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Article

Motional properties of unfolded ubiquitin: a model for a random coil protein

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Abstract

The characterization of unfolded states of proteins has recently attracted considerable interest, as the residual structure present in these states may play a crucial role in determining their folding and misfolding behavior. Here, we investigated the dynamics in the denatured state of ubiquitin in 8 M urea at pH2. Under these conditions, ubiquitin does not have any detectable local residual structure, and uniform ^{15}N relaxation rates along the sequence indicate the absence of motional restrictions caused by residual secondary structure and/or long-range interactions. A comparison of different models to predict relaxation data in unfolded proteins suggests that the subnanosecond dynamics in unfolded states depend on segmental motions only and do not show a dependence on the residue type but for proline and glycine residues.

Introduction

The detection of an increasing number of protein misfolding diseases with polypeptide chains forming fibrils and toxic aggregates under specific conditions (Dobson, 2003), and the observation that some of these proteins lack a persistent globular fold under native conditions, has renewed the interest in the investigation of non-native, often unfolded states of proteins. Heteronuclear NMR spectroscopy has emerged as a powerful tool to investigate aspects of residual structure in non-native states of proteins (Dobson and Hore, 1998; Dyson and Wright, 2002, 2004; Wirmer et al., 2005). The lack of strong secondary chemical shifts and the absence of long-range NOEs and coupling constant values consistent with random coil predictions (Smith et al., 1996a) have supported the notion that large parts of the polypeptide chain in these non-native states can be well described by a random coil (Fiebig et al., 1996, Schwalbe et al., 1997). A protein in a random coil state is described as a polymer consisting of 20 different monomers, the amino acids. The polymer possesses no structure except that inherent to its different monomers. However, residual secondary and tertiary structure has been detected in a surprisingly large number of proteins in non-native states. Significant deviations from random coil behaviour have been observed in the denatured states of lysozyme (Buck et al., 1994; Schwalbe et al., 1997; Klein-Seetharaman et al., 2002; Wirmer et al., 2004, 2006; Schlorb et al., 2005), staphylococcal nuclease (Shortle and Abeygunawardana, 1993; Gillespie and Shortle, 1997; Shortle and Ackerman, 2001; Choy and Kay, 2003; Choy et al., 2003; Ohnishi and Shortle, 2003), apomyoglobin (Eliezer et al., 1998, Yao et al., 2001; Lietzow et al., 2002; Mohana-Borges

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et al., 2004), barstar (Wong et al., 1996, Bhavesh et al., 2004), barnase (Arcus et al., 1994; Arcus et al., 1995; Wong et al., 2000), protein G (Frank et al., 1995; Sari et al., 2000), FK506BP (Logan et al., 1994), acyl-CoA-binding protein (ACBP) (Teilum et al., 2002; Fieber et al., 2004; Lindorff-Larsen et al., 2004), 434 repressor (Neri et al., 1992), α-lactalbumin (Wirmer et al., 2006), SH3 (Farrow et al., 1997; Blanco et al., 1998; Mok et al., 1999, Tollinger et al., 2001; Crowhurst et al., 2002; Crowhurst and Forman-Kay, 2003) and β-2-microgobulin (McParland et al., 2000; McParland et al., 2002). Interestingly, also for so-called natively unfolded proteins that do not adopt a persistent tertiary structure under native conditions such as tau (Smet et al., 2004; Mukrasch et al., 2005; Landrieu et al., 2006), α-synuclein (Bussell and Eliezer, 2001; Bertoncini et al., 2005; Bernado et al., 2005; Bertini et al., 2005), a 130 amino acid fragment of the fibronectin binding protein (Penkett et al., 1997; Penkett et al., 1998) and the pro-peptide of subtilisin (PPS) (Buevich and Baum, 1999; Buevich et al., 2001) residual structure could be detected.

The nature of the interactions that cause parts of the polypeptide chain to deviate from random coil behavior is still under debate. Under some conditions (low pH and high concentrations of denaturant), electrostatic interactions do not dominate the formation of non-random structure. Rather, this residual structure is due to the formation of clusters of aromatic residues, particularly tryptophans (Neri et al., 1992; Ropson and Frieden, 1992, Saab-Rincon et al., 1996; Schwalbe et al., 1997; Klein-Seetharaman et al., 2002; Wirmer et al., 2004; Schlorb et al., 2005; Schlorb et al., 2006). Further analysis of these clusters suggested that they are stabilized by both native and non-native hydrophobic long-range interactions (Wirmer et al., 2004).

NMR is such a powerful tool for the detection of residual structure in denatured proteins that it is surprisingly difficult to find a protein lacking residual non-random structure and dynamics. Therefore, it is of interest to identify a model system that lacks any detectable non-random structure in its unfolded state. We show here that ubiquitin, an extremely stable, small (76 amino acids) and monomeric protein without disulfide bridges, is such a protein in its low pH, urea-denatured state.

Ubiquitin is present in all eukaryotic systems and is a very important marker for protein degradation. Covalent binding of poly-ubiquitin chains with their C-terminal glycine to lysine residues of other proteins labels proteins for degradation (Rechensteiner, 1988; Hershko and Ciechanover, 1998). In its native state, ubiquitin is composed of a five stranded β-sheet (β1–β5), a long (residues 23–34) helix (H1) and two short 3^{10} helices. It is very stable: the protein remains folded at temperatures up to 80 °C and between pH 1.2 and pH 13 (Lenkinski et al., 1977; Jenson et al., 1980; Nash and Jonas, 1997). The stability of the native protein can be attributed to its strong hydrophobic core: hydrophobic residues are buried in the interior of the protein, formed by the long helix and the five stranded β-sheet.

The so-called A-state (acid state) of ubiquitin is formed in the presence of 60% methanol at pH 2 (Wilkinson and Mayer, 1986; Brutscher et al., 1997). NMR spectroscopic investigations revealed that while the N-terminal part comprising β1, β2 and H1 of the protein is conserved in the A-state, the structure of the C-terminal changes dramatically (Brutscher et al., 1997). Based on chemical shift analysis, it is known that the three β-strands β3–β5 are converted into an α-helical conformation (Brutscher et al., 1997).

Here, we investigate the low pH urea-denatured state of ubiquitin and compare it to the other states of ubiquitin. We show that denatured ubiquitin resembles closely a polypeptide in a random coil conformation. On this basis, we test the validity of models describing the random coil behavior of a polypeptide devoid of any secondary and tertiary structure (Levinthal, 1969).

Materials and methods

Sample preparation

5 mg of uniformly ^{13}C, ^{15}N labeled (^{15}N labeled) human ubiquitin (VLI Research, Malvern, PA) were dissolved in 300 μl of 90% H_2O, 10% D_2O, 8 M urea at pH 2.

Chemical shifts

Resonance assignment was taken from Peti et al. (2001). Random coil chemical shifts (δ^{rc}) using the

"Wishart peptide" scale (Wishart et al., 1995) were extracted using the program NMRView (version 5.0.4) including sequence specific corrections (Schwarzinger et al., 2001). Chemical shift deviations ($\Delta\delta$) were calculated according to $\Delta\delta = \delta^{exp} - \delta^{rc}$ (δ^{exp} = experimental chemical shift taken from Peti et al. (2001)). Significant deviations are defined according to the chemical shift index (CSI) (Wishart et al., 1992; Wishart and Sykes, 1994).

NMR measurements

All NMR measurements were performed on a four-channel Bruker DRX 600 spectrometer equipped with an actively shielded TXI z-gradient probe at a temperature of 25 °C.

$^1J(N_i,C_{\alpha i})$ and $^2J(N_i,C_{\alpha(i-1)})$ coupling constants were measured and determined as described previously (Wirmer and Schwalbe, 2002).

^{15}N longitudinal (R_1) and transverse (R_2) relaxation rates and the heteronuclear NOE (hetNOE) were measured as described in the literature (Kay et al., 1989; Palmer and Case, 1992; Akke and Palmer, 1996) using sensitivity enhancement in the back transfer (Schleucher et al., 1994). All spectra were acquired with 1024 points in the direct dimension at a sweep width (^1H) of 8389 Hz, and 128 complex points in the indirect dimension (^{15}N) at a sweep width of 1520 Hz. For the measurement of R_1 and R_2 relaxation rates nine experiments each were recorded using seven different mixing times between 30 and 1500 ms (R_1) and 17 and 340 ms (R_2 relaxation rates). Two experiments were recorded in an interleaved manner for the measurement of the hetNOE, one with proton presaturation (applied by a series of 120° high power proton pulses during the recycle delay) and one without proton presaturation. The recycle delay (RD) was 3 s for the hetNOE experiment and 2 s for R_1 and R_2 experiments.

Processing of the relaxation data and measurement of peak heights was carried out using the program Felix 98.0 (Biosym/MSI San Diego, CA). Data were fourier transformed applying zerofilling and apodization by a 90° shifted sinebell window function; only the $^1H^N$ region of the spectra was retained, the final matrix size was 1024*256 points. R_1 and R_2 relaxation rates were fitted as single exponential decays ($I = A^*\exp(-t/R)$) to the peak height data (I) with t = relaxation delay, A = Amplitude and R = either R_1 or R_2 (Stone et al., 1992; Stone et al., 1993) (http://www.cpmc-net.columbia.edu/dept/gsas/biochem/labs/palmer/software.html).

The hetNOE was determined by dividing the peak heights in the spectra without presaturation by the ones with presaturation.

Spectral density mapping

Spectral density mapping was performed as described in the literature using the following formulae (Buevich et al., 2001):

$$J(0.87\omega_H) = \frac{4}{5d^2}\frac{(NOE-1)R_1\gamma_N}{\gamma_H} \quad (1)$$

$$J(\omega_N) = \left(4R_1 - 5\frac{(NOE-1)R_1\gamma_N}{\gamma_H}\right) \Big/ \left(3d^2+4c^2\right) \quad (2)$$

$$J(0) = \left(6R_2 - 3R_1 - 2.72\frac{(NOE-1)R_1\gamma_N}{\gamma_H}\right) \Big/ \left(3d^2+4c^2\right) \quad (3)$$

with $d = \frac{\mu_0 h \gamma_N \gamma_H}{8\pi^2}\frac{1}{r_{NH}{}^3}$ and $c = \frac{\omega_N}{\sqrt{3}}\Delta\sigma$. μ_0 is the permeability of free space, h is Planck's constant, R_{NH} = 1.02Å, $\delta\sigma = -172$ ppm and γ_H and γ_N are the gyromagnetic ratios of ^{15}N and ^1H, respectively and ω_N is the Larmor frequency of ^{15}N.

Hydrophobicity calculations

Hydrophobicity was calculated using the protscale tool from the ExPAsy (Appel et al., 1994) (Expert Protein Analysis System) molecular biology server (http://www.us.expasy.org/cgi-bin/protscale.pl). The Abraham and Leo (1987) scale was applied. The window size (length of the interval used for profile computation in units of residues) was 7 (as this is the persistence length found in a number of unfolded proteins (Schwalbe et al., 1997; Schwarzinger et al., 2002)) and the weight at the edge of the window was set to 100%; the hydrophobicity shown is normalized from 0–1.

Results

Absence of secondary structure in the unfolded state of ubiquitin

Ubiquitin is unfolded in 8 M urea at pH 2 as shown by CD and 1D-NMR spectroscopy (data not shown). We previously investigated the urea-denatured state of ubiquitin in terms of non-random secondary structure by chemical shift deviations (Peti et al., 2001), *J* coupling constants (Peti et al., 2001; Wirmer and Schwalbe, 2002) and cross-correlated relaxation rates (Peti et al., 2000).

The chemical shifts of urea-denatured ubiquitin (Peti et al., 2001) are very close to those of the random coil (Wishart et al., 1995), as expected for an unfolded protein; only five residues show significant deviations of H^{α} chemical shifts: K11 and Y59 (negative H^{α} deviations), F4, L8 and I13 (positive H^{α} deviations). The averaged absolute deviation in H^{α} chemical shift in ubiquitin is $<|\Delta\delta|>_{ubi} = 0.04 \pm 0.04$ ppm. This value is significantly smaller than that observed for the averaged absolute deviations in other denatured proteins such as unfolded lysozyme ($<|\Delta\delta|>_{hewl} = 0.11 \pm 0.09$ ppm (Klein-Seetharaman et al., 2002; Wirmer et al., 2004)). As the average deviations in ubiquitin are very small and randomly distributed along the sequence, we concluded that urea-denatured ubiquitin does not possess any residual secondary structure, as judged from chemical shift data.

Another method that can be used for the identification of residual secondary structure in unfolded proteins involves the measurement of scalar coupling constants such as $^{3}J(H^{N},H_{\alpha})$. For ubiquitin, a remarkable correlation between experimentally determined coupling constants (Peti et al., 2001) and data predicted by the random coil model (Fiebig et al., 1996; Smith et al., 1996a, b; Schwalbe et al., 1997; Hennig et al., 1999) is observed, except for all histidine (H), phenylalanine (F), aspartate (D) and glutamate (E) residues of the sequence (Figure S1). For the charged residues, these differences can be attributed to the different pH and the different solvation of the measured data (pH2, 8 M urea) in comparison with the structural data used by the random coil model (pH7, no denaturant). $^{3}J(H^{N},H^{\alpha})$ coupling constants depend only on the protein backbone angle ϕ. For a good differentiation between a protein in the random coil state and a protein in which residual secondary structure elements are present, coupling constants that also depend on the backbone angle ψ need to be measured. $^{1}J(N_i,C_{\alpha(i-1)})$ and in particular $^{2}J(N_i,C_{\alpha(i-1)})$ coupling constants are dependent on the backbone conformation, predominantly on the protein backbone angle ψ and are therefore particularly well suited for this purpose (Wirmer and Schwalbe, 2002). The experimental $^{2}J(N_i,C_{\alpha(i-1)})$ coupling constants agree well with the predicted coupling constants for the random coil indicating, in agreement with the analysis of the chemical shifts that the random coil is a good model for ubiquitin in the denaturing conditions used for this study (Figure S2). Measured $^{3}J(C_{\alpha i-1},C_{\alpha i})$ coupling constants support this conclusion (Peti et al., 2000).

From the analysis of chemical shifts deviation and a variety of *J* coupling constants data, we conclude that ubiquitin in 8 M urea at pH2 is completely unfolded and devoid of residual non-random structure.

Dynamics

The prediction of backbone dynamics in unfolded proteins is more challenging than that of torsions presented in the previous section. One particularly complex feature of relaxation rates in proteins is that they are affected by both the degree of restricted motion, i.e. structure, and by the hydrodynamic properties of the protein molecule as a whole. This convolution of effects renders the interpretation of relaxation rates especially cumbersome in unfolded proteins with fluctuating residual structure.

The end-to-end distance is a measure for the global chain conformation that can be related to the hydrodynamic properties of polypeptides. In a freely jointed chain, the averaged end-to-end distance ($<r^2>$) is given by the square of the bond length times the number of bonds. Such a chain would follow the isolated-pair hypothesis, that assumes each pair of rotation angles (ϕ, ψ) to be independent of the neighboring pair. This hypothesis has been shown to fail for short polypeptides of up to 7 residues (Pappu et al., 2000). This indicates that in a polypeptide each residue is influenced by its neighbours. The chain is therefore not as flexible as the freely jointed chain and thus, has a longer end-to-end distance (Flory, 1969). The end-to-end distance reflects on a number of

properties e.g. (1) the hydrodynamic radius (R_h) which is a global property of polypeptide chains and (2) the rotational tumbling which monitors local dynamics and global tumbling.

