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# Dynamics of a De Novo Designed Three-Helix Bundle Protein Studied by $^{15}\text{N}$ , $^{13}\text{C}$ , and $^2\text{H}$ NMR Relaxation Methods<sup>†</sup>

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**ABSTRACT:** Understanding how the amino acid sequence of a polypeptide chain specifies a unique, functional three-dimensional structure remains an important goal, especially in the context of the emerging discipline of de novo protein design.  $\alpha_3\text{D}$  is a single chain protein of 73 amino acids resulting from a de novo design effort. Previous solution nuclear magnetic resonance studies of  $\alpha_3\text{D}$  confirm that the protein adopts the designed structure of a three-helix bundle. Furthermore,  $\alpha_3\text{D}$  has been previously shown to possess all of the major thermodynamic and structural characteristics of natural proteins, though it shares no sequence homology to any protein sequence in the database. In this work, the backbone and side-chain dynamics of  $\alpha_3\text{D}$  were investigated using  $^{15}\text{N}$ ,  $^{13}\text{C}$ , and  $^2\text{H}$  nuclear magnetic resonance relaxation methods with the aim of assessing the character of the internal motions of this native-like protein of de novo design. At the backbone level, both  $^{15}\text{N}$  and  $^{13}\text{C}_\alpha$  relaxation studies indicate highly restrictive motion on the picosecond to nanosecond time scale in the  $\alpha$ -helical regions of  $\alpha_3\text{D}$ , with increasing mobility at the ends of the  $\alpha$ -helices and in the two loop regions. This is largely consistent with what is seen in proteins of natural origin. Overall, the view provided by both  $^2\text{H}$  and  $^{13}\text{C}$  methyl relaxation methods suggest that the side chains of  $\alpha_3\text{D}$  are more dynamic compared to natural proteins. Regions of relative flexibility bound clusters of rigid methyl-bearing side-chain groups that are interspersed with aromatic and  $\beta$ -branched amino acids. The time scale of motions associated with methyl-bearing side chains of  $\alpha_3\text{D}$  are significantly longer than that seen in natural proteins. These results indicate that the strategies underlying the design of  $\alpha_3\text{D}$  have largely, but not completely, captured both the structural and dynamic character of natural proteins.

The robust de novo design of conformationally specific hydrophobic cores of proteins such as those typically seen in proteins of natural origin remains a significant challenge. More often than not, proteins of de novo design have adopted structural, dynamic and thermodynamic features more characteristic of the protein molten globule than of native-state proteins (*1*). To overcome this deficiency, there has been an intense effort in the development of computational repacking algorithms that can assist in the design of protein interiors [for a review see Lazar and Handel (*2*)]. Several studies have shown the importance of including  $\beta$ -branched and aromatic residues within the hydrophobic core with the aim of restricting side-chain mobility (*3–6*). Recently, de novo designed coiled coils (*7, 8*), several four-helix bundle proteins (*5, 9–11*) and a three-helix bundle protein (*12*) have been

shown to have native-like structure indicating that robust design principles are beginning to emerge.

Our view of the internal dynamics of natural proteins is also evolving. Extensive nuclear magnetic resonance (NMR)<sup>1</sup>-based studies have indicated that motion on the main chain of natural proteins is generally of high frequency and small amplitude with exceptions being restricted to loops and elements lacking classical secondary structure (e.g., refs *13–16*). In contrast, a theme emerging from NMR relaxation studies of methyl-bearing side chains of proteins is that the hydrophobic cores of natural proteins are quite dynamic and heterogeneously so (*17–23*). Most recently, it has been suggested that the subnanosecond time-scale motions of methyl-bearing side chains in native proteins tend to cluster into three distinct classes of angular order (*24*). The presence of significant dynamics brings into play the role of residual conformational entropy in stabilizing the folded state of the protein (*25*). Understanding the side-chain dynamics in the cores of natural proteins using NMR relaxation methods should reveal the principles necessary to allow for the successful design of more native-like hydrophobic cores. In this regard, similar studies of de novo designed proteins will further enrich our understanding of the detailed success of

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<sup>1</sup> Abbreviations: fid, free induction decay; LRCC, long-range coupling constant; NOE, nuclear Overhauser effect;  $T_1$ , spin lattice relaxation time;  $T_2$ , spin-spin relaxation time.

de novo protein design.  $\alpha_3$ D is a particularly appropriate candidate for such an analysis as it has proven to be a de novo designed three-helix bundle protein having both native-like thermodynamic and structural characteristics (12). Here the internal dynamics of both the backbone and side chains of  $\alpha_3$ D are investigated using  $^{15}\text{N}$ ,  $^{13}\text{C}$ , and  $^2\text{H}$  NMR relaxation methods.

## EXPERIMENTAL PROCEDURES

**Sample Preparation.** A uniformly  $^{15}\text{N}$  labeled  $\alpha_3$ D sample was prepared for the  $^{15}\text{N}$  relaxation experiments by growing BL21(DE3) *Escherichia coli* cells on  $^{15}\text{NH}_4\text{Cl}$  (1 g/L, Isotec). A specifically labeled  $^{13}\text{C}_\alpha$  carbon at alanine and leucine residues in  $\alpha_3$ D was prepared by growth on amino acids supplemented with  $^{13}\text{C}_\alpha$  labeled alanine and leucine (Isotec). A uniformly  $^{15}\text{N}$ ,  $^{13}\text{C}$ , and randomly fractionally  $^2\text{H}$  (50%)  $\alpha_3$ D sample was prepared using M9 minimal media with  $^{15}\text{NH}_4\text{Cl}$  (1 g/L),  $[\text{U-}^{13}\text{C}]$ glucose (2 g/L, Isotec) grown in 50%  $\text{H}_2\text{O}:\text{H}_2\text{O}$ . The alanine, leucine, valine, and isoleucine  $\gamma$  methyl groups were specifically labeled with  $^{13}\text{C}$  by growing the cells in 0.3%  $^{13}\text{C}(3)$ -sodium pyruvate (w/v, Cambridge) as the sole carbon source with  $^{15}\text{NH}_4\text{Cl}$  in M9 minimal media as described (26). All samples were purified as described previously (12). All samples were 2 mM except the pyruvate grown sample, which was 1.7 mM. All samples were prepared in 50 mM  $d_3$ -sodium acetate (pH 5.0), 0.05% sodium azide, and 92%:8%  $\text{H}_2\text{O}:\text{H}_2\text{O}$ .

**NMR Spectroscopy.** NMR experiments were conducted using Varian Inova 500 (11.7 T), 600 (14.1 T), and 750 (17.6 T) spectrometers equipped with three-axis pulsed field gradient triple resonance probes. Also, a  $^{15}\text{N}$   $T_1$  data set was collected on a Bruker DMX-400 (9.4 T) spectrometer equipped with  $^1\text{H}/^{15}\text{N}/^{13}\text{C}/^{31}\text{P}$  probe and  $z$ -axis pulsed field gradients (at NMRFAM, University of Wisconsin, Madison, WI). The temperature was calibrated to  $30.0 \pm 0.5$  °C using a 100% methanol sample (27).

