Millisecond Protein Folding Studied by NMR Spectroscopy

Markus Zeeb and Jochen Balbach

Laboratorium für Biochemie, Universität Bayreuth, D-95440 Bayreuth, Germany.

Abstract: Proteins are involved in virtually every biological process and in order to function, it is necessary for these polypeptide chains to fold into the unique, native conformation. This folding process can take place rapidly. NMR line shape analyses and transverse relaxation measurements allow protein folding studies on a microsecond-to-millisecond time scale. Together with an overview of current achievements within this field, we present millisecond protein folding studies by NMR of the cold shock protein CspB from Bacillus subtilis.

Keywords: Millisecond protein folding, NMR line shape analysis, R₂ relaxation, R₂ relaxation dispersion, cold shock protein, CspB.

1. INTRODUCTION

The mechanism by which polypeptides fold to their native conformation remains an area of active research in structural biology. Our understanding of the protein folding reaction and the deduction of protein folding models has been strongly influenced by the data accessible at the time when they were formulated. During all stages of progression in our understanding of protein folding, NMR spectroscopy played an important role. NMR combines high spatial resolution with dynamic and kinetic analyses on an enormous time scale ranging from picoseconds to days (Fig. 1). Therefore, NMR has emerged as an especially fruitful technique to study the protein folding reaction on molecular grounds.

The first milestones were certainly the use of NMR to define the distributions of deuterons at labile sites of proteins. Site specific H/D exchange experiments [1,2] and the calculation of protection factors revealed the thermodynamics of local and global unfolding of the native state as well as protein folding intermediates [3]. These states show distinct protection factors at different denaturant concentrations [4]. The determination of proton occupancies on a residue level by NMR following quenched-flow pulse labeling experiments provided important information about the structure formation during the first steps in protein folding [5-7].

Early direct NMR investigations of protein folding included studies of the equilibrium conversion between the native and the unfolded state at elevated temperatures, high denaturant concentrations, or extreme pH values [8,9]. These experiments provided insights into the cooperativity and thermodynamics of protein folding. Additionally, NMR spectroscopy of non-native states emerged. Although the
Most folding reactions are too fast to be monitored directly by real-time NMR spectroscopy [19-21]. Because the time required to record one 1D $^{1}H$ spectrum of a protein is at least 100 ms. Therefore, fast protein folding reactions have been measured under equilibrium conditions. Fluctuations on a 10 ms to 10 µs time scale between two or more states, where the NMR active nuclei experience different chemical environments, have profound effects on the shape and position of their resonances [22]. For proteins, the analysis of line shapes have been used to study ligand binding, and local fluctuations as well as global fluctuations such as complete un- and refolding of the polypeptide chain (a very good overview and references are given in [10]). One major drawback during quantifying NMR line shapes in terms of intrinsic unfolding and refolding rates is that a folding model has to be assumed. In most cases simple two-state protein folding systems have been studied or an apparent two-state model involving free and complexed protein has been applied to analyze the line shapes. The first protein folding system, which could be quantitatively studied by dynamic NMR spectroscopy on the above mentioned time scale was the N-terminal domain of phage λ repressor, λ$_{6.5}$ [11]. The urea dependence of the intrinsic un- and refolding rate constants could be determined between 1.3 M and 3.2 M urea with a maximum value for $k_u = 1200$ s$^{-1}$ and a minimum value for $k_u = 100$ s$^{-1}$ at 1.3 M urea. From a linear extrapolation of the chevron plot to 0 M urea, $k_u^0 = 3600\pm400$ s$^{-1}$ and $k_u^0 = 27\pm6$ s$^{-0}$ have been obtained. Later, Oas et al. verified these extrapolated rates by $^{1}H$ transverse relaxation experiments (see below) [12]. Typically, the resonances of aromatic protons are analyzed in $^{2}H$O samples with completely exchanged amide protons, because only then the former resonance between 6 ppm and 11 ppm can be assigned for both folded and unfolded state. These chemical shifts are needed at any urea concentration for an accurate line shape analysis. According to a two-state folding mechanism, only aromatic resonances of the folded and unfolded state are present within this region. In the case of λ$_{6.5}$, which contains only two tyrosine and two phenylalanine residues, the entire aromatic region of the 1D spectra could be simulated at any urea concentration (for example by using the program ALASKA [23]), which significantly increases the reliability of the extracted folding rates [12].

Figure 2. Stacked plot of 1D $^{1}H$-NMR spectra of a complete urea induced unfolding transition of CspB from B. subtilis in $^{2}H$O at 25 °C. Experimental 1D spectra of the His29$^\alpha$ resonance in the low field region is shown in the left panel. In the right panel, simulations of the 1D spectra using the extracted and extrapolated folding rates from the line shape analysis described in Materials and Methods at the respective urea concentration are depicted.
resonance, according to the extracted parameters, resembles very well the experimental data at every urea concentration and is depicted in the right panel of Fig. 2. The extrapolated folding rates from the dynamic NMR experiment ($k_0 = 1490 \pm 370 \text{ s}^{-1}$ and $k_0^\prime = 16 \pm 3 \text{ s}^{-1}$) correspond nicely to the rates derived from the stopped-flow fluorescence experiments. The Tanford-factors $\beta_T = m/(m + m_u)$ obtained from the linear slopes of $\log k_f$ and $\log k_u$ versus urea concentration (Fig. 3) are 0.9 from both the NMR and fluorescence experiment and indicate that the activated state of unfolding of CspB still resembles the native state in the accessibility to the solvent [24].

Most current applications facilitate CPMG-based $^15$N-relaxation measurements to determine the chemical exchange contribution $R_{ex}$ to $R_2$, which can be converted into micro-to-millisecond unfolding and refolding rates. The advantage of this approach is that 2D $^1H$-$^15$N correlation spectra are used to extract the relaxation rates. Therefore, $R_{ex}$ can be determined on a residue-by-residue basis to discriminate for example local and global unfolding of the peptide chain. The calculation of $k_f$ and $k_u$ from $R_{ex}$ is only possible for proteins that follow a simple two-state folding model. Additionally, the difference in the Larmor frequency (in Hz) of the respective nucleus in both conformations and the populations of both states have to be known beforehand. Alternatively, these parameters can be determined directly from the relaxation experiments, if experiments at different magnetic field strength are available [25]. Among several approaches to determine the $R_{ex}$ contributions to $R_2$ (such as an extended Lipari-Szabo approach, $R_{ex}$ measurements, or the interference of dipolar $^1H$-$^15$N and $^15$N chemical shift anisotropy relaxation), $R_2$ dispersion experiments are the most popular ones (for an overview and further literature see [10]). The basic idea is that the $R_{ex}$ contributions to $R_2$ can be modulated in a CPMG-based sequence by varying the delay time $\tau_{cp}$ between consecutive 180° pulses on the $^15$N nuclei (see Materials and Methods). The so called dispersion curve is a plot of $R_2$ over $1/\tau_{cp}$ (Fig. 4). At very small $1/\tau_{cp}$ values, the transverse $^15$N relaxation rate $R_2$ contains the full contribution of $R_{ex}$ and at high $1/\tau_{cp}$ values no $R_{ex}$ contributions remain. Therefore, the difference between $R_2$ at

2. RESULTS

The cold shock protein CspB from Bacillus subtilis follows a two-state folding mechanism with $k_0 = 1070 \pm 20 \text{ s}^{-1}$ and $k_0^\prime = 12 \pm 7 \text{ s}^{-1}$ at 25°C and 0 M urea [24]. From a urea transition monitored by 1D NMR in $^2$H$_2$O, the resonance of His29 could be used to determine unfolding and refolding rates between 2.9 M and 5.6 M urea (Fig. 2 and Fig. 3). The simulation of the line shape and the position of this

![Figure 3](image1.png)

Figure 3. Urea dependence of the folding rates of CspB determined by the line shape analysis approach applied to the 1D $^1H$-NMR spectra of His29 in $^2$H$_2$O (Fig. 2). The logarithm of $k_f$ and $k_u$ in the transition region (between 2.9 ppm and 5.6 ppm) is plotted versus the urea concentration. The linear slope of the continuous lines represents the $m$-value of refolding and unfolding ($m, m_u$) from which the Tanford value $\beta_T$ is derived with 0.9. Extrapolation to the absence of denaturant reveals folding rates of $k_f^0 = 1490 \pm 370 \text{ s}^{-1}$ and $k_u^0 = 16 \pm 3 \text{ s}^{-1}$.

![Figure 4](image2.png)

Figure 4. Relaxation dispersion curves of A32 of CspB from B. subtilis in 90% H$_2$O/10% $^2$H$_2$O at 25°C. Values for $R_2/(1/\tau_{cp})$ in the presence of (a) 1.2 M, (b) 2.0 M, and (c) 3.1 M urea at $B_0 = 14.1$ T (60.8 MHz $^15$N frequency) are depicted. The solid lines represent fits of the data to eq. 10 with the known difference in $^15$N chemical shifts between the cross peaks of A32 in native and unfolded CspB (119.2 Hz, 122.3 Hz, and 126.7 Hz for 1.2 M, 2.0 M, and 3.1 M urea, respectively) as well as the population of the native state $p_N$ (0.96, 0.89, and 0.73, respectively). It revealed $k_{ex} = k_f + k_u$ rates ($227 \pm 13 \text{ s}^{-1}$ at 1.2 M urea, $138 \pm 6 \text{ s}^{-1}$ at 2.0 M urea, and $74.9 \pm 5.3 \text{ s}^{-1}$ at 3.1 M urea). $k_f$ can be calculated by $k_f = k_{ex}/(1+(1-p_N)/p_N)$ and $k_u = k_{ex} - k_f$. 

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the lowest and highest $1/\tau_{cp}$ value provides an estimate for $R_{ex}$. Fitting of the entire dispersion curve reveals the apparent folding rate $k_{ex}$, which is the sum of $k_l$ and $k_u$. If the two-state model is valid, and the equilibrium constant under the respective conditions is known, explicit values for $k_l$ and $k_u$ can be calculated [10,12,13]. $R_2$ dispersion curves depend on the magnetic field strength [26] and therefore a joint fit of data obtained at various field strengths decreases the errors of the derived dynamic parameters.

Fig. 4 shows the $^{15}$N $R_2$ dispersion curves for A32 of the cold shock protein CspB at 1.2 M urea, 2.0 M urea and 3.1 M urea with native populations of 0.96, 0.89 and 0.73 [24], respectively. From a urea transition under NMR detection and from ZZ-exchange spectra [27] the difference in chemical shifts of the A32 backbone amide resonances between the native and unfolded state at the three urea concentrations are known (119, 112 and 127, respectively). The analysis of the dispersion curves reveals $k_u$ (227 s$^{-1}$ at 1.2 M urea 139 s$^{-1}$ at 2.0 M urea, and 75 s$^{-1}$ at 3.1 M urea) and subsequently $k_l$ (217 s$^{-1}$ at 1.2 M urea, 124 s$^{-1}$ at 2.0 M urea, and 55 s$^{-1}$ at 3.1 M urea) and $k_0$ (9.8 s$^{-1}$ at 1.2 M urea, 15 s$^{-1}$ at 2.0 M urea, and 20 s$^{-1}$ at 3.1 M urea) in good agreement with the rates from conventional stopped-flow folding experiments under fluorescence detection [24]. In principle this kind of NMR relaxation experiments can be used to measure chevron plots of proteins (such as depicted in Fig. 3) on a residue-by-residue basis, which should be very useful to map the transition state of folding in terms of homogeneity or heterogeneity and to determine the cooperativity within the folding peptide chain.

3. DISCUSSION

The presented dynamic NMR analysis of the line shapes of the His29$^F_1$ resonances at various urea concentrations revealed unfolding and refolding rates in good agreement with earlier stopped-flow fluorescence data. Both experiments reflect a native-like solvent accessibility of the transition state of folding, because the derived Tanford factors $\beta_w$ were close to 0.9. It is generally assumed that the tryptophan fluorescence is a very sensitive reporter for structural rearrangements of all sites of the polypeptide chain, which occur during the protein folding process. In the case of CspB, we could verify this assumption, because His 29 located at the opposite site of the first three stranded $\beta$-sheet reported similar folding rates compared to Trp 8 (Fig. 5).

