and the CD-loop cluster, for which reliable fits could not be obtained from ^{15}N dispersion data. ^1H proton dispersion is more sensitive to these fast processes than ^{15}N dispersion, with a larger number of curves that turn over, allowing more robust fitting of exchange models (k_{ex} , p_B). These observations agree with the previous theoretical work of Kovrigina et al., where it was observed that combined fitting of relaxation dispersion data measured at multiple field strengths improved the estimates of the conformational exchange parameters.²⁸ Related work from Ishima and Torchia has shown that global fitting of data leads to improved estimates of the conformational exchange parameters.²⁹ An alternative to measuring dispersion for multiple nuclei is the measurement of dispersion at multiple temperatures; while this approach has been applied with success,^{26, 30} there is always a concern that additional processes may be observed at different temperatures. Similarly it has been observed that global fitting of data from zero,

single, double, and multiple quantum ^{15}N and ^1H relaxation dispersion experiments increases the accuracy of the globally determined exchange parameters.³¹ Throughout this work we have fitted data from two static magnetic fields as it has previously been shown that large variability in fitted parameters results when only data from a single field are fitted.^{28, 32} Even with two field data, we observed that the range of exchange parameters that could be fitted was large when either ^1H or ^{15}N probes were fitted individually, as illustrated in figure 3 for residue Y128. The situation is improved somewhat by fitting both nuclei simultaneously, however there remains a large variability in the exchange parameters caused by the significant degree of correlation between k_{ex} and p_B . Thus, when fitting dispersion data for individual residues, even a small amount of error in the measured dispersion curves can give rise to an inability to determine precise exchange parameters. However, by simultaneously fitting dispersion profiles for clusters of residues that experience the same exchange process, the exchange parameters can be defined more robustly and with greater precision as also pointed out by Ishima and Torchia.²⁹ This is clearly the case for the fitted parameters of the C-terminal associated cluster, where the variability in k_{ex} and p_B observed in fitting data for single residues is greatly diminished when cluster fits are performed, as is typified by Y128 (figure 3). Additional benefits to routine measurement of ^1H relaxation dispersion are that higher effective pulsing frequencies can be used, which increases the sensitivity of CPMG measurements to faster processes.^{8, 31} As was seen for the CD-loop process, the ^{15}N dispersion data alone was unable to estimate the global k_{ex} or p_B , while for the active-site region the p_B could not be precisely estimated. The ability to fit the parameters p_B and k_{ex} accurately, and thus obtain reliable measures of ω for each residue, is due to two factors: the higher effective pulsing frequencies that were used for the ^1H dispersion experiments, and also the relatively larger ω values (in Hz) associated with ^1H probes. For example, a 1 ppm chemical shift difference corresponds to a frequency difference of 50 Hz for ^{15}N and 500 Hz for ^1H at a field of 11.7 T. The typical residue specific chemical shift deviation in folded proteins for ^{15}N is ~ 4 ppm, while for ^1H it is ~ 0.7 ppm.³³ Combining this with the frequency differences, we see that a 0.7 ppm chemical shift change at 11.7 T is equivalent to 350 Hz, while the corresponding chemical shift change of 4 ppm at 11.7 T is equivalent to 200 Hz change in frequency. Because ω values (in Hz) associated with conformational changes are on average larger for ^1H than for ^{15}N , proton relaxation dispersion curves are often better shaped and more intense (larger R_{ex}), allowing more robust fitting of the kinetic parameters. We note that this approximate two-fold increase for ^1H over ^{15}N is consistent with our observed greater number of residues in slow exchange for ^1H .

Structural insights from ω

The differing sensitivity of ^{15}N and ^1H chemical shifts to molecular environment means that ^1H and ^{15}N often report on quite different processes. While ^{15}N chemical shifts are relatively more sensitive to the ϕ and ψ dihedral angles,^{34–37} ^1H chemical shifts are more sensitive to ring currents.^{38–41} That ^1H and ^{15}N are sensitive to different processes may mean that multiple processes are detected. This in turn may require three or four state models to fit the dispersion profiles.^{31, 42, 43} Both ^1H and ^{15}N chemical shifts are also very sensitive to hydrogen bonding through direct and indirect effects.^{34, 44} Much insight can be gained from consideration of the absolute chemical shift changes in the context of the

known structure. For this purpose we consider chemical shift hypersurfaces to be invaluable as they directly relate chemical shift changes to specific structural perturbations.

Changes in backbone dihedral angles

Historically, the majority of relaxation dispersion studies have relied on ^{15}N relaxation only, despite the technical advancements that allow the measurement of dispersion data for many different nuclei throughout proteins. One reason for this is that the structural determinants of ^{15}N chemical shifts are well understood from both theoretical and statistical studies. In general the major contribution is from the backbone conformation characterized by the ϕ and ψ angles, with hydrogen bonding, side-chain orientation, and neighboring residue effects being relatively less important.^{34–37} Changes in ^{15}N chemical shift are largely dominated by backbone dihedral angle changes, and once the random coil contribution is factored out, the contribution is estimated to be 50 % of the observed chemical shift variation.⁷ For the E:folate:NADP⁺ complex of N23PP/S148A DHFR, the largest changes in ^{15}N chemical shift are observed within the C-terminal associated cluster. The changes in ^{15}N ω of >1 ppm for residues 128, 129, 131, 133, 134, and 158, in combination with relatively small changes in ω_{H} for all residues except D131, indicate that there are large changes in the backbone dihedral angles during the exchange between the ground and excited states. In contrast, large changes in ω_{H} , especially in conjunction with little or no change in ^{15}N chemical shift, would be indicative of modulation of hydrogen bonding or possibly ring current effects. The majority of dispersing residues in the C-terminal associated cluster are within the extended region of the Ramachandran plot with the only exception being 131, which is in the proximity of the 3_{10} helix region. Examples of hypersurfaces for the ^{15}N and ^1H backbone chemical shifts projected onto the Ramachandran diagram are shown in figure 7. Even relatively small changes in the ϕ or ψ dihedral angles between the ground and excited state will result in substantial changes in ^{15}N chemical shift that will be reflected in the dispersion profiles.

Changes in hydrogen bonding

Even though, ^{15}N dispersion essentially reports on changes in backbone dihedrals, the ^{15}N nucleus is also sensitive to hydrogen bonding. The contribution from hydrogen bonding to the observed chemical shift is small, and once the random coil contribution is factored out, hydrogen bonding contributes an estimated 10 % to ^{15}N chemical shift variation.⁷ However both making and breaking of hydrogen bonds as well as changes in hydrogen bond geometry can lead to changes in ^{15}N chemical shifts. Direct hydrogen bond chemical shift effects are mediated via the amide proton and can be on the order of 1–2 ppm, while indirect hydrogen bond effects mediated by hydrogen bonding to the carbonyl within the same peptide plane can result in changes in ^{15}N chemical shift as large as 5 ppm.³⁴ Both direct and indirect effects are highly distance dependent.³⁴ The effects of hydrogen bonding on amide proton chemical shifts can be large and have a strong dependence on the hydrogen bond length^{40, 45} and angle.⁴⁴ Even a change in NH---O hydrogen bond length as small as 0.2–0.3 Å can result in amide ^1H chemical shift changes of 1 ppm or greater. Chemical shift hypersurfaces showing the dependence of the amide proton chemical shift on hydrogen bond distance and angular properties can be found in Moon and Case.⁴⁴

In the active-site cluster, the largest changes in chemical shift are for residues 7–12 and residues 30–31 and 111–112 in the ligand-binding region. Even though large nitrogen dispersion is observed, it is unlikely that large changes in the backbone dihedral angles are involved in the exchange process, given that many of the residues in this cluster form part of the central β -sheet. The chemical shifts changes are unlikely to be caused by modulation of the ring currents as ring current contributions are very small for nitrogen.⁴¹ Instead, it seems that likely, based on the pattern of amides that exhibit dispersion (figure 5B), that small fluctuations in hydrogen bond geometry are involved. All of the peptide groups that exhibit dispersion are involved in hydrogen bonding interactions within the β -sheet or to the carboxamide group of the NADP⁺ cofactor. All of the amide protons that exhibit ¹H dispersion are involved in direct hydrogen bonding interactions (figure 5B). The observed changes in chemical shift, $|\omega_H| = 0.1$ to 0.3 ppm, are consistent with small fluctuations in hydrogen bond length of the order of $0.1 \text{ \AA}$ ⁴⁴ or subtle changes in hydrogen bond angle between ground and excited states. The amide ¹⁵N chemical shift changes associated with fluctuations in the direct hydrogen bond are relatively small; for example, $|\omega_N| = 0.3$ ppm for I115, 1.1 ppm for A6, and 1.0 ppm for A7 (figure 5B). In contrast, changes in hydrogen bonding to the carbonyl oxygen lead to much larger changes in ¹⁵N chemical shift for the amide nitrogen in the same peptide plane.³⁴ This is exemplified by the ¹⁵N chemical shift changes observed for L8, A9, and L112 ($|\omega_N| = 1.6, 2.6, \text{ and } 1.6$ ppm, respectively), which probably reflect subtle changes in the A6 NH – Y111 CO, L8 CO – I115 NH, and A7 CO – carboxamide hydrogen bonds during the transition to the excited state (figure 5B). Thus, the observed changes in ¹⁵N and ¹H chemical shifts can be rationalized in terms of modulation of the hydrogen bonding and are consistent with the previous observations of Ishima and Torchia who observed proton and nitrogen dispersion consistent with hydrogen bond modulation within the β -sheet of HIV-1 protease.^{8, 46}

Changes in ring current shielding

¹H chemical shifts are highly sensitive to ring currents, in contrast to the ¹⁵N chemical shift.⁷ As a consequence, ¹H dispersion can be mediated by changes in ring currents associated with fluctuations in the position of aromatic side-chains. Robusteilli et al.⁴⁷ observed that conformational heterogeneity for aromatic rings is largely responsible for the disagreement between chemical shifts calculated from molecular dynamics simulations and from static X-ray crystal structures. The ring-current contribution is well understood and can be calculated using the empirical Haigh-Mallion model that is accurate to ~ 0.1 ppm for amide protons.^{48, 49}

The relaxation dispersion observed for residues in the CD-loop is particularly striking. The backbone amides of R52, G67, and T73 show ¹H dispersion ($\omega_H = 0.2 - 0.4$ ppm) but their ¹⁵N dispersion profiles are flat, suggesting that the transition between ground and excited state does not involve a change in the average backbone conformation. ¹H dispersion ($\omega_H = 0.9$ ppm) is also observed for the W47 indole NεH. Since none of these NH protons are hydrogen bonded in the X-ray structure, fluctuations in hydrogen bond interactions would appear to be an unlikely source of the chemical shift modulation. A more likely origin of the ¹H chemical shift changes is fluctuation in ring currents. All of these NH protons lie within the ring current fields associated with the aromatic rings of W47 and W74 (figure

6B), such that even subtle changes in the orientation of one or both of the tryptophan side-chains would lead to altered chemical shifts in the excited state.

Conclusions

Although the N23PP/S148A mutation abrogates the closed –occluded conformational transition observed in wild type *E. coli* DHFR, the combined application of both ^{15}N and ^1H relaxation dispersion reveals a rich landscape of conformational fluctuations. The use of multiple spin probes improves our ability to detect conformational changes by relaxation dispersion measurements because the sensitivity of different nuclei to changes in the molecular environment differ. In addition the combined analysis of dispersion data for multiple nuclei, here ^{15}N and amide ^1H , within the same peptide plane increases both the accuracy and precision of the globally fitted exchange parameters, allows more robust separation of dynamic processes into appropriate clusters of residues, and provides more detailed insights into the structural changes associated with the exchange processes. Given the generally larger value of ω (in Hz) for ^1H , measurement of ^1H dispersion provides access to faster time scales that tend towards the μs time regime. Finally, using the wealth of knowledge from theoretical studies that have established empirical relationships between structure and chemical shifts, it is possible to start to dissect out the separate processes to determine the structural features of the higher energy species and physical nature of the barriers that separate them.

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Figure 1.

Representative curves for the identified clusters showing that the ^1H probe is more sensitive to the exchange processes, often with a larger R_{ex} term and larger degree of “turning over” consistent with slow exchange. A9-amide (active-site cluster), W47-indole and G67 (CD-loop cluster), G95 and G121 (closed to occluded transition markers), and Y128-amide (C-terminal associated cluster). Fitted curves shown are for the cluster fits. Color code: red 500MHz- ^{15}N , black 800MHz- ^{15}N , blue 500MHz- ^1H , green 800MHz- ^1H .

Figure 2.