**$^{15}\text{N}$  Relaxation Measurements.**  $^{15}\text{N}$   $T_1$ ,  $T_2$ , and  $\{^1\text{H}\}$ - $^{15}\text{N}$  NOE experiments employed pulse sequences as described by Farrow et al. (28).  $^{15}\text{N}$   $T_1$  experiments were carried out at 9.4, 11.7, and 14.1 T. At 9.4 T, the  $^{15}\text{N}$   $T_1$  relaxation delays were 30\*, 50, 80, 120\*, 170.0, 220.0, 290.0, 350.0, 430.0\*, 510.0, 600.0, and 700.0 ms. Asterisks indicate duplicated measurements. At 11.7 T,  $^{15}\text{N}$   $T_1$  delay periods were 55, 90\*, 125, 195.0, 275.0\*, 355.0, 445.0, 545.0, 655.0, 755.0\*, 850.0, and 1005.0 ms. At 14.1 T,  $^{15}\text{N}$   $T_1$  delay periods were 54\*, 84, 114.0, 164.0\*, 234.0, 314.0, 424.0, 544.0\*, 684.0, 844.0, 1014.0, and 1214.0 ms.  $^{15}\text{N}$   $T_2$  experiments were carried out at 11.7 and 14.1 T with a CPMG pulse train of 12.6\*, 24.6, 47.6, 72.6, 83.6\*, 100.6, 114.6\*, 126.6, 141.6, and 153.6 ms with a 5.1 kHz field strength, and 9.0\*, 34.0, 59.0\*, 84.0, 109.0, 134.0\*, and 160.0 ms with a 6.25 kHz field strength, respectively.  $^{15}\text{N}$   $T_1$  and  $T_2$  experiments were run with 8–24 transients/fid and with a total recycle delay of 1.1–1.5 s. In the  $\{^1\text{H}\}$ - $^{15}\text{N}$  NOE experiments, a steady state was reached after at least 3 s of  $120^\circ$   $^1\text{H}$  saturation pulses placed every 5 ms, and a total recycle of 5 s was used to allow for the longitudinal  $^{15}\text{N}$  magnetization to reach equilibrium. Spectra with and without the NOE were collected in an interleaved manner. For all experiments, the  $^{15}\text{N}$  carrier was positioned at 116 ppm with a typical spectral width of 33 ppm, while the  $^1\text{H}$  carrier was placed on  $\text{H}_2\text{O}$  with a spectral width of

$\sim 12$  ppm. Experiments were collected with 1024 ( $^1\text{H}$ ) by 120–160 ( $^{15}\text{N}$ ) complex points.

**$^{13}\text{C}$   $\alpha$  Relaxation Measurements.**  $^{13}\text{C}_\alpha$   $T_1$ ,  $T_2$ , and  $\{^1\text{H}\}$ - $^{13}\text{C}_\alpha$  NOE experiments were carried as described (28) adapted for the methine carbon. At 11.7 T,  $^{13}\text{C}_\alpha$   $T_1$  relaxation delays were 80.5\*, 115.5, 150.5, 220.5, 300.5, 380.5\*, 470.5, 570.5, 685.0, 780.5\*, 910.5, and 1030.5 ms with 16 transients/fid.  $^{13}\text{C}_\alpha$   $T_2$  experiments consisted of a CPMG pulse trains of 11.4\*, 20.4, 27.4, 36.4\*, 43.4, 51.4, 66.4, 82.4\*, 98.4, and 114.4 ms with a 20.0 kHz field strength and 16 transients/fid. The  $\{^1\text{H}\}$ - $^{13}\text{C}_\alpha$  NOE experiment was collected in a similar manner as described for the  $\{^1\text{H}\}$ - $^{15}\text{N}$  NOE experiment, with 44 transients/fid. For all the  $^{13}\text{C}_\alpha$  experiments the  $^{13}\text{C}$  carrier was positioned at 52.5 ppm with a spectral width of 10 ppm, and the  $^1\text{H}$  carrier was placed on  $\text{H}_2\text{O}$  with a spectral width of 13 ppm. Experiments were collected with 1024 ( $^1\text{H}$ ) by 120 ( $^{13}\text{C}$ ) complex points.

**$^2\text{H}$  Methyl Relaxation Measurements.** Measurements for multiple spin coherence involving deuterium were measured for  $\text{R}(\text{I}_z\text{C}_z)$ ,  $\text{R}(\text{I}_z\text{C}_z\text{D}_z)$ , and  $\text{R}(\text{I}_z\text{C}_z\text{D}_y)$  at 11.7 and 14.1 T. The pulse sequences used involved the increased resolution experiments developed by Kay and co-workers (29). At 11.7 T, relaxation delays were 11.0\*, 18.4, 25.7, 33.1\*, 40.5, 47.9, 55.2, 62.1\*, and 70.0 ms for the  $\text{I}_z\text{C}_z$ ; 2.2\*, 6.3, 11.5, 17.7\*, 24.7, 32.5, 50.0\*, 59.7, and 70.0 ms for the  $\text{I}_z\text{C}_z\text{D}_z$ ; and 1.0\*, 2.7, 4.9\*, 10.6, 14.0\*, 17.6, 21.5, 25.6\*, and 30.0 ms for the  $\text{I}_z\text{D}_z\text{D}_y$ . Recycle delays of 1.6, 1.8, and 2.1 s and 32, 48, and 48 transients/fid were collected for the  $\text{I}_z\text{C}_z$ ,  $\text{I}_z\text{C}_z\text{D}_z$ , and  $\text{I}_z\text{C}_z\text{D}_y$ , respectively. The spectra were collected with 1024 ( $^1\text{H}$ ) by 56 ( $^{13}\text{C}$ ) complex points. At 14.1 T, relaxation delays were 2.0\*, 9.4, 16.7, 24.1\*, 31.5, 38.9, 46.2, 53.6\*, and 61.0 ms for the  $\text{I}_z\text{C}_z$ ; 2.2\*, 6.3, 11.5, 17.7\*, 24.7, 32.5, 40.9, 50.0\*, 59.7, and 70.0 ms for the  $\text{I}_z\text{C}_z\text{D}_z$ ; 1.0\*, 2.7, 4.9\*, 7.6, 10.6, 14.0\*, 17.6, 21.5, 25.6\*, and 30.0 ms for the  $\text{I}_z\text{D}_z\text{D}_y$ . Recycle delays of 2.1, 2.1, and 2.3 s and 24, 32, and 32 transients/fid were collected for the  $\text{I}_z\text{C}_z$ ,  $\text{I}_z\text{C}_z\text{D}_z$ , and  $\text{I}_z\text{C}_z\text{D}_y$ , respectively. The spectra were collected with 1024 ( $^1\text{H}$ ) by 68 ( $^{13}\text{C}$ ) complex points. The  $^{13}\text{C}$  carrier was placed at 20 ppm with a spectral width of 16 ppm, and the  $^1\text{H}$  carrier was placed on  $\text{H}_2\text{O}$  with a spectral width of 12–13 ppm.

**$^{13}\text{C}$  Methyl Relaxation Measurements.**  $^{13}\text{C}$  methyl  $T_1$  and  $\{^1\text{H}\}$ - $^{13}\text{C}$  methyl NOE experiments were collected at 11.7, 14.1, and 17.6 T as described (21) with the exception at 17.6 T, at which the  $T_1$  experiment incorporated the WATERGATE sequence (30) for  $\text{H}_2\text{O}$  suppression. At 11.7 T,  $^{13}\text{C}$  methyl  $T_1$  relaxation delays were 40.5\*, 100.5, 190.5, 300.5\*, 420.5, 550.5, 690.5\*, 840.5, and 1005.0 ms. Enhancement from the NOE was developed for 2.3 s and the total recycle delay was 3.0 s. The spectrum was collected with 1024 ( $^1\text{H}$ ) by 120 ( $^{13}\text{C}$ ) complex points with 8 transients/fid. At 14.1 T,  $^{13}\text{C}$  methyl  $T_1$  relaxation delays were set to 48.5\*, 120.5, 228.5, 360.5\*, 504.5, 660.5, 828.5\*, 1010.5, and 1205.0 ms. Enhancement from the NOE was developed for 2.3 s and the total recycle delay was 3.0 s. The spectrum was collected with 1024 ( $^1\text{H}$ ) by 110 ( $^{13}\text{C}$ ) complex points with 8 transients/fid. At 17.6 T,  $^{13}\text{C}$  methyl  $T_1$  relaxation delays were set to 45.0\*, 125.5, 231.5, 355.5\*, 496.5, 652.5, 822.5\*, 1005.5, and 1205.0 ms. Enhancement from the NOE was developed for 2.5 s and the total recycle delay was 3.3 s. The spectrum was collected with 1024 ( $^1\text{H}$ ) by 100 ( $^{13}\text{C}$ ) complex points with 8 transients/fid.  $\{^1\text{H}\}$ - $^{13}\text{C}$  methyl NOE experiments were collected with a reference experiment without  $^1\text{H}$  saturation

and with  $^1\text{H}$  saturation for approximately 10  $T_1$  values (6, 7, and 7 s for the 11.7, 14.1, and 17.6 T, respectively) in an interleaved manner. At 11.7, 14.1, and 17.6 T, the  $\{^1\text{H}\}$ - $^{13}\text{C}$  methyl NOE spectra were collected with 12, 16, and 12 transients/fid, respectively. The  $^{13}\text{C}$  carrier in all spectra was placed at 20.0 ppm with a spectral width of 16 ppm and the  $^1\text{H}$  carrier was placed on  $\text{H}_2\text{O}$  with a spectral width of 12–14 ppm.

**Data Analysis.** The  $^{15}\text{N}$  and  $^{13}\text{C}_\alpha$  data set were processed into 1024 ( $^1\text{H}$ ) by 1024 ( $^{15}\text{N}$ ,  $^{13}\text{C}$ ) point matrixes and the  $^{13}\text{C}$  methyl and  $^2\text{H}$  data were processed into 1024 by 512 ( $^{13}\text{C}$ ) point matrixes using the program Felix (Biosym Technologies). Cross-peak intensities were extracted and fit to a two-parameter exponential fit using the Levenberg–Marquardt algorithm (31). The  $\chi^2$  residuals were less than the number of measurements taken, signifying a reasonable “goodness of fit”. Uncertainty in the measurements was determined from duplicate experiments for the  $T_1$  and  $T_2$  measurements. The steady-state NOE values were determined from the intensity of the NOE divided by the reference experiment. The error was estimated from the root-mean-square of the baseline noise, and to be conservative, this value was doubled. Typical errors in the  $^{15}\text{N}$ ,  $^{13}\text{C}_\alpha$ ,  $^{13}\text{C}$  methyl, and  $^2\text{H}$  relaxation parameters ranged from 1 to 4%. A duplicate  $\{^1\text{H}\}$ - $^{13}\text{C}$  methyl NOE experiment was collected at 17.1 T, and the error was approximately 1%.

**Characterization of Global and Local Motion.** The anisotropy of global tumbling was assessed using the “local site approach” implemented in the programs Quadric 1.1 program (32) and an in-house program (33). The simple (34, 35) and extended (36) model-free spectral densities were used as described below. Model-free internal dynamic parameters were determined using the local site treatment, using a nonlinear least-squares Powell minimization (31) as described previously (21, 33). Parameter errors were estimated from 150 Monte Carlo simulations. The  $^{15}\text{N}$  and  $^{13}\text{C}$  chemical shift anisotropy ( $\Delta\sigma$ ) was fixed at  $-170$  (33, 37, 38) and 25 ppm (39), respectively. The bond lengths used for the N–H of 1.02 Å,  $\text{C}_\alpha$ –H of 1.095 Å (40), and C methyl–H of 1.115 Å (40). The quadrupolar coupling constant ( $e^2qQ/h$ ) of 165 kHz (41) was used for the  $^2\text{H}$  methyl analysis.

**Determination of Side-Chain Coupling Constants.** The  $\chi^1$  angles for the isoleucine, valine, and threonine residues were calculated from  $^3J_{\text{NC}\gamma}$  and  $^3J_{\text{COC}\gamma}$  coupling constants obtained from quantitative  $J$ -correlation experiments (42, 43). The  $\chi^2$  angles of leucine and isoleucine residues were calculated from  $^3J_{\text{C}\delta\text{C}\alpha}$  coupling constants obtained from a three-dimensional long-range carbon coupling (LRCC) experiment (44). The LRCC pulse sequence was modified from the original experiment to include continuous SEDUCE-1 carbonyl decoupling during  $t_1$  and  $t_2$  evolution periods and included a WATERGATE sequence for water suppression (30).

## RESULTS

**Characterization of Global Tumbling.** A critical first step in the analysis of NMR relaxation data obtained on macromolecules is the assessment of the degree of anisotropy of molecular reorientation. Analysis of the lowest energy solution structure of  $\alpha_3\text{D}$  reveals principal moments of inertia of 1.00:0.94:0.34, suggesting the possibility of significant

anisotropy of tumbling in solution. This was addressed by analysis of obtained  $^{15}\text{N}$  relaxation data using the “local site approach” implemented in the programs Quadric 1.1 program (32) and an in-house program (33).  $^{15}\text{N}$   $T_1$  relaxation data were collected at field strengths of 9.4, 11.7, and 14.1 T and  $^{15}\text{N}$   $T_2$  and NOE data were collected at 11.7 and 14.1 T, for a total of seven data sets. The rotational correlation time ( $\tau_m$ ) of  $\alpha_3\text{D}$  was determined using a reduced set of  $^{15}\text{N}$  sites. Sites were excluded if the obtained  $^{15}\text{N}$  NOE value was less than 0.60, if there was an unanticipated field dependence of the  $^{15}\text{N}$   $T_2$  measurements, indicating contamination by chemical exchange processes ( $R_{\text{ex}}$ ), or if the cross-peak was ill-resolved. These criteria resulted in a set of 35 of 73 residues being used to characterize global tumbling. Using these data, the solution structure of  $\alpha_3\text{D}$  was fit to isotropic, axially symmetric, and a fully anisotropic models. The global effective correlation time ( $\tau_{m,\text{eff}}$ ) was determined as  $\tau_{m,\text{eff}} - 1 = 6D_{\text{eff}}$ , where  $D_{\text{eff}}$  is the sum of the diagonal components from the diffusion tensor ( $D_{\text{eff}} = 1/3(D_{xx} + D_{yy} + D_{zz})$ ) (32). The best fits obtained for  $\alpha_3\text{D}$  are to an axially symmetric diffusion tensor. Using only the  $^{15}\text{N}$   $T_1$  and NOE data, the  $D_{\parallel}/D_{\perp}$  is  $1.48 \pm 0.14$  with a  $\tau_{m,\text{eff}}$  of 6.54 ns compared to the value of 5.20 ns determined from the isotropic model. Anisotropic fits are too demanding when only using  $T_1$  and NOE data. Incorporation of the  $^{15}\text{N}$   $T_2$  data, however, shows a dramatic smoothing in the fitting of the diffusion tensor due to the  $J(0)$  information from the  $^{15}\text{N}$   $T_2$ . The  $D_{\parallel}/D_{\perp}$  is  $1.26 \pm 0.06$  and an  $\tau_{m,\text{eff}}$  of 5.59 ns similar to the value of 5.50 ns determined for the isotropic model. Incorporation of the  $^{13}\text{C}_\alpha$  data set, yields  $D_{\parallel}/D_{\perp}$  value of  $0.57 \pm 0.03$ . Similar behavior has been seen for ubiquitin (33) and cytochrome  $c_2$  (45) exhibiting two minima corresponding to prolate and oblate shapes. The  $\tau_{m,\text{eff}}$  from the  $^{15}\text{N}$  ( $T_1$ ,  $T_2$ , and NOE) and  $^{13}\text{C}_\alpha$  ( $T_1$ ,  $T_2$ , and NOE) data yields a value of 5.16 ns very similar to the value of 5.20 ns determined using only  $^{15}\text{N}$   $T_1$  and NOE data fit to an isotropic model. In summary, using the  $^{15}\text{N}$   $T_1$  and NOE data, an isotropic  $\tau_m$  of 5.2 ns was obtained and inflates to 5.5 ns when  $T_2$  are included, similar to what has been seen previously (33). Finally, calculations of the simple model-free squared generalized order parameters ( $S^2$ ) and the effective correlation times ( $\tau_e$ ) using an axially symmetric diffusion tensor yield values within experimental error of those derived from the isotropic model.