Such a line shape analysis is limited to well-resolved resonances in 1D NMR spectra of proteins and explicit folding rates can only be determined at denaturant concentrations, where the population of unfolded and folded protein molecules are above 10% (Fig. 3). For a more comprehensive analysis of the folding peptide chain, 2D NMR spectra are required. As a proof of principle, we analyzed $^{15}$N transverse relaxation data of Ala32 obtained from 2D $^1$H-$^1$N correlation spectra at three urea concentrations. Ala32 is located at the beginning of a long loop between $\beta$-strand 3 and $\beta$-strand 4 of the cold shock protein. Its backbone nitrogen reports a urea dependence of the unfolding and refolding rates as expected from the neighboring His29. Together with Trp8, Ala32 and His29 experience the same solvent accessibility of the transition state of folding. A global description of the transition state will be possible after an analysis of relaxation data of $^{15}$N nuclei from all sites of CspB at least at 8 different urea concentrations, which is current work in progress. We expect from these data evidences about the homogeneity of the transition state of folding and the cooperativity of protein folding reactions on a millisecond time scale. Most applications of $R_2$ dispersion curves found in the literature focus on local motions of backbone amides and rates and populations of these local breathing reactions are discussed. With CspB, we could show that these methods are also sensitive to global folding and unfolding reactions.

The advantage of 1D dynamic NMR experiments is that a wide range of conditions can be sampled because only one 1D spectrum has to be recorded. The folding and unfolding kinetics of the N-terminal domain of the ribosomal protein L9 for example have been measured at temperatures between 7 °C and 85 °C and between 0 M and 6 M guanidinium chloride. The joint analysis of stopped-flow fluorescence (between 7 °C and 55 °C) and dynamic NMR data (between 55 °C and 85 °C) revealed thermodynamic parameters of the activated state of folding [28,29]. The dynamic NMR data were required in this case, because the apparent folding rate constant for the latter temperature range was, at 700 s$^{-1}$–3000 s$^{-1}$, too high for traditional stopped-flow experiments. Following the same lines a small all-helix protein psb41 could be analyzed by the line shape approach revealing maximum folding rates of 21,600 s$^{-1}$ and maximum unfolding rates of 24,600 s$^{-1}$ [30].

The upper time limit for dynamic NMR to study protein folding reactions is tens of microseconds if the difference in chemical shift of an NMR nuclei between the unfolded and native state is sufficient (at least 500 Hz). Raleigh and
coworkers found in a thermal unfolding transition of the villin headpiece refolding rates between 3·10^4 s^{-1} and 2·10^5 s^{-1}, which experimentally confirmed recent all atoms molecular dynamics calculations to simulate protein folding reactions (see [31] and references therein). A similar link between theory and experiment was possible for the three helix bundle forming B-domain of staphylococcal protein A. Dynamic NMR analyses revealed folding rate constants of 120.000 s^{-1}, which are in good agreement with predictions from diffusion-collision theory [32].

As mentioned above, the major limitation of dynamic NMR to study fast protein folding rates is that reliable rates can only be obtained from the central region of denaturant- or temperature-induced unfolding transitions, where at least 10% of both states are populated. The wide linear extrapolation of log(k) to conditions with a maximal population of the native state might miss non-linear effects such as a ‘roll-over’ under strong native conditions. One way to bypass this problem is the use of NMR relaxation data to determine protein folding rates. If the rates of interconversion are on the millisecond-to-microsecond time scale and large chemical shift differences between the states are present, transverse relaxation rates can be sensitive to the presence of the minor conformation with populations as low as 1% [10].

One early application was the direct determination of folding rates of the λ repressor head piece λ_{6,85} under strong native conditions by ^1H transverse relaxation using a 1D Carr-Purcell-Meiboom-Gill (CPMG)-based spin echo pulse sequence [12]. The chemical exchange contribution (R_c) to the transverse relaxation rate (R_T) leads to an apparent increase of the latter from which the folding rates can be extracted. At 0 M urea, the authors found for λ_{6,85} k_f = 4000±340 s^{-1} and k_u = 32±3.2 s^{-1}, which is in good agreement with the values obtained by extrapolation from the line shape analysis (k_i = 49000±600 s^{-1} and k_u = 30±4.6 s^{-1}).

Several applications of ^15N and ^13C R_T dispersion curves have been reported to determine rates of local motions under strong native conditions [25,26,33-36]. In this case, two conformations have to be assumed in this region of the protein to gain apparent rates of interconversion. Additionally, the populations of the two conformations and the difference in chemical shifts of the respective nuclei have to be extracted entirely from the NMR relaxation data, which makes this kind of analysis very difficult and might limit the interpretation of the data.

Studies of global two-state folding-unfolding reactions allow an accurate determination of folding rates from R_T dispersion experiments, because the populations of the native and unfolded state under the respective conditions can be determined experimentally [13,15,37]. Additionally, the differences of their chemical shifts are accessible from independent NMR experiments or can be at least extrapolated (see Materials and Methods). For global unfolding reactions all resonances of the protein should show R_T dispersion curves as long as these differences are above 2 Hz, which makes joint fittings possible. In the case of the N-terminal SH3 domain of the Drosophila protein drk, on top of the global unfolding reaction, a local conformational exchange process of the unfolded state could be revealed by a systematic analysis of ^15N R_T dispersion data [15]. For the de novo designed dimeric four-helix bundle protein α_2D, refolding rates of (4.7±0.9)-10^5 M^{-1} s^{-1} and unfolding rates of 15±3 s^{-1} could be determined for this bimolecular reaction from ^13C R_T dispersion curves [37]. The ^13C δ chemical shifts for unfolded and folded forms of α_2D indicate that the ensemble of unfolded states include transiently structured helical conformations.

Very recent developments allowed extremely long MD simulations of proteins by using clusters of thousands of computers [38,39]. On the other hand, dynamic NMR spectroscopy can reveal experimentally folding rates of proteins up to 200.000 s^{-1} [31]. At such high rates, several folding-unfolding interconversions occur during the now accessible length of one MD simulations. Therefore the gap between experiments and simulations has been closed and many exciting new insights into the elementary steps of protein folding can be expected in the very near future.

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5. MATERIALS AND METHODS

For an accurate line shape analysis the system under investigation has to fulfill several requirements. Most important is the existence of a two-state folding process where only folded (N) and unfolded (U) protein molecules are present at equilibrium and in the kinetics at all denaturant concentrations (eq. 1). This has been shown for CspB by equilibrium and stopped-flow fluorescence spectroscopy [24]. Additionally it is important that the analyzed resonance signals are well resolved throughout the entire equilibrium transition and do not overlap with other resonances. In rare cases, where only a few resonances are in a narrow region of the 1D spectra under all conditions, the entire 1D spectrum can be simulated to overcome signal overlap problems [12,23]. The His29^{15}N proton of CspB is in fast exchange between the two states in respect to the NMR chemical shift time scale ((k_i + k_u) > Δν). Therefore, only one resonance is detectable over the entire transition with a line shape and resonance frequency that represents the relative populations.

\[
\frac{k_f}{k_u} \rightarrow \nu_N \rightarrow \nu_U \quad (eq. 1)
\]

All NMR samples contained 0.7 mM CspB, 50 mM Na-cacodylate/HCl, pH 7, various amounts of urea and all spectra were recorded at 25 °C. CspB and ^15N isotope enriched CspB was purified as described previously [14,24]. 1D spectra were recorded in 100% ^2H_2O with 256 scans and 4096 complex points and were processed with a single-exponential window function. To prevent saturation of ^1H with long spin-lattice relaxation times the recycle delay between individual transients was 3 s. The 1D spectra were...
separately phase corrected, referenced against TSP, and the baseline correction of each spectrum in the region around the His29 resonance was performed manually.

The line shape analysis was performed with Grafit (Erithacus software) using the Levenberg-Marquardt algorithm for non-linear least square fittings of eq. 2 [11]. $I(\nu)$ represents the intensity as a function of the frequency $\nu$ and $C_0$ is a normalization constant, which is proportional to the protein concentration. $I(\nu)$ depends also on the population of the native and unfolded state ($P_{N}$, $P_{U}$), the apparent spin-spin relaxation times of the resonance in the respective state under non-exchanging conditions ($T_{2N}$, $T_{2U}$), the resonance frequency of both states ($\nu_{N}$, $\nu_{U}$) and the refolding and unfolding rate constant ($k_1$, $k_2$).