Structure of the N23PP/S148A knockout mutant and the clustering of residues. The active-site cluster and the top cluster from visual clustering of residues (left). Probes pairs with $R_{ex} > 3.0$ Hz shown as spheres and the color of the sphere indicates the magnitude of R_{ex} (white/green for proton and blue/red for nitrogen). The C-terminal associated cluster (right) is shown for the reverse side of the protein.

Figure 3.

Two dimensional histograms showing the distribution of p_B and k_{ex} for Y128 using ¹H and ¹⁵N dispersion alone and together from 200 Monte Carlo simulations. In the last panel the improvement of clustering residues is shown with the distribution of p_B and k_{ex} determined by fitting all probes and residues within the C-terminal associated cluster together.

Figure 4.

Two dimensional histograms showing the distribution of p_B and k_{ex} for the C-terminal associated cluster, the CD-loop and the active-site cluster from 200 Monte Carlo simulations showing the improved accuracy in fitted parameters and the reduction in correlation between the fitted parameters when multiple probes are fitted together.

Figure 5.

Structure of the C-terminal associated region showing the loop region and the active-site showing where chemical shift changes are observed. The chemical shift range is shown from 0 to 0.5 ppm for proton atoms and 0 to 2.5 ppm for nitrogen atoms as this range is visually helpful to discern the approximate changes in the structure within the backbone of the protein. Chemical shifts that are larger than 0.5 or 2.5 ppm take the maximum value for proton and nitrogen respectively. This range is also consistent with the range of δ observed for empirical chemical shift hypersurfaces for these nuclei.⁵⁰

Figure 6. Structure of the CD-loop cluster showing the positions of the dispersing residues and the approximate extent ring currents caused by side-chains of residues W47 and W74 on the surrounding atoms. Coloring of the spheres is the same as for figure 4.

Figure 7.

Ground state dihedral angles of the C-terminal associated cluster residues with large values of $|\omega_{\text{N}}|$ mapped to the ^{15}N (a) and ^1H (b) empirical chemical shift hypersurfaces taken with permission from.⁵⁰ The green shaded regions indicate the allowed region of the Ramachandran plot.

Table 1

Classification of dispersion curves for the amide pairs of *E. coli* DHFR N23PP/S148A. R_{ex} calculated from the highest and lowest CPMG frequencies at spectrometer frequency of 800 MHz. The value of α' in the range 0 – 2 indicates the approximate chemical shift timescale. Counts for the indoles are given in brackets.

	Number of dispersing residues		
	^{15}N only	^1H only	^{15}N and/or ^1H
$R_{ex} > 3.0$ Hz	14(2)	23(3)	30(3)
$R_{ex} > 5.0$ Hz	8(2)	17(3)	20(3)
$\alpha' < 2.0$	7(0)	7(3)	11(3)
$\alpha' < 1.0$	1(0)	0(1)	1(1)

Table 2

Fitted parameters for clustered fitting of the CPMG data. Error values (in brackets) were determined from Monte Carlo simulations.

	Clusters			
	all	C-terminal associated	CD-loop	active-site
<i>fitting only ^{15}N dispersion curves</i>				
k_{ex}	910 (20)	610 (20)	--	4600 (300)
p_B	2.95 (0.05)	3.69 (0.08)	--	10 (2)
χ^2	2.79	1.98	--	1.58
$\chi^2_{\text{clust}}/\chi^2_{\text{indiv}}$	2.00	1.63	--	1.12
<i>fitting only ^1H dispersion curves</i>				
k_{ex}	2970 (80)	920 (40)	3000 (200)	4200 (200)
p_B	2.24 (0.05)	2.36 (0.08)	2.13 (0.06)	4.1 (0.6)
χ^2	1.81	0.87	0.78	0.84
$\chi^2_{\text{clust}}/\chi^2_{\text{indiv}}$	3.06	1.48	1.12	1.48
<i>simultaneous fitting of both ^{15}N and ^1H dispersion curves</i>				
k_{ex}	1850 (30)	630 (20)	3100 (200)	4200 (100)
p_B	2.37 (0.03)	3.32 (0.07)	2.13 (0.06)	4.2 (0.7)
χ^2	2.72	1.56	1.28	1.13
$\chi^2_{\text{clust}}/\chi^2_{\text{indiv}}$	2.59	1.39	1.03	1.17