**Backbone Dynamics.** Simple model-free parameters obtained for the backbone N–H bonds of  $\alpha_3\text{D}$  are shown on a per residue basis in Figure 1. They are similar to those seen for natural proteins in regions of secondary structure, with  $S^2$  values ranging from 0.80 to 0.85. As with natural proteins, the N–H  $S^2$  values decrease at the ends of the  $\alpha$ -helices and in the two loop regions. The effective correlation times ( $\tau_e$ ) average 45 ps in the  $\alpha$ -helices to 150 ps in the more flexible regions of  $\alpha_3\text{D}$ , again typical values for a natural protein.

The W4 indole N–H is resolved in the  $^{15}\text{N}$ –HSQC spectrum, which allows for determination of its relaxation parameters. The indole N–H has a  $S^2$  value of 0.61 (using a CSA of  $-170$  ppm) while the backbone N–H of W4 has a  $S^2$  value of 0.74. The solution structure of  $\alpha_3\text{D}$  shows that the indole N–H bond points toward solvent (12). Using a probe sphere radius of 1.4 Å, the W4 side chain is 34% solvent exposed. The indole N–H has similar mobility to



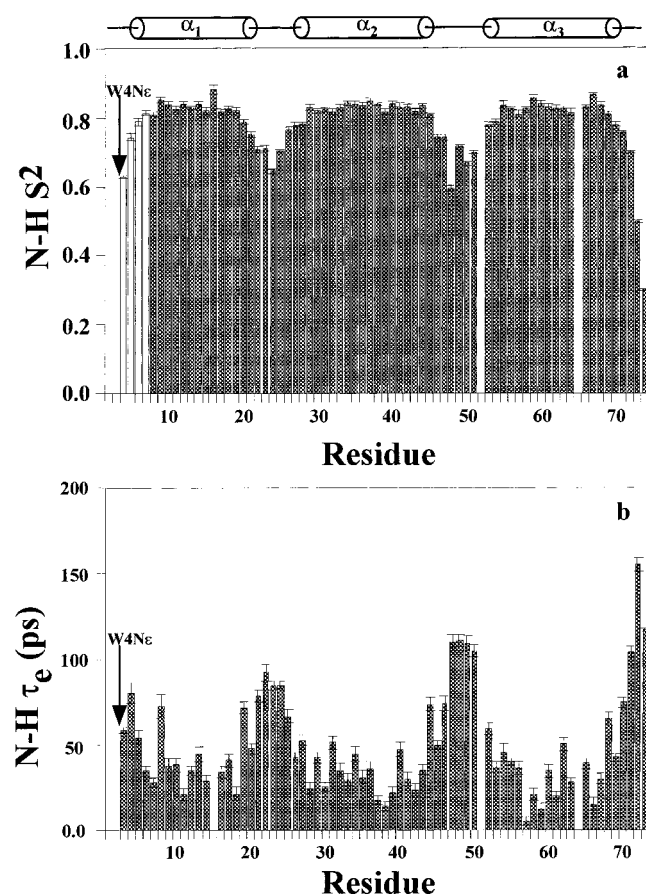


FIGURE 1: (a)  $^{15}\text{N}$ -derived amide N–H squared generalized order parameters determined for  $\alpha_3\text{D}$  using  $^{15}\text{N}$   $T_1$  (9.4, 11.7, and 14.1 T) and NOE (11.7 and 14.1 T) data at 30 °C. (b) The corresponding effective correlation times,  $\tau_e$ . An isotropic rotational correlation time ( $\tau_m$ ) of 5.2 ns was used.

the two loop regions (G22–G23 and K46–P51) of  $\alpha_3\text{D}$  (Figure 1).

The dynamics of the polypeptide backbone were also studied using  $^{13}\text{C}_\alpha$  relaxation of alanine and leucine residues.  $T_1$ ,  $T_2$ , and NOE experiments were carried out in 92:8%  $\text{H}_2\text{O}$ :  $^2\text{H}_2\text{O}$  at 11.7 T, to match solution conditions to the  $^{15}\text{N}$  sample. The experiments were implemented in identical fashion to the  $^{15}\text{N}$ -based experiments, using gradient coherence selection as an effective method of water suppression (28). There are 15 alanine and 7 leucine residues in  $\alpha_3\text{D}$ . Twelve of the 15 alanine residues were sufficiently resolved to allow accurate quantification of the relaxation parameters.  $^{13}\text{C}_\alpha$ -H  $S^2$  and  $\tau_e$  values were determined for six of seven leucine residues (L42 did not yield a unique solution). Figure 2 shows the obtained  $\text{C}_\alpha$ -H  $S^2$  and  $\tau_e$  values. The uniformly high  $\text{C}_\alpha$ -H  $S^2$  values ( $\sim 0.90$ ) are indicative of extremely restricted motion of the  $\text{C}_\alpha$ -H vectors in the ps–ns time regime. The relatively low  $\text{C}_\alpha$ -H  $S^2$  values of alanine and leucine residues are in close proximity to the loop regions (A20, L21, and A44) and the C-terminus (L67 and A69). The  $^{13}\text{C}_\alpha$ -H  $S^2$  values are higher than the corresponding N–H  $S^2$  value by  $\sim 0.10$ . Similar behavior has been seen for  $^{15}\text{N}$ - and  $^{13}\text{C}$ -relaxation studies of cyclosporin A (46), ubiquitin (14, 18) and the snake toxin  $\alpha$  from *Naja nigricollis* (47, 48). Molecular dynamics simulations suggest that this behavior can result from anti-correlated fluctuations of the torsion angles of  $\Psi_{i-1}$  and  $\Phi_i$  (48, 49). The  $^{13}\text{C}_\alpha$   $\tau_e$  times

