Synthesis, Structural and Biological Studies of Ubiquitin Mutants Containing (2*S*, 4*S*)-5-Fluoroleucine Residues Strategically Placed in the Hydrophobic Core

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Understanding protein folding is currently a major research challenge and multidimensional NMR spectroscopy is increasingly being employed in its solution.[1] Although fluoroaromatic and derivatised amino acids have long been used as reporter groups for NMR spectroscopic investigations of proteins,[2] the first study with fluorinated aliphatic amino acids appeared only in 1996 when we reported incorporation of (2*S*,4*S*)-5-fluoroleucine (FLeu) in place of all 13 Leu residues in dihydrofolate reductase using biological methods.[3] Since then, trifluoromethionine and fluoroproline have been incorporated into proteins and recently synthetic[4, 5] and in vivo[6] techniques were used to incorporate mixtures of diastereoisomers of 5,5,5-trifluoroleucine[4, 5, 6] and 5,5,5,5′,5′,5′-hexafluoroleucine[6, 7] into the leucine zipper peptide GCN4-p1.

Use of auxotrophic bacteria in our original studies had the disadvantage of labeling all Leu residues in the protein and incorporation was variable at each position. For folding studies, site-specific labeling in the hydrophobic core of a protein with minimal disruption to the natural fold is the goal. We therefore set out to incorporate FLeu[3, 8] in place of strategically selected Leu residues in the core of the protein ubiquitin (Ub). Ubiquitin was chosen, not only because it is a small, conserved protein, with five Leu residues within its hydrophobic core, but also because we have experience with the synthesis, folding, crystallography and biological studies of this protein and its mutants.[9, 10] We selected pairs of Leu residues for substitution with the aim of establishing two proximal ^{19}F reporter groups within the hydrophobic core. FLeu-mutants for residues 50 and 67, FLeu50/67-Ub, and residues 43 and 67, FLeu43/67-Ub, were chosen on this basis (distance between the *pro-R* methyl carbon atoms, $d = 4.18$ and 4.49 Å, respectively). Mutants were synthesised using standard Fmoc/*t*Bu strategy[11] on Wang resin, purification was achieved as described[10, 12] and products were judged to be homogeneous by reversed-phase(RP)-HPLC (C8), MALDI-TOF/MS and amino acid analysis. One-dimensional ^{1}H NMR spectra of both mutants showed similar dispersion to the spectrum of bovine ubiquitin, suggesting native folding. The far-UV CD spectra (Figure 1) also indicated that the mutants were very similar to the wild-type.

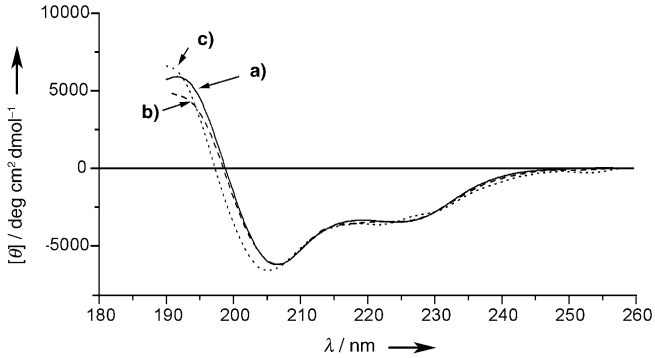

Figure 1. *CD spectra of ubiquitin and fluorinated derivatives. Spectra were recorded in 50 mM sodium borate buffer at pH 7; a) wild type ubiquitin (solid line); b) FLeu50/67 ubiquitin (dashes); c) FLeu43/67 ubiquitin (dots).*

The ^{19}F NMR resonances (Table 1) displayed a large (ca. 10 ppm) chemical shift variation. Assignment of ^{1}H and ^{19}F chemical shifts to residues 50 and 67 derived from sequential backbone assignment based on TOCSY and NOESY experiments combined with a ^{1}H-^{19}F HMQC experiment. Sequential and short-

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Table 1. *^{19}F chemical shifts of FLeu and FLeu-mutants of ubiquitin.*[a]

Compound	^{1}H NMR	^{19}F-67	^{19}F-50	^{19}F-43	^{19}F-other
FLeu					-223.7
FLeu50/67-Ub (folded)	native	-221.0	-217.1		
FLeu50/67-Ub (denatured)					-221.3
FLeu43/67-Ub (folded)	native	-222.0		-226.3	
FLeu43/67-Ub (denatured)					-221.4

[a] At 564.3 MHz and 25 °C in 25 mM CD_3CO_2Na, pH 4.8, referenced to $CFCl_3$ assigned as zero.

range homonuclear NOEs and interstrand NOEs observed for the mutant were indistinguishable from those seen for the bovine protein. Long range NOEs confirmed that the side chains of FLeu50 and 67 are buried within a well-packed hydrophobic core that is similar to the core of the wild-type protein. Preliminary calculations based on 674 inter-residue NOEs produced structures of the FLeu50/67-Ub mutant that showed no significant differences from the native structure.