Here, we use backbone ^{15}N relaxation data as reporter of chain dynamics. ^{15}N backbone relaxation rates are modulated by the overall rotational tumbling of the polypeptide chain and are sensitive to motions on a subnanosecond time scale and to slow conformational exchange in the millisecond time scale (Wagner, 1993). ^{15}N heteronuclear relaxation rates (R_1, R_2) and heteronuclear NOE (hetNOE) of backbone amides of unfolded ubiquitin are shown in Figure 1. The relaxation

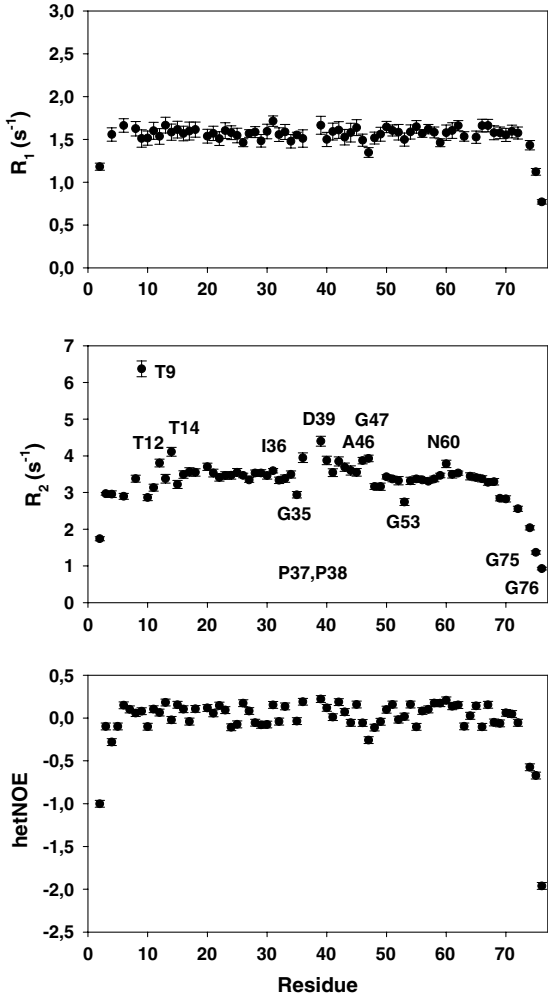

Figure 1. Backbone ^{15}N heteronuclear relaxation data of unfolded ubiquitin at 600 MHz. Top: R_1 relaxation rates in the range of 0–3 s^{-1}, middle: R_2 relaxation rates in the range of 0–7; bottom: heteronuclear NOE in a range of −2.5–0.5 a.u.

data show only small variations along the sequence; values approach a plateau at the middle of the polypeptide chain while lower values are found at the termini of the sequence. These results imply that the relaxation properties of a given amide are not influenced by the nature of its neighbours but are dominated by its sequence position in the polypeptide chain. The only exception from this behavior is the R_2 relaxation rate of T9, which shows large deviation.

The relaxation rates and heteronuclear NOEs at the N-terminus of ubiquitin are not as low as those at the C-terminus where the hetNOE values are particularly low, most likely due to the presence of two consecutive glycine residues at the very C-terminus of the protein.

Plateau values in the central part of the ubiquitin sequence, taking into account residue 6 to residue 71, are as follows: hetNOE $= 0.05 \pm 0.11$, $R_1 = 1.57 \pm 0.063\ s^{-1}$ and $R_2 = 3.48 \pm 0.50\ s^{-1}$. All these values are considerably lower than those expected for a structured protein, and reflect the unfolded character of ubiquitin at pH 2 and 8 M urea: e.g. for a rigid protein, a hetNOE near 1 is expected. The plateau value of the R_2 s ($R_2^{plat} = 3.48 \pm 0.5\ s^{-1}$) is very similar to plateau values found in other unfolded proteins in 8 M urea, e.g. $R_2^{plat} = 3.5\ s^{-1}$ in reduced and methylated lysozyme in urea (Schwalbe et al., 1997). However, the fluctuations of the ^{15}N relaxation rates in the central part of unfolded ubiquitin differ considerably from those observed in other proteins. R_2 relaxation rates in unfolded ubiquitin range from 3.2 to 4 s^{-1} (with exception of T9), while they range from 2.8 to 6.5 s^{-1} in unfolded apomyoglobin (Schwarzinger et al., 2002), from 3 to $7s^{-1}$ in unfolded lysozyme (Schwalbe et al., 1997) and from 3.5 to 8 s^{-1} in unfolded FK506BP (Logan et al., 1994) only to name some other urea-denatured protesins (see also Table 1).

The most significant difference between the unfolded state of these proteins as compared to ubiquitin is the lack of residual secondary structure in the latter. Thus, the question arises why the combination of low pH and urea diminishes residual structure in ubiquitin while it fails to eliminate residual structure in the other proteins. Close observation suggests that α-helical structure is more persistent against the combination of urea and low pH than β-sheet structure. Native ubiquitin has a number of β-sheets and only one

Table 1. Comparison of published R_1 and R_2 relaxation rates and hetNOE of unfolded proteins. Length indicates the number of residues of the used protein/fragment. Low indicates the lower level of the relaxation data, high the highest observed relaxation parameter. Values were extracted from graphical material in the literature.