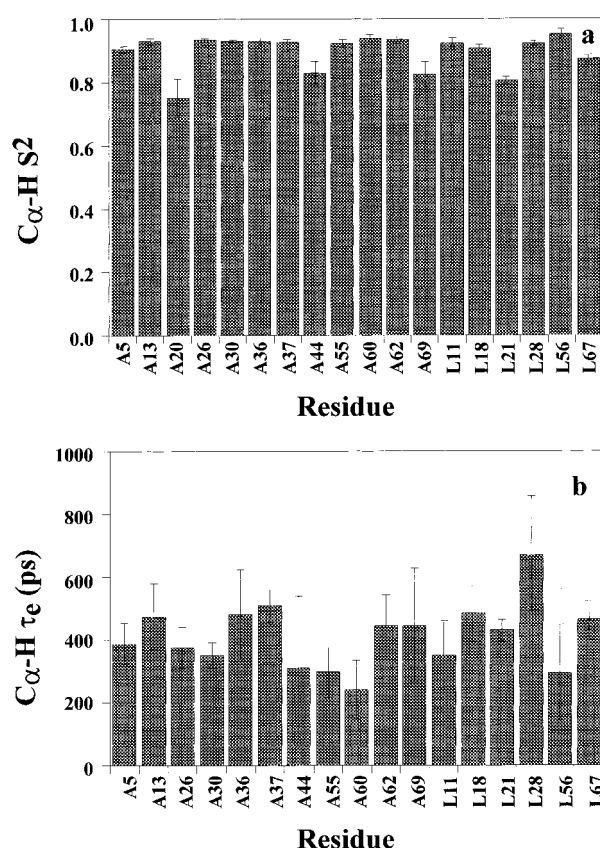


FIGURE 2: (a)  $^{13}\text{C}$ -derived  $\alpha$  C–H squared generalized order parameters determined for alanine and leucine residues of  $\alpha_3\text{D}$  at 30 °C. (b) The corresponding effective correlation times,  $\tau_e$ . A12, A29, and A61 were not determined due to spectral overlap. An isotropic correlation time ( $\tau_m$ ) of 5.2 ns was used.

average 400 ps, which is approximately 100 ps longer than the average of 300 ps determined for ubiquitin (18).

**Side-Chain Dynamics.** Deuterium  $T_1$  and  $T_{1\rho}$  relaxation times were determined at 11.7 and 14.1 T for the majority of methyl groups of  $\alpha_3\text{D}$ . Exceptions include alanine  $\beta$  methyl groups where the  $^1\text{H}$ - $^{13}\text{C}$  methyl correlations were not sufficiently resolved. The  $\delta$  methyl groups of L11, L18, and L21 have minor resonance overlap and therefore their relaxation parameters should be interpreted with caution. The symmetry axis squared generalized order parameters ( $S_{\text{axis}}^2$ ) and the corresponding effective correlation times are listed in Table 1 and illustrated in Figures 3 and 4. Also provided in Table 1 are the corresponding effective cone angles derived from the “diffusion in a cone” model (34, 35) using the following equation:

$$\theta = \cos^{-1} \left[ \frac{1}{2} \left( (1 + 8S_{\text{axis}}^2)^{\frac{1}{2}} - 1 \right) \right] \quad (1)$$

The  $S_{\text{axis}}^2$  values of the methyl groups of  $\alpha_3\text{D}$  are on average lower than the average  $S_{\text{axis}}^2$  values determined for natural proteins (22). The alanine  $\beta$  methyl groups have  $S_{\text{axis}}^2$  values ranging from 0.57 to 0.79. They have an average  $S_{\text{axis}}^2$  value of 0.64, which is approximately 0.20 lower than that seen in natural proteins (22). A60 $^\beta$  has the highest  $S_{\text{axis}}^2$  value of 0.79, which correlates well with this side chain being buried in the hydrophobic core. Interestingly, the  $S_{\text{axis}}^2$  values for A20 $^\beta$ , A26 $^\beta$ , and A44 $^\beta$  are significantly different and lower (Table 1) than their corresponding  $\text{C}_\alpha$ -H  $S^2$  values (Figure

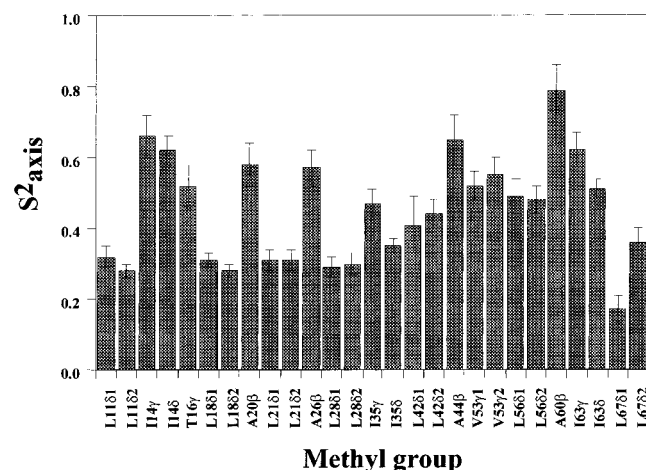


FIGURE 3:  $^2\text{H}$ -derived methyl symmetry axis squared generalized order parameters ( $S_{\text{axis}}^2$ ) determined for  $\alpha_3\text{D}$  at 30  $^{\circ}\text{C}$ .

Table 1:  $\alpha_3\text{D}$  Methyl Simple Model-Free (SMF) Parameters from the  $^2\text{H}$  Relaxation at 30  $^{\circ}\text{C}$

	$S_{\text{axis}}^2$	$\tau_e$ (ps)	$\chi_{M-n}^2$	$\theta$ (deg)
L11 $\delta^1$	$0.32 \pm 0.03$	$49.9 \pm 1.8$	5.0	47.6
L11 $\delta^2$	$0.28 \pm 0.02$	$53.9 \pm 2.1$	0.1	50.0
I14 $\gamma$	$0.66 \pm 0.06$	$38.1 \pm 4.0$	0.4	29.6
I14 $\delta$	$0.62 \pm 0.04$	$18.8 \pm 2.2$	0.0	31.9
T16 $\gamma$	$0.52 \pm 0.06$	$59.9 \pm 2.5$	2.4	36.7
L18 $\delta^1$	$0.31 \pm 0.02$	$51.5 \pm 1.9$	0.1	48.4
L18 $\delta^2$	$0.28 \pm 0.02$	$54.2 \pm 2.1$	0.6	50.1
A20 $\beta$	$0.58 \pm 0.06$	$44.6 \pm 2.8$	0.5	33.8
L21 $\delta^1$	$0.31 \pm 0.03$	$53.1 \pm 2.0$	0.6	48.0
L21 $\delta^2$	$0.31 \pm 0.03$	$51.7 \pm 3.6$	6.5	48.1
A26 $\beta$	$0.57 \pm 0.05$	$46.8 \pm 2.4$	0.1	34.1
L28 $\delta^1$	$0.29 \pm 0.03$	$60.6 \pm 2.4$	0.3	49.2
L28 $\delta^2$	$0.30 \pm 0.03$	$58.1 \pm 1.9$	0.7	49.0
I35 $\gamma$	$0.47 \pm 0.04$	$55.0 \pm 2.1$	1.5	39.1
I35 $\delta$	$0.35 \pm 0.02$	$30.0 \pm 1.9$	0.9	45.8
L42 $\delta^1$	$0.41 \pm 0.08$	$57.7 \pm 7.2$	0.1	42.8
L42 $\delta^2$	$0.44 \pm 0.04$	$55.7 \pm 2.5$	0.1	41.0
A44 $\beta$	$0.65 \pm 0.07$	$43.1 \pm 3.0$	0.4	30.2
V53 $\gamma^1$	$0.52 \pm 0.04$	$50.8 \pm 2.0$	0.3	36.9
V53 $\gamma^2$	$0.55 \pm 0.05$	$49.4 \pm 2.3$	0.3	35.3
L56 $\delta^1$	$0.49 \pm 0.05$	$53.9 \pm 4.8$	0.3	38.4
L56 $\delta^2$	$0.48 \pm 0.04$	$43.2 \pm 3.2$	0.2	39.0
A60 $\beta$	$0.79 \pm 0.07$	$41.2 \pm 2.3$	0.3	22.6
I63 $\gamma$	$0.62 \pm 0.05$	$39.9 \pm 3.6$	0.2	31.7
I63 $\delta$	$0.51 \pm 0.03$	$27.0 \pm 2.1$	0.5	37.4
L67 $\delta^1$	$0.17 \pm 0.04$	$75.0 \pm 6.0$	3.2	57.7
L67 $\delta^2$	$0.36 \pm 0.04$	$56.1 \pm 2.5$	0.7	45.4

2). This indicates that the  $\text{C}_\alpha\text{C}_\beta$  vector is not collinear with the effective director axis of the motion.