We opted to synthesise the FLeu50/67-Ub mutant in greater quantities for further study. Crystals isomorphous with the native were grown and the X-ray structure was determined by using techniques described previously.[9, 10, 13] Superposition of the mutant structure with that of native ubiquitin showed an excellent agreement (Figure 2 a). The main discernible difference

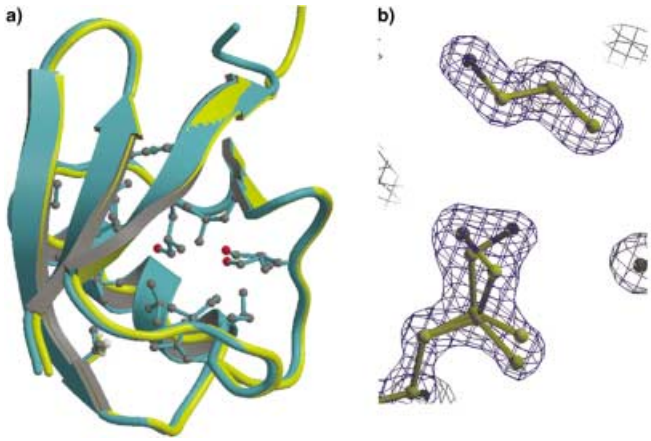

Figure 2. a) *Cartoon showing superposition of the FLeu50/67-Ub mutant (cyan) with the native structure (yellow). The core residues of the mutant protein (Val5, 17, 26; Ile3, 13, 23, 30, 36, 61; Leu15, 43, 56, 69 FLeu50, 67) are represented as ball-and-stick with fluorine atoms in red. The alternative positions of FLeu50 can be seen. Both mutant and native side chains for the typical Val17 (bottom left) are shown. The small changes between the two proteins do not represent a significant change in the overall fold. b) Electron density for the two FLeu residues in the core of ubiquitin. The map was calculated with sigmaA-weighted coefficients 3|Fo|−|2Fc| and is contoured at a level of 1.3{σ}. The alternative positions for the fluorine atom on FLeu50 can be clearly seen in contrast to the unique position for the fluorine atom in FLeu67.*

occurs at the C terminus, where conformational flexibility is known to occur, thus allowing ubiquitin to conjugate to other proteins.[14] The two fluorine atoms in the core were clearly visible and well-defined (Figure 2 b). Two conformations exist for the side chain of FLeu50 ($\chi_1 = -51°$, $\chi_2 = -166°$, χ_3F $= -48°$ and $\chi_1 = -47°$, $\chi_2 = +162°$, χ_3F $= +171°$). The former rotamer has an occupancy of 37 % and the fluorine is in a *gauche* arrangement to C-β, while the latter rotamer (occupancy 63 %) is *anti* to C-β. The side chain in FLeu67 ($\chi_1 = -44°$, $\chi_2 = -174°$, χ_3F $= -65°$) is in a single conformation similar to that of the *gauche* form of FLeu50. Despite the presence of two distinct rotamers of FLeu50, the distances from its fluorine atom to that of FLeu67 were similar (4.6 and 4.5 Å for the *gauche* and *anti* arrangements respectively).

Comparison of thermal stability in solution of natural (bovine) and FLeu50/67-Ub was achieved by differential scanning calorimetry(DSC; Figure 3). This showed that natural ubiquitin

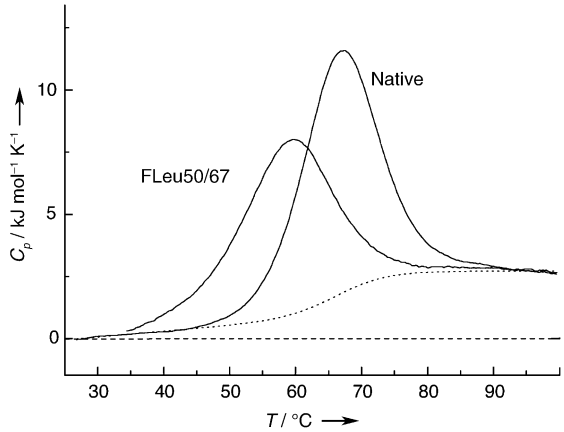

Figure 3. *Comparison of the thermal stability in solution of natural (bovine) ubiquitin and FLeu50/67-Ub (1 mg mL^{-1}, pH 3.0) obtained using a Microcal VP-DSC instrument under standard operating conditions.*[17] *Normalised DSC excess heat capacity data are consistent with cooperative unfolding:* $T_m = 66.9$ °C, $\Delta H_{VH} = 238$ kJ mol^{-1} *(natural) and* $T_m = 58.9$ °C, $\Delta H_{VH} = 217$ kJ mol^{-1} *(FLeu50/67). The dotted line indicates the heat capacity increment (ΔC_p) upon unfolding.*