Protein	$R_2(s^{-1})$ low	$R_2(s^{-1})$ high	$R_1(s^{-1})$ low	$R_1(s^{-1})$ high	hetNOE low	hetNOE high	length	pH	T (°C)	Denaturant	field (MHz)	citation
Lysozyme-S^{ME}	3	7	1.55	1.75	0.1	0.4	129	2	20	8 M urea	600	(Schwalbe et al., 1997, Klein-Seetharaman et al., 2002)
Lysozyme-S^{ME}	2.5	8	na	na	na	na	129	2	20	–	600	(Klein-Seetharaman et al., 2002, Wirmer et al., 2004)
BLA-S^{ME}	4	7	na	na	na	na	123	2	20	8 M urea	600	(Wirmer et al., 2006)
All-Ala-HLA	3.5	7.5	na	na	na	na	123	2	20	8 M urea	600	(Wirmer et al., 2006)
Staphylococcal nuclease Δ 131Δ	2	4	na	na	–0.4	0.1	131	3	32	–	600	(Ohnishi and Shortle, 2003)
Staphylococcal nuclease Δ 131Δ	1.5	3	na	na	–0.2	0.1	131	3	32	8 M urea	600	(Ohnishi and Shortle, 2003)
Staphylococcal nuclease Δ131Δ	3	7	na	na	–0.1	0.2	131	5.2	32	8 M urea	600	(Ohnishi and Shortle, 2003)
apomyoglobin	2.8	6.5	1.4	1.7	0.2	0.45	153	2.3	20	8 M urea	750	(Schwarzinger et al., 2002)
barnase	1.6	3.5	1.3	2	–1.6	–1.2	110	4.5	30	5.5 M urea	500	(Wong et al., 2000)
SH3 of drkN	3	7.4	1.5	1.7	0.1	0.2	59		14	2 M GdnCl	600	(Farrow et al., 1997)
α-synuclein	3	6	1.6	2.7	–0.2	0.4	140	7.4	10	–	600	(Bussell and Eliezer, 2001)
Fibronectin bp	3	7.5	1.5	1.7	0	0.4	130	6	5	–	600	(Penkett et al., 1998)
Pro-peptide subtilisin	3.3	6	1.5	1.7	0	0.1	77	6	9	–	600	(Buevich et al., 2001)
FK506BP	3.5	8	1.5	2 (5)	na	na	107	6.3	25	6.3 M urea	500	(Logan et al., 1994)
Ubiquitin	3.2	4 (6.5)	1.6	1.7	0.1	0.2	76	2	25	8 M urea	600	here

na: not applicable; values in parenthesis: single outlying points

long α-helix. The other proteins where the combination of low pH and urea fails to diminish the residual structure have a higher number of α-helices in there native states. Residual secondary structure in the unfolded states of these proteins often is α-helical and coincides with positions of α-helices in their native states (e.g. α-lactalbumin (Wirmer et al., 2006), hen lysozyme (Schwalbe et al., 1997) and apomyoglobin (Yao et al., 2001)).

Interestingly, the values defining the baseline of the R_2 relaxation rates are similar in all the urea-denatured proteins. The baseline values of R_2 relaxation rates in unfolded proteins therefore seem to be a general property of unstructured polypeptide chains (at a given viscosity), while highest R_2 relaxation rates arise from the presence of residual structure. Another interesting feature is revealed from comparison of relaxation data in the different proteins: R_2 rates in all proteins show larger variations than the other two relaxation parameters (Logan et al., 1994; Schwalbe et al., 1997). The observed variations in R_2 relaxation rates cluster in certain regions of the protein while they seem to be more randomly distributed in R_1 relaxation rates and the hetNOE. Hence, R_2 rates are a more sensitive tool for the investigation of motional properties of the (unfolded) polypeptide chain.

Discussion

Description of relaxation rates in a random-coil

The interpretation of relaxation rates in unfolded proteins is different from the interpretation of relaxation data in folded proteins. Internal motions in folded proteins are on a different time

scale than global motions. These motions therefore can be separated (Lipari and Szabo, 1982a, b). In contrast, global and internal motions in unfolded proteins are strongly coupled rendering the use of the model-free approach difficult. Reduced spectral density mapping, however, is possible (Peng and Wagner, 1992; Farrow et al., 1995, 1997; Peng and Wagner, 1995). We have used the experimental R_1, R_2 relaxation rates and the hetNOE for the calculation of the spectral density $J(\omega)$ characterizing the motion of the ^{1}H–^{15}N bond vector (see Methods section). This approach permits the direct evaluation of $J(\omega)$, at the three frequencies 0, ω_N and $0.87\omega_H$ (Figure 2).

$J(\omega_N)$ and $J(0.87\omega_H)$ reflect the uniform distribution observed in R_1 relaxation rates and the hetNOE. Only very small variations are observed along the sequence. This is not the case for $J(0)$, which is much larger than $J(\omega_N)$ and $J(0.87\omega_H)$. This analysis reveals that the motions associated with the relaxation in unfolded proteins are on time scales slower than ω_N. In addition, significant variations are present in $J(0)$. These variations agree remarkably well with the deviations in R_2 relaxation rates, as shown in Figure 2b plotted as correlation of R_2 and $J(0)$: it is therefore sufficient to concentrate on R_2 relaxation rates when characterizing the residual structure present henceforth. Different models for the description of R_2 relaxation rates in unfolded states of proteins based on segmental motions have been discussed in the literature (Schwalbe et al., 1997; Schwarzinger et al., 2002).

Regions of the sequence that exhibit increased relaxation rates are often hydrophobic (Neri et al., 1992; Ropson and Frieden, 1992; Saab-Rincon et al., 1996; Wirmer et al., 2004). The R_2 rates in methylated WT-lysozyme (WT-S^{ME}) in water, for example, were observed to be correlated with hydrophobicity. However, upon a single point mutation (W62G) the hydrophobicity remains largely unchanged while R_2 rates change dramatically all over the sequence (Klein-Seetharaman et al., 2002; Wirmer et al., 2004). Also in the case of ubiquitin, variations of hydrophobicity (Figure 3a) are uncorrelated with variations in R_2 relaxation rates: while the R_2 relaxation rates are rather uniform along the sequence, large variations are found in hydrophobicity. Normalized hydrophobicities range from 0.33 to 0.75 a.u. with a mean value of 0.53 ± 0.09 a.u. (see methods). Mean value and spread in hydrophobicities compare well with other proteins, e.g. lysozyme (mean: 0.51 ± 0.10 a.u., spread: 0.25 to 0.74 a.u.). Therefore, in the case of unfolded proteins without residual structure, hydrophobicity does not seem to influence the relaxation properties of the protein both in water and in urea.

The simplest model for the description of R_2 relaxation rates in a random coil is the segmental motion model (Schwalbe et al., 1997). The segmental motion model assumes that the influence of the neighboring residues is independent of side chain volume or hydrophobicity, and decays exponentially as the distance (in number of peptide bonds) from a given residue increases. This is described by the following expression:

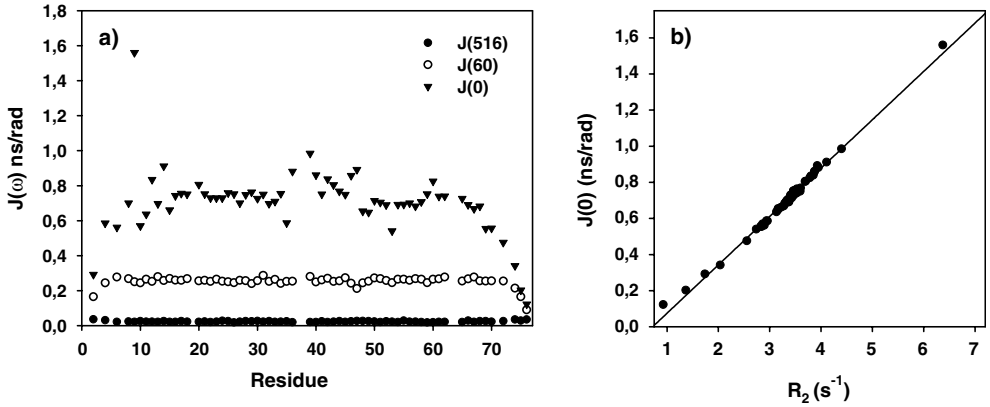

Figure 2. (a) Spectral densities, $J(\omega)$, at 0, 60 and 516 MHz as a function of residue number in unfolded ubiquitin. (b) Correlation of $J(0)$ with R_2 relaxation rates.

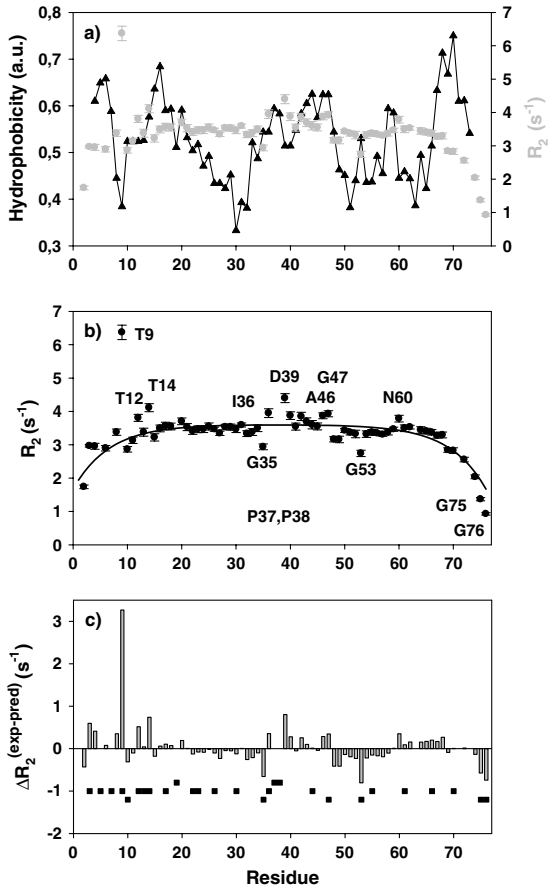

Figure 3. (a) Hydrophobicity profile of ubiquitin in black linked triangles. Hydrophobicity was calculated from the sequence using the Abraham and Leo scale (Abraham and Leo, 1987) as described in the methods section. R_2 relaxation rates are shown in gray for comparison. (b) Experimental R_2 relaxation rates fitted with the segmental motion model (Equation 4) for the dynamics of an unbranched polymer chain $R_{int}=0.27$, $\lambda_0=6.67$. (c) Deviations of the experimental R_2 relaxation rates from the fit in (b). Black boxes indicated the position of proline residues (level = −0.8), β-branched residues (level = −1) and glycine residues (level = −1.2) are indicated.