The leucine  $\delta$  methyl groups of  $\alpha_3\text{D}$  also display a greater amplitude of motion than in natural proteins, with an average difference of approximately 0.12 [ $\langle S_{\text{axis}}^2 \rangle$  of 0.32 for  $\alpha_3\text{D}$  versus  $\langle S_{\text{axis}}^2 \rangle$  of 0.44 for natural proteins (22)]. The obtained  $S_{\text{axis}}^2$  values for each of the methyl groups of each leucine residue agree well with each other except for L67 ( $\delta^1$  of 0.17 and  $\delta^2$  of 0.36, Table 1). Thus, in nearly all cases, the effective director of the motion appears to lie in a plane that bisects the  $\text{C}_\gamma\text{—C}_\delta$  vectors, i.e., director axis projects equal components onto both symmetry axes. The L67 $\delta^1$  methyl group has the largest amplitude of motion, having a  $S_{\text{axis}}^2$  value of 0.17, corresponding to a free diffusion cone angle of  $\sim 58^\circ$  (Table 1). The  $\gamma$  methyl groups of the three isoleucine residues (I14, I35, I63) have an average  $S_{\text{axis}}^2$  value of 0.57. This is about 0.20 lower than that seen in natural

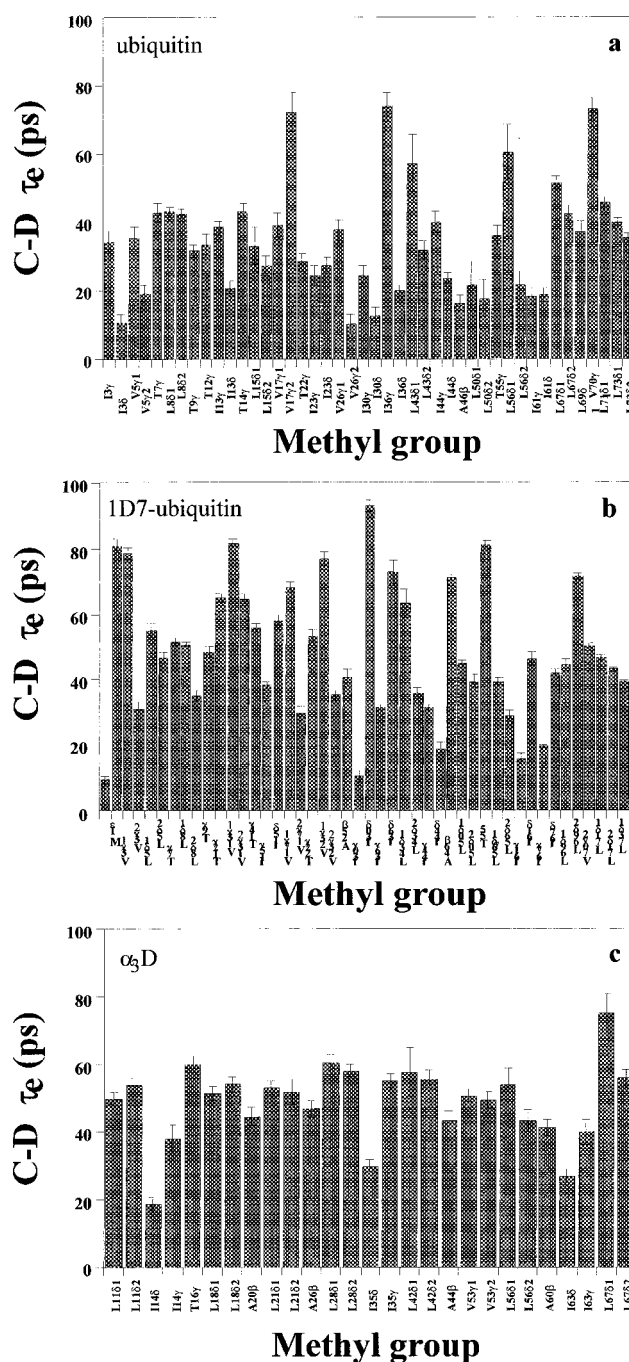


FIGURE 4: (a) Effective correlation times ( $\tau_e$ ) determined from the  $^2\text{H}$  methyl analysis fit to the simple model-free formalism (SMF, eq 8) for ubiquitin at 30  $^{\circ}\text{C}$  (21). (b) The  $\tau_e$  times determined from the  $^2\text{H}$  methyl analysis for a repacked ubiquitin variant, 1D7, at 30  $^{\circ}\text{C}$  (50, 51). (c) The  $\tau_e$  times determined from the  $^2\text{H}$  methyl analysis for  $\alpha_3\text{D}$  at 30  $^{\circ}\text{C}$ .

proteins (21–23). On the other hand, the average  $S_{\text{axis}}^2$  value of 0.47 seen for the  $\delta$  methyls of isoleucines in  $\alpha_3\text{D}$  is similar to that observed in natural proteins (21, 22). The  $^2\text{H}$ -derived effective correlation times ( $\tau_e$ ) of the methyl groups of  $\alpha_3\text{D}$  range from 18.8 to 75.0 ps, with an average value of 47.7 ps (Table 1).

Notwithstanding the inherent limitations of  $^2\text{H}$ -derived  $\tau_e$  values (21), the distribution of  $\tau_e$  times of methyl groups in ubiquitin (21), a repacked ubiquitin variant, 1D7 (50, 51) and in  $\alpha_3\text{D}$  show a similarly broad distribution (Figure 4). The results obtained for  $\alpha_3\text{D}$  were sufficiently compelling for us to undertake a more exhaustive study using carbon





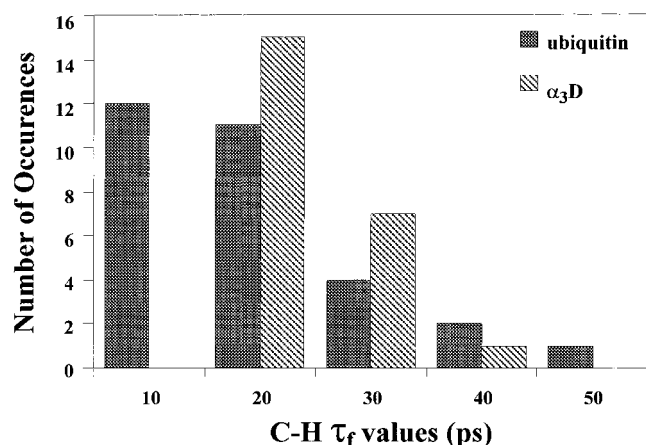


FIGURE 6: Histogram of the distribution of  $^{13}\text{C}$ -derived  $\tau_f$  values for methyl groups of ubiquitin (2I) and  $\alpha_3D$  (see Table 2).