$$I(\nu) = \frac{C_0}{P_{N}^{2} + R_{\nu}^{2}} \left[ P_{N} \left( 1 + \tau \left( \frac{P_{N}}{T_{2N}} + \frac{P_{U}}{T_{2U}} \right) \right) + Q \cdot R \right]$$

$$P = \tau \left[ \left( \frac{1}{T_{2N}T_{2U}} \right) - 4\pi^2 \Delta\nu^2 + \delta\nu^2 \left( \delta\nu^2 \right)^2 \right] + \frac{P_{N}}{T_{2N}} + \frac{P_{U}}{T_{2U}}$$

$$Q = \tau \left( 2\pi \cdot \Delta\nu^2 - \pi \cdot \delta\nu \cdot (P_{N} - P_{U}) \right)$$

$$R = 2\pi \Delta\nu \left[ 1 + \tau \left( \frac{1}{2N} + \frac{1}{2U} \right) + \Delta\nu \cdot \tau \cdot \left( \frac{1}{T_{2N}} - \frac{1}{T_{2U}} \right) \right] + \pi \cdot \delta\nu \cdot (P_{N} - P_{U})$$

$$\Delta\nu = (\nu_{N} + \nu_{U}) / 2$$

$$\delta\nu = (\nu_{N} - \nu_{U})$$

$$\tau = (k_1 - k_2)^{-1}$$

(Eq. 2).

Firstly, the apparent transverse relaxation rate of the native and unfolded state ($T_{2N}$, $T_{2U}$) have to be determined. Therefore, the signal of the fully native or unfolded protein, respectively, is simulated with a function corresponding to an absorptive Lorentzian signal ($I(\nu) = C_0R_{\nu}f(R_{\nu})$) where $R_{\nu} = 1/T_{2}$ and $\nu$ represents the chemical shift of the signal. Secondly, the dependence of the resonance frequency of the signal of the native and the unfolded state from the denaturant concentration is required. Thus, the resonance frequency of the respective signal in the spectra under non-exchanging conditions (that is in the beginning of the baselines of the transition under strong native conditions (between 0 M and 2 M urea) and strong non-native conditions (between 6 M and 8 M urea)) is measured. Assuming a linear dependence of the resonance frequency on the denaturant concentration the actual resonance frequency of the signal of the native and the unfolded state ($\nu_{N}$, $\nu_{U}$) under all conditions can be calculated by linear extrapolation with the determined parameters ($T_{2N}$, $T_{2U}$, $\nu_{N}$, $\nu_{U}$) fitting of the resonance signals in the transition region with eq. 2 reveals the normalization constant $C_0$ and the folding rates ($k_1$, $k_2$) for every individual 1D spectrum (Fig. 2 and Fig. 3). The error of $k_1$ and $k_2$ was estimated by a systematic variation of $k_1$ and $k_2$ around their fitted values and a comparison of the residuals between the calculated and experimental line shapes [11,28,31]. It never exceeded 10%.

As described previously the determination of explicit unfolding and refolding rates requires a protein that follows a two-state folding model. One of the first applications of $R_2$ relaxation dispersion curves to study fast folding reactions was performed by homonuclear 1D $^{1}$H NMR spectroscopy [12]. To overcome the limitations of 1D NMR like severe spectral overlap, more recently heteronuclear 2D relaxation methods have been established [10], which provide many site specific probes. One common approach is the measurement of so called $^{15}$N $R_2$ dispersion curves. Despite the high spectral resolution of the 2D $^{1}H/^{15}$N correlation spectra used to determine the $R_2$ relaxation rates this method is applicable over all exchange regimes in respect to the NMR chemical shift time scale. Additionally, the determination of the $R_2$ rates is straightforward and robust so that instrument effects such as magnetic field inhomogeneities might not introduce significant errors, which are present in 1D line shape analyses. The most important advantage of the $R_2$ dispersion approach is that folding rates can be extracted down to a population of one state of about 1%. The major drawback of this method is the time consuming acquisition. $R_2$ rates are usually measured with pulse sequences based on the Carr-Purcell-Meiboom-Gill (CPMG) spin echo element [40,41]. Principally, the spin echo element contains a single 180° pulse on the nuclei of interest, which is separated by two equivalent delays $\tau = \tau_{\text{ex}}/2$. Contributions from chemical exchange ($R_{\text{ex}}$) to $R_2$ can be effectively eliminated by the CPMG sequence if the exchange process is on a slower time scale in respect to the delay time $\tau_{\text{ex}}$. For the fast pulsing limit (very short $\tau_{\text{ex}}$ values) only very fast processes on a picosecond to nanosecond time scale contribute to the apparent $R_2$ value, which is $R_2^{0}$. However, if $\tau_{\text{ex}}$ is long enough to allow multiple folding/unfolding events during $\tau_{\text{ex}}$, chemical exchange $R_{\text{ex}}$ contributes to $R_2$: $R_2 = R_2^{0} + R_{\text{ex}}$. Therefore, a dispersion of $R_2$ regarding $R_{\text{ex}}$ can be generated by varying $\tau_{\text{ex}}$.