$$R_2^{rc}(i) = R_{int} \sum_{j=1}^{N} e^{\frac{|i-j|}{\lambda_0}} \quad (4)$$

where R_{int} is the intrinsic relaxation rate, which depends also on the temperature and viscosity of the solution, λ_0 is the persistence length of the polypeptide chain (in numbers of residues) and N is the total chain length of the polypeptide. The model does not take any amino-acid specific properties of the residues into account.

Only few deviations are observed using the segmental motion model (Equation 4). The segmental motion model thus describes the R_2 relaxation rates reasonably well (Figure 3b). A best fit is found using an intrinsic relaxation rate of $R_{int} = 0.27\ s^{-1}$ and a persistence length of $\lambda_0 = 6.67$ (Figure 3b). Since this persistent length agrees with the persistence length found in other unfolded proteins with $\lambda_0 = 7$ (Schwalbe et al., 1997; Klein-Seetharaman et al., 2002; Wirmer et al., 2004) we propose that the persistence length of seven residues is a general property of unfolded polypeptide chains with a normal composition of amino-acids (e.g. neither glycine rich or proline rich). Interestingly, this value agrees with the maximum size of peptides for which the isolated-pair hypothesis fails (Pappu et al., 2000). The average deviation of the relaxation rates from the model is $<\Delta R_2^{1\text{-}76}> = 0.27\ s^{-1}$. A significant deviation from the fit is found for T9. Leaving out T9 the average deviation is only $0.22\ s^{-1}$. The N-terminus is slightly less well represented by the model than the C-terminus with $<\Delta R_2^{1\text{-}15}> = 0.34\ s^{-1}$ (leaving out T9) and $<\Delta R_2^{62\text{-}76}> = 0.22\ s^{-1}$. Small ($>0.5\ s^{-1}$) positive deviations are found for residues I3, T12, T14 and for residue D39. I36 ($\Delta = 0.36\ s^{-1}$ only) and D39 flank two proline residues (P37, P38). These deviations are in agreement with previous studies of this unfolded state: Earlier investigations on unfolded ubiquitin (Peti et al., 2000) revealed restricted ψ sampling of P38 (no data are available for P37 due to overlap) and non-random $^3J(C_\alpha,C_\alpha)$ coupling constants values (1.8 Hz instead of 0.8 ± 0.1 Hz) for P19 and P38. Thus P37 and P38 induce motional restrictions onto this part of the polypeptide chain. The deviations in I3 also can be explained by non-random secondary structure in this region: the $^3J(C_\alpha, C_\alpha)$ coupling constant of I3 (1.1 Hz instead of 0.8 ± 0.1 Hz) indicates a residual preference for sampling β-sheet conformations, the H^α chemical shift of F4 is one of only three residues displaying positive deviations from random coil behaviour. The other residues that deviate from the baseline (T9, T12 and T14) are all threonine residues, suggesting that motional restrictions are found for these β-branched residues. The presence of additional threonine residues (T22, T55 and T66) in ubiquitin that do not deviate from the expected random coil relaxation rates cannot be explained using this argument. H^α chemical shift values for

L8 and I13 show positive deviations, $^3J(C_\alpha,C_\alpha)$ for I13 is slightly higher than the average (1.0 Hz) and also cross-correlation rates in the region are a little higher than average. This small bias of the conformational space towards β-sheet conformations is consistent with the present data and may explain the conformational restrictions indicated by the relaxation rates. In addition, the fact that the distance, in sequence, between T9 and T12 and between T12 and T14 is shorter than λ_0 whereas that between T22, T55 and T66 and any other T residue in the protein is longer than λ_0 suggests that the presence of clusters of these polar β-branched residues in unfolded proteins can lead to baseline distortions. A general rule for the appearance of motional restrictions in regions with high proportion of β-branched residues and/or proline residues however can be ruled out. No deviations are observed for the region from residue P19 to V26 despite the presence of three β-branched residues (T22, I23 and V26) near the obviously bulky P19. Negative deviations (< -0.5) are only found for four out of the six glycine residues (G35, G53, G75 and G76). Glycine residues thus show greater mobility than the other residues, due to the absence of any side chain. Overall, only a few deviations from the segmental motion model are found. This suggests that the segmental motion model describes the ^{15}N relaxation rates of unfolded ubiquitin well.

A combination of the segmental motion model (Equation 4) with a volume-dependent model (Schwarzinger et al., 2002) as shown in Equation 5 was also tested.

$$R_2(i) = \underbrace{R_{\text{int}} \sum_{j=1}^{N} e^{\frac{|i-j|}{\lambda_0}}}_{R_2^{\text{rc}}} + \underbrace{k \sum_{j=1}^{N} \tau_j e^{\frac{|i-j|}{\lambda_0}}}_{R_2^{R_g}} \qquad (5)$$

where τ_j = is proportional to the intrinsic correlation time of a residue j given as $\tau_j = (R_g)^3$ (R_g = radius of gyration of the respective amino acid side chain), N is the total chain length of the polypeptide, k is a scaling constant and λ_j is the persistent length of the polypeptide chain (in numbers of residues). The best fit, however, was found for the R_g independent case (with $k = 0$), yielding the same equation as for the segmental motion model alone.

Comparison of the different states of ubiquitin

The data presented here and in earlier work (Peti et al., 2000, 2001; Wirmer and Schwalbe, 2002) clearly reveal that ubiquitin is a protein devoid of residual structure in its low pH urea-denatured state. Figure 4 shows a comparison of R_2 relaxation rates in the three states of ubiquitin (Tjandra et al., 1995; Brutscher et al., 1997). R_2 relaxation rates in the native state of ubiquitin range from 4.8 s^{-1} to 6.4 s^{-1} but for residues D21 (R_2 = 6.9 s^{-1}) and I23 (R_2 = 8.3 s^{-1}) and at the unstructured C-terminus, where lower relaxation rates resembling closely those of the denatured state are observed. A relaxation rate of 6.4 s^{-1} thus marks the highest possible relaxation rate in ubiquitin that can be observed caused by motional restrictions due to persistent secondary and tertiary contacts in the native state of this very rigid protein.

The relaxation rates in urea-denatured ubiquitin are considerably lower than this value, reflecting the flexibility of the protein in this state. In contrast, relaxation rates in the A-state of ubiquitin are of the same order of magnitude (around 5.5 s^{-1}) than those in the native state for the structured very N-terminus of the protein (except for residue T9); however, considerably larger in the two helices ranging from residue 23 to residue 34 and residue 39 to residue 72. The highest relaxation rate in the A-state is 14.9 s^{-1}.

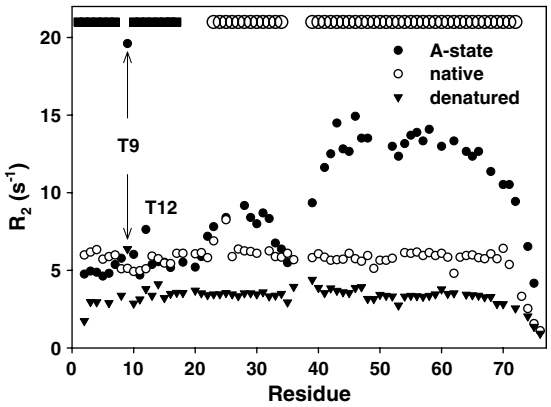

Figure 4. Comparison of R_2 relaxation rates in the A-state (Brutscher et al., 1997), native (Tjandra et al., 1995) and denatured state of ubiquitin. Structural elements identified in the A-state are indicated (sheet as black bars, α-helices as overlapping open circles).

The high relaxation rates observed in the A-state cannot be attributed to rigidity caused by persistent tertiary contacts, to locally restricted motions of segments of an unfolded conformation, or to conformational exchange. Rather, Brutscher et al. argued that these high relaxation rates in the helices are caused by anisotropic rotational motions (Brutscher et al., 1997). This conclusion is in agreement with their observed lack of slow exchange contributions to the ^{15}N R_2 relaxation rates (Brutscher et al., 1997, supplementary material).

Conclusions

The data presented here show that unfolded ubiquitin is a good model protein for unfolded states of proteins without any residual structure, thus for a protein in a random coil. $^3J(H^N,H^\alpha)$, $^1J(N_i,C_{\alpha i})$ and $^2J(N_i,C_{\alpha(i-1)})$ coupling constants and chemical shift deviations reveal the absence of any secondary structure elements. Uniform relaxation data along the sequence indicate the absence of motional restriction caused by residual secondary structure and long-range interactions.

The criteria used here for the identification of a protein in the random coil state, e.g. chemical shifts, coupling constants and relaxation rates, are easily accsessible in labeled proteins. Thus, they can be used as a general tool for the identification of proteins in their random coil or to identify residual structure. Particularily R_2 relaxation rates are very sensitive to any deviation from the random coil.

Relaxation data in unfolded states without residual structure are best predicted independently from residue types involved, solely depending on segmental motions. Therefore, the segmental motion model can be used to predict residual structure in unfolded proteins. Deviations from the segmental motions are due to residual structure rather than due to residue specific relaxation properties but for glycine and proline residues. This is different for J couplings, chemical shifts or cross-correlated relaxation rates (our previous work) or residual dipolar couplings (M. Blackledge, personal communication) that show clear variation between amino acids due to the amino-acid specific sampling of ϕ,ψ-conformational space. In the absence of chemical exchange contributions, relaxation rates are influenced by faster and therefore more restricted motions; with the exception of glycine and proline residues, those motions do not depend on amino-acid type. Comparison of R_2 relaxation rates in the three different states of ubiquitin highlights the distinct characteristica of the protein states reflecting on their motional properties.

Supplementary Material

Two figures showing (i) Residue specific correlation between experimental $^3J(H^N,H^\alpha)$ coupling constants with predicted coupling constants from the random coil model and (ii) $^1J(N_i,C_{\alpha i})$ and $^2J(N_i,C_{\alpha(i-1)})$ coupling constants in denatured ubiquitin. This electronic supplementary material is available at http://dx.doi.org/10.1007/s10858-006-9026-9.

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