analysis was therefore conducted on the 50% deuterated  $\alpha_3D$  protein. The linear extrapolation method of guanidine hydrochloride denaturation indicates that the 50% deuterated  $\alpha_3D$  is destabilized by  $\sim 1.0 \text{ kcal mol}^{-1}$  relative to fully protonated  $\alpha_3D$ . Furthermore, the cooperativity of unfolding ( $2.08 \text{ kcal mol}^{-1} \text{ M}^{-1}$ ) is less than the fully protonated  $\alpha_3D$  ( $2.41 \text{ kcal mol}^{-1} \text{ M}^{-1}$ ), and the thermal denaturation midpoint ( $T_m$ ) of the 50% deuterated  $\alpha_3D$  is  $5^\circ\text{C}$  lower than the fully protonated form of  $\alpha_3D$ . This suggests that the presence of a 50% mixture of deuterons and hydrogens in  $\alpha_3D$  is destabilizing and, in principle, could be also manifested in the internal dynamics of the protein.

## DISCUSSION

$^2\text{H}$  and  $^{13}\text{C}$  methyl relaxation studies offer complementary information about the dynamics of methyl groups (21). Although there is good agreement between fast time scale parameters ( $^{13}\text{C}$ -derived  $\tau_f$  and  $^2\text{H}$ -derived  $\tau_e$ ), the corresponding  $S_{\text{axis}}^2$  parameters do not correlate well. An in depth discussion of the potential reasons why  $^2\text{H}$  and  $^{13}\text{C}$  methyl analysis may differ is provided elsewhere (21). Briefly,  $^2\text{H}$  methyl relaxation is useful for the determination of the  $S_{\text{axis}}^2$  parameter due to the inherently low sampling frequencies of the deuteron. For example, at 14.1 T, frequencies of 0, 92, and 184 MHz are sampled, which puts most motions in the extreme narrowing limit ( $\omega\tau \ll 1$ ). On the other hand,  $^{13}\text{C}$  methyl relaxation is useful for determining the time scales of methyl dynamics due to the breadth of sampling frequencies. For example at 14.1 T, frequencies of 150, 450, 600, and 750 MHz are sampled. If the slow time scale motion ( $\tau_{\text{axis}}$ ) is in the approximate range of  $150 \text{ ps} \leq \tau_{\text{axis}} \leq 800 \text{ ps}$  at 14.1 T, then accurate determination of the  $S_{\text{axis}}^2$  parameter compared may be achieved with  $^{13}\text{C}$  relaxation at currently available field strengths.

**Time Scale of the Methyl Group Dynamics.** The time scale of methyl rotation and of motion of the methyl symmetry axis provides a unique insight into the local environments of side chains. Recent studies of natural proteins such as ubiquitin (21) reveal that methyl groups of interior (i.e., packed) side chains show a range of  $\tau_e$  values while methyl groups of exterior (i.e., solvent accessible) side chains show a tighter and more uniform distribution of  $\tau_e$  values. The methionine methyl groups in calmodulin perhaps most

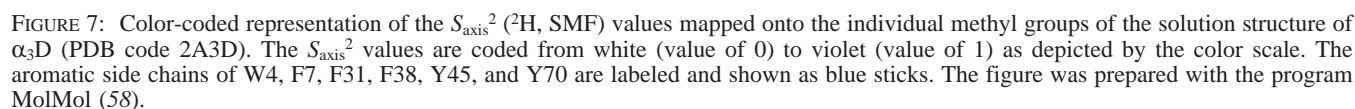
vividly exemplify this. In the uncomplexed form of calmodulin, the methionines are located on the surface of the protein and have a uniform profile of  $\tau_e$  values (23). Upon binding to the calmodulin binding domain of the smooth muscle myosin light chain kinase peptide, the  $^2\text{H}$   $\tau_e$  times of the methionine methyl groups adopt a significantly broader range of values, characteristic of the individual methyl groups undergoing a conformational change from being solvent exposed to a more ordered locally distinct environment (23).

The narrowness of the distribution of the time scales of methyl dynamics in  $\alpha_3D$  may indicate that the local environments around the methyl groups of  $\alpha_3D$  are not locked down in structurally unique environments in the picosecond to nanosecond time regime, in a manner observed in natural proteins. Moreover, taken together with the low  $S_{\text{axis}}^2$  value, the substantially long  $\tau_{\text{axis}}$  times (Tables 2) appear to be reporting higher amplitude, slower frequency motions ( $\sim 0.5$ – $1 \text{ ns}$ ) relative to natural proteins. This behavior would have been overlooked without the  $^{13}\text{C}$  methyl analysis.

**Structural Correlates of  $S_{\text{axis}}^2$  Values.** The mapping of  $S_{\text{axis}}^2$  values determined from the  $^2\text{H}$  analysis onto the  $\alpha_3D$  structure shows characteristics that have been seen in natural proteins as well as features that appear unique to  $\alpha_3D$  (Figure 7). As in natural proteins, there is heterogeneity and a significant range of  $S_{\text{axis}}^2$  values of the methyl groups throughout the hydrophobic core of  $\alpha_3D$ , indicating substantial motion on the ps-ns time scale (Figure 7). However, unlike natural proteins studied to date, there appears to be recognizable clustering of rigid and fluid methyl groups (Figure 7). At the N-terminal end of  $\alpha_3D$ , there is a relatively rigid region involving the methyl groups of L42, V53, and L56 (Figure 7). The  $^3J_{\alpha\beta}$ ,  $^3J_{N\beta}$ ,  $^3J_{NC\gamma}$ , and  $^3J_{COC\gamma}$  coupling constants, and  $^1\text{H}$ - $^1\text{H}$  NOEs indicate the preferred  $\chi^1$  angle for an  $\alpha$ -helix of  $180^\circ$  for V53 (53). Leu56 has  $\chi^1$  ( $^3J_{\alpha\beta}$ ,  $^3J_{N\beta}$  coupling constants, and  $^1\text{H}$ - $^1\text{H}$  NOEs) and  $\chi^2$  ( $^3J_{C\delta C\alpha}$  coupling constants) angles of  $-60^\circ$  and  $60^\circ$ , respectively. The  $\delta$  methyl groups of L56 also have the highest leucine  $S_{\text{axis}}^2$  values in  $\alpha_3D$ . Leu56 packs next to a cluster of several aromatic residues: W4, F7, and Y45. Interestingly, L42 has a  $\chi^1$  ( $^3J_{\alpha\beta}$ ,  $^3J_{N\beta}$  coupling constants, and  $^1\text{H}$ - $^1\text{H}$  NOEs) angle of  $-60^\circ$ , but the  $\chi^2$  ( $^3J_{C\delta C\alpha}$  coupling constants) angle undergoes rotamer averaging, yet still has the second highest leucine  $S_{\text{axis}}^2$  value. The packing of the aromatic groups may help reduce the motions of L42, V53, and L56 in the picosecond to nanosecond time regime.