All NMR samples contained 0.7 mM uniformly labeled $^{15}$N CspB in 50 mM Na-cacodylate/HCl pH 7.0 in H$_2$O/H$_2$O (90%/10%) in the presence of 1.2 M, 2.0 M and 3.1 M urea, respectively. Heteronuclear 2D NMR spectra were recorded at 25 °C with a Bruker Avance600 NMR spectrometer using pulse sequences published by Palmer and coworkers [26,42]. Spectra were acquired with 2048 complex points and 16 transients were averaged for each of the 192 $\tau_1$-increments. A recycle delay of 1 s was employed. All 2D spectra were...
processed identically by zero filling the FID once in both directions and applying a skewed quadratic cosine bell window function. Data matrices contained 2048 x 512 real points. The relaxation delay used a fixed spin echo delay $\tau_{cp}$ was varied between 2.6 ms and 208 ms by multiple repetition of the spin echo sequence. The decay of the cross peak intensity was defined by 5 to 8 different relaxation delays. For long $\tau_{cp}$ values multiple spectra with identical relaxation delays were acquired. Dispersion curves were generated by evaluating $R_2$ rates for $\tau_{cp}$ values between 650 $\mu$s and 21.6 ms.

An accurate determination of $R_2$ was performed by fitting the data with a simple exponential function without offset ($I = A \exp(-R_2 t)$) using Grafit (Erithacus software). The so derived $R_2$ values are plotted on the ordinate of the $R_2$ dispersion curve (see Fig. 4). For the analyses of the latter the program CPMGfit (kindly provided by A. G. Palmer, Columbia University) was used. The exchange rate $k_{ex}$ and the chemical exchange contribution $R_{ex}$ to $R_2$ was derived by fitting eq. 3 to the $R_2$ dispersion curves [10,43]. In eq. 3 $\Delta \omega$ is the difference of the $^{15}$N chemical shift of the cross peak between the native and the unfolded state ($\Delta \omega = 2 \Delta \omega N$ with $\Delta \omega$ given in Hz), $p_N$ and $p_U$ the population of the respective state and $\tau_{cp} = 2 \tau$ as well as $k_{ex} = k_e + k_\omega$. $\Delta \omega$ defines the exchange regime for a given $k_{ex}$ ($k_{ex} < \Delta \omega$: slow exchange; $k_{ex} = \Delta \omega$: intermediate exchange; $k_{ex} > \Delta \omega$: fast exchange). This formalism represents the general phenomenological description of $R_2$ of site N in eq. 1. It is independent of the exchange regime (slow, intermediate or fast exchange in respect to the NMR chemical shift time scale) and the present equilibrium population ratio.

The precise determination of $\Delta \omega$ is essential for the reliability of the extracted parameters. Therefore, we recorded a series of $^1$H/$^15$N HSQC spectra at various urea concentrations to monitor the denaturant induced unfolding transition and to determine the urea dependence of the chemical shifts. Therein, nuclei in the slow exchange regime depict a cross peak for each state. A straightforward assignment of the backbone amide cross peak of A32 in the unfolded state was performed by correlating the cross peaks of the native and the unfolded state via the respective exchange cross peaks in 2D ZZ-exchange spectra [27]. Additionally, the spectra revealed the equilibrium populations of the native and the unfolded state from cross peak intensities.

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