undergoes cooperative two-state thermal unfolding transition under the conditions used ($T_m = 66.9$ °C, $\Delta H = 238 (\pm 10)$ kJ mol^{-1}), comparable to previous observations[15] with a positive ΔC_p typical of the unfolding of globular proteins.[16] The FLeu50/67 mutant shows a similar unfolding transition but with a midpoint T_m about 8 °C lower than the natural protein. Thus the mutant retains the typical unfolding thermodynamic characteristics of a globular protein, but with reduced stability. This may reflect fundamental changes in solvation thermodynamics (hydrophobicity) arising from H-F substitutions. By comparison, perfluorination *increases* the stability of the dimer produced by Leu-Leu interactions in the GCN4 zipper protein.[4–7]

The biological activity of ubiquitin involves covalent conjugation of multi-ubiquitin chains or monomers of ubiquitin to target proteins, signaling their proteolysis or regulating other non-degradative cellular functions, respectively. Samples of the FLeu50/67 mutant and bovine ubiquitin were separately incubated with a preparation from rabbit reticulocyte lysate enriched in elements of the ubiquitin conjugation pathway but depleted of ubiquitin.[18] The newly formed ubiquitin-protein conjugates were detected by Western blotting with anti-ubiquitin antibodies. The FLeu50/67 mutant formed high molecular weight

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conjugates with a similar efficiency to natural ubiquitin. This suggests that the mutant is recognised by the reticulocyte activating and conjugating enzymes, thus lending support to the proposal that no significant perturbation of the native structure occurs when FLeu residues are incorporated into ubiquitin.

The ^{19}F NMR spectrum of the 50/67 mutant in buffer (Figure 4a) showed one main signal for each of the FLeu residues. The FLeu50 ^{19}F signal is broader than the FLeu67 signal. The

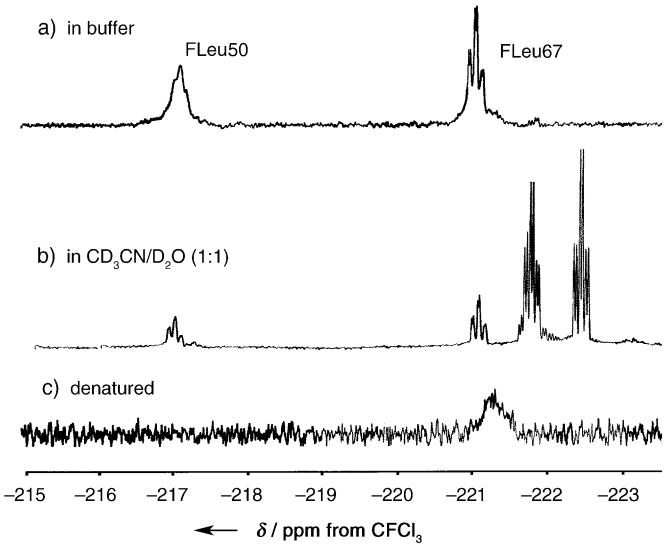

Figure 4. *^{19}F NMR spectrum of FLeu50/67-Ub; a) in 25 mM CD_3CO_2Na at pH 4.5 b) folded protein in CD_3CN/D_2O (1:1); c) denatured protein in 25 mM CD_3CO_2Na at pH 4.5.*

^{19}F NMR spectrum in a mixture of acetonitrile and water (1:1; Figure 4b), used to disrupt hydrophobic contacts in the core, showed the two resonances corresponding to the native form of the mutant, together with a much sharper pair of discrete multiplets at −221.8 and −222.5 ppm. The fully denatured mutant, obtained directly from the synthesis before folding, had ^{19}F resonances as a broad hump, consistent with multiple conformations in intermediate exchange (Figure 4c). The very sharp peaks observed in acetonitrile indicate highly mobile entities consistent with loss of any residual structure, and slow (or no) exchange with the remaining natively folded material. These preliminary studies illustrate the potential of the ^{19}F reporter nuclei for monitoring folding and unfolding of the protein.

In summary, we have shown that preparation of a mutant of ubiquitin with fluorine introduced stereospecifically into leucine residues 50 and 67 by solid phase protein synthesis gives a product with a very similar structure to the natural product both in solution and in the solid state, and with the biological properties of the natural protein. Despite differences in the arrangement of side chains within the hydrophobic interior and small changes in thermodynamic stability, we have produced a minimally disturbed homogeneous protein that contains reporter labels of unambiguous structure and stereochemistry lying close together within the core. Initial studies suggest that the provision of these samples will enable ^{19}F NMR spectroscopy to be used as a spectroscopic probe to explore the folding process. This enabling technology, based as it is on synthetic organic chemistry, has promise for the study of folding in this and other proteins.

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