The next layer contains the L11 $\delta$ s, A60 $\beta$ , and F38. A60 $\beta$  has the highest  $S_{\text{axis}}^2$  value of 0.79 in  $\alpha_3D$ . The methyl groups of L11 show more dynamic  $S_{\text{axis}}^2$  values relative to the methyl groups of L56 that pack to the left of it (Figure 7). Moreover, the  $\delta$  methyl groups of I35 and I63 that pack next to the A60 $\beta$  layer show increased dynamics (Figure 7). A60 is the sole alanine residue and smallest side chain in the hydrophobic core of  $\alpha_3D$ . The dynamic behavior around this region suggests that it may not have been the ideal residue for this position. Furthermore, the replacement of A60 with leucine or isoleucine increases the thermodynamic stability by 1.8 to  $1.5 \text{ kcal mol}^{-1}$  ( $\Delta\Delta G_u$ ) relative to the wild-type  $\alpha_3D$  (unpublished results).  $^3J_{\alpha\beta}$ ,  $^3J_{N\beta}$ ,  $^3J_{C\delta C\alpha}$  coupling constants, and  $^1\text{H}$ - $^1\text{H}$  NOEs indicate that both the  $\chi^1$  and  $\chi^2$  angles of L11 undergo rotamer averaging, which may also contribute to the low  $S_{\text{axis}}^2$  value. On the other hand, the  $^3J_{\alpha\beta}$ ,  $^3J_{N\beta}$





The C-terminal end of  $\alpha_3$ D contains the hydrophobic layers with the most dynamic methyl groups in  $\alpha_3$ D (Figure 7). The C-terminal end contains all leucine residues with two aromatic residues F31 and Y70 (Figure 7). The C-terminus of  $\alpha_3$ D contains dynamic residues at both the backbone (Figure 1a) and side-chain level (Figure 3). The dynamic behavior of the C-terminal residues, Y70 to H73, may offer

Kay and co-workers, investigating the methyl side-chain dynamics of the free and bound states of the pLCC- $\gamma$  SH2 domains, showed that several leucine residues with low  $S_{\text{axis}}^2$  values also experience rotameric averaging, even with the leucine residues being completely buried in the core (54, 55). In a repacked hydrophobic core variant of ubiquitin, Handel and co-workers have shown that there was a good correlation between the dynamic methyl groups and side chains in unfavorable or averaged rotamers (50, 51). This recurring theme is now seen for  $\alpha$ -D where five of the seven

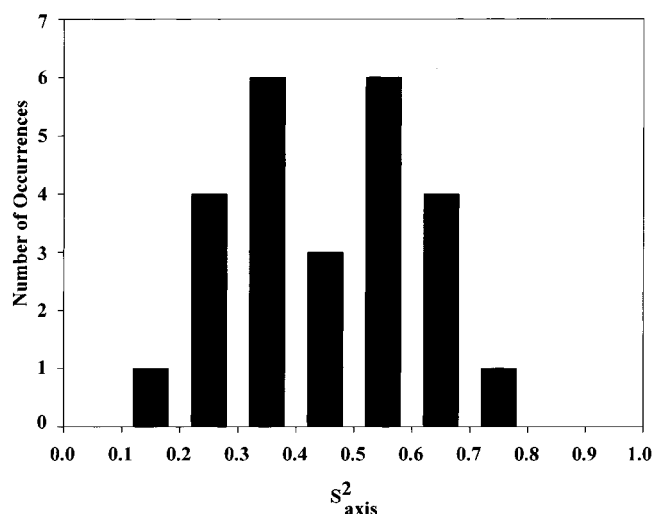


FIGURE 8: Histogram of the distribution of  $^2\text{H}$ -derived  $S_{\text{axis}}^2$  values obtained for the methyl groups of  $\alpha_3\text{D}$  (see Table 2).

leucine residues have  $\chi^2$  angles undergoing rotamer averaging with the majority displaying low  $S_{\text{axis}}^2$  values (Table 1). In contrast, all three isoleucine residues in  $\alpha_3\text{D}$  adopt the preferential  $\chi^1$  and  $\chi^2$  angles for an  $\alpha$ -helix (53), in addition to having high  $S_{\text{axis}}^2$  values (Table 1 and Figure 6).

**Implications for de novo Protein Design.** Though it is now relatively straightforward to design protein sequences that adopt a desired secondary structural element, the design of large tertiary folds with well-defined hydrophobic cores remains a challenge (56). The reasons for this are only beginning to emerge. Site-resolved analysis of the main chain and side-chain dynamics may offer unique insights into the nature and therefore design of nativelike hydrophobic cores. From the NMR relaxation studies,  $\alpha_3\text{D}$  has proven to be a successful design at the backbone level insofar as the backbone of the protein displays the dynamical properties of a native protein. The uniformly high squared generalized order parameters of the amide N–H and C $\alpha$ –H support this view. Interestingly, however, certain features of the dynamics of methyl-bearing side chains of  $\alpha_3\text{D}$  are distinct from that seen in studies of native state proteins and their complexes.

On average, the angular order of methyl-bearing side chains in  $\alpha_3\text{D}$  is lower than that seen in natural proteins. Recently, Lee and Wand have suggested that a general feature of native proteins is the partitioning of subnanosecond motion of methyl bearing side chains into three classes of motion centered on  $S_{\text{axis}}^2$  values of 0.3, 0.6, and 0.8 (24). Interestingly,  $\alpha_3\text{D}$  also appears to have a nonuniform distribution of  $S_{\text{axis}}^2$  values. Indeed, the two lower order classes appear to be represented in  $\alpha_3\text{D}$  (Figure 8). Because of the effects of having only a limited number of samplings within the  $\alpha_3\text{D}$  molecule it is difficult to discern a definitive distribution. Nevertheless, these data seem to suggest that  $\alpha_3\text{D}$  is underrepresented in the most rigid class of methyl-bearing side chains seen in native proteins (24). Furthermore, the methyl rotation ( $\tau_r$ ) and methyl symmetry axis ( $\tau_{\text{axis}}$ ) time scales are larger than what has been observed for natural proteins.

There are lessons from the NMR relaxation studies of  $\alpha_3\text{D}$  relating to the de novo design of proteins. One involves packing hydrophobic cores of proteins with several  $\beta$ -branched and aromatic amino acids and avoiding an overabundance

of leucine residues. As observed in  $\alpha_3\text{D}$ , aromatic residues apparently restrict the motion of methyl groups to adopt a conformationally specific hydrophobic core. Studies of de novo designed four-helix bundle proteins have also shown the benefits of packing cores with  $\beta$ -branched and aromatic amino acids (3, 5, 57). When packing hydrophobic cores using computational algorithms, potentially more weight should be given for rotamer preferences relative to optimizing for optimal van der Waals interactions.

## CONCLUSIONS

The backbone and side-chain dynamics of  $\alpha_3\text{D}$ , a de novo designed three-helix bundle protein, have been investigated. At the backbone level, both the  $^{15}\text{N}$ –H and  $^{13}\text{C}_\alpha$ –H bond vectors display rigid motion in the  $\alpha$ -helices of  $\alpha_3\text{D}$ , with increasing motions at the ends of the  $\alpha$ -helices and in the loop regions. Thus, the backbone dynamics of  $\alpha_3\text{D}$  are similar to natural proteins. In contrast, methyl-bearing amino acids of  $\alpha_3\text{D}$  display lower order than their counterparts in natural proteins. Additionally, the  $^{13}\text{C}$  methyl data show that the methyl rotation rates ( $\tau_r$ ) to be more uniform and slightly longer than native proteins. The increased dynamics of the methyl groups of  $\alpha_3\text{D}$  may be the consequence of several side chains switching between unfavorable or averaged rotamer states, thus reflecting rotameric strain in the hydrophobic core. Thus, the core of  $\alpha_3\text{D}$  appears to have a higher degree of fluidity or malleability than natural proteins of similar size.

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