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SOLID PHASE PEPTIDE SYNTHESIS OF UBIQUITIN+

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Summary: The synthesis of ubiquitin has been achieved using N^G-Pmc protection of the Arg residues. Throughout the synthesis the N^α-Fmoc deprotection was followed by UV monitoring. Authenticity of the primary structure was confirmed by automated Edman sequencing and enzymatic degradation.

Ubiquitin[1] is a 76α-amino acid polypeptide and, as the name implies, it is thought to be present in all eukaryotic cells. It has the most conserved sequence in evolutionary terms; indeed both human and bovine ubiquitin are identical and are shown below. This fact, combined with the vital biological functions requiring ubiquitin,[2] makes it a fascinating target for chemical synthesis. Although ubiquitin has been produced by gene expression the chemical approach to synthesis would allow the introduction of unnatural α-amino acids, and thence to an investigation of the structural factors controlling tertiary structure development. In this respect ubiquitin, having no S-S bonds, is an excellent model since the crystal structure[3] at 1.8 A resolution shows it to be a compact, globular protein with a hydrophobic core and important secondary structure features such as α-helix, mixed β-sheet, reverse turns and β-bulges.

HUMAN/BOVINE UBIQUITIN

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 1               5                  10                  15
Met Gln Ile Phe Val Lys Thr Leu Thr Gly Lys Thr Ile Thr Leu Glu Val Glu Pro
 20                  25                  30                  35
Ser Asp Thr Ile Glu Asn Val Lys Ala Lys Ile Gln Asp Lys Glu Gly Ile Pro Pro
     40                  45                  50                  55
Asp Gln Gln Arg Leu Ile Phe Ala Gly Lys Gln Leu Glu Asp Gly Arg Thr Leu Ser
         60                  65                  70                  75
Asp Tyr Asn Ile Gln Lys Glu Ser Thr Leu His Leu Val Leu Arg Leu Arg Gly Gly
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For the Merrifield solid phase synthesis[4] of ubiquitin we used the protecting group strategy (Figure 1), based on the Fmoc group designed by Carpino,[5] which has been developed by Sheppard.[6]

The loading of Fmoc.Gly.OH to *p*-alkoxybenzyl alcohol/polystyrene resin[7] (1.05 mmol/g) was effected by either the acid chloride[8] in methylene chloride (DCM)/pyridine, or using the diphenylphosphinic mixed anhydride[9]

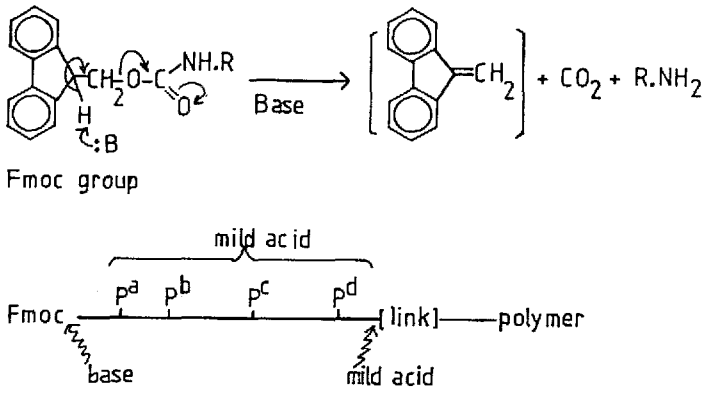

Figure 1

in DCM/DMF, outwith the automated ABI 430A peptide synthesiser using ultrasonication to achieve mixing. No Gly.Gly could be detected and the degree of loading (55%, 0.44 mmol/g) was established by U.V. analysis of a loaded resin sample deprotected by 20% piperidine/DMF. The guanidino functions of the four arginine residues were protected by the 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc) group[10] and the imidazole ring of histidine was protected by the trityl group. Most of the N^{α}-Fmoc amino acids were coupled twice by sequential symmetric anhydride (2 mmole) followed by hydroxybenzotriazole (HOBt) active ester (1 mmole) methods of activation employing N:N'-diisopropylcarbodiimide (DIC) alone and DIC/HOBt respectively. The exceptions to this coupling protocol were (i) Fmoc.Gly.OH single coupling (2 mmole), (ii) Fmoc.Arg(Pmc).OH, Fmoc.Asn.OH and Fmoc.Gln.OH involved only DIC/HOBt activation in double coupling (2 x 1 mmole). After each second coupling, acetic anhydride in DCM was added and the capping reaction continued for 6 min. Each N^{α}-Fmoc deprotection stage involved successive treatments with 20% piperidine in dimethylformamide (DMF) for periods of 3, 3, 3 and 1 min. In order to afford a real-time assessment of the progress of the synthesis the effluent, containing the chromophoric Fmoc degradation product, was degassed then pumped through a UV cell (313 nm monitor) linked to a recorder to give a continuous record of the deprotection stages. In this way we could establish the efficiency of each cycle and identify problem couplings and relatively slow deprotections for subsequent syntheses. For example it was found that couplings to N-terminal Gln and Lys(Boc) residues were slightly less than optimum and that the minimal conditions used for incorporation of Fmoc.Gly required enhancement.

On completion of the assembly of protected ubiquitin on the initial polystyrene resin (1.14 g), the final weight (6.25 g) was found to be 90% of the expected value. The N^{α} terminal-Fmoc group of the peptidyl resin was removed, as required, with 20% piperidine-DMF in a sonicator for 10 mins. In order to cleave the protecting groups and remove the peptide from the

resin, typically the peptidyl resin (2.0 g) was treated with thioanisole (3 ml, 5%), ethylmethylsulphide (3 ml, 5%) for 20 mins followed by the addition of 95% aq. TFA (60 ml) at room temperature for 1.5 h. In this initial synthesis in the ubiquitin programme, a considerable number of experiments were undertaken with resin-bound peptides to establish a general purification protocol. After the TFA was removed *in vacuo*, the residue was stirred with 2% 2-mercaptoethanol in diethylether (60 ml) then filtered to afford the insoluble peptide, (1.23 g), 50% of which was immediately dissolved in N_2-saturated 25 mM NH_4OAc-8M urea (pH 4.5) for 2 h before applying to a high resolution Sephadex G50 column (1.6 x 70 cm), previously equilibrated with 25 mM NH_4OAc-8M urea (pH 4.5). The fractions obtained were sequentially dialysed against N_2-saturated 25 mM NH_4OAc-4M urea (pH 4.5) for 18 h (1 litre); followed by N_2-saturated 25 mM NH_4OAc-2M urea (pH 4.5) for 12 h (1 litre); N_2-saturated 25 mM NH_4OAc (pH 4.5) for 18 h (4 x 1 litre). These fractions were examined by isoelectricfocusing (IEF) (PHAST, Pharmacia) on phast gel pI range 3-9. The desired fractions were further purified by cation exchange chromatography on CM-52 cellulose (Whatman) column (2.5 x 50 cm) eluting with 25 mM NH_4OAc (pH 4.5 1 bed volume), followed by gradient elution up to 0.1 M NH_4OAc (pH 5.5). This gave material which was further purified by anion-exchange chromatography on DEAE-52 (Whatman) column (1.5 x 36 cm) eluting with 20 mM NH_4HCO_3 (pH 9.0), which was then submitted to chromatofocusing using a Mono P column (HR5/20 Pharmacia) at pH range 8-5. With 25 mM triethanolamine/iminodiacetic acid (pH 8.3) as start buffer, the material was eluted with polybuffer 96 (3 ml)/polybuffer 74 (7 ml) (Pharmacia) in H_2O-iminodiacetic acid (pH 5.0) at the pre-gradient (a similar pattern was observed for bovine ubiquitin even under pH range 7-4 and 9-6 conditions) at a flow rate 0.75 ml/min. The pre-gradient fractions were pooled, desalted (*Sephadex G15*) and concentrated.

Analysis of the synthetic peptide by IEF and HPLC on C-8 column (0.46 x 22 cm) judged this to be >80% pure. Finally semi-preparative HPLC purification gave synthetic ubiquitin (4 mg) in >95% purity. The synthetic material so obtained, exhibited identical HPLC and IEF behaviour in comparison with authentic bovine ubiquitin. The primary sequence of the synthetic ubiquitin was confirmed by automated Edman degradation through to residue 74. Also the synthetic ubiquitin was oxidised then subjected to trypsin which gave 13 fragment peptides which were eluted in 1 ml fractions after high voltage paper electrophoresis (HVPE) at pH 6.5 (pyridinium acetate buffer). The N-terminus of these peptides was established by Dansylation. These eluates were then processed by total hydrolysis in 6 M HCl and resubmitted to HVPE at pH 2.1 (acetic acid-formic acid) when amino acid composition was determined by comparison with amino acid standards. The pattern of electrophoretic bands obtained from synthetic ubiquitin was identical to authentic erythrocytic bovine ubiquitin except for the addition of an extra fragment. It is of interest that the extra tryptic action,

which is entirely consistent with the ubiquitin sequence, occurred at positions 63-64 in the synthetic material and is indicative of a difference in tertiary structure between synthetic and natural ubiquitin at the C-terminus. A parallel for this may be found in the difference in immunospecificity involving the same region of the C-terminus of erythrocyte ubiquitin and the ubiquitin component of the lymphocyte-homing receptor. A detailed structural analysis of the synthetic ubiquitin and large fragments of the sequence is currently under investigation.

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References and Notes

+ This paper is dedicated to the memory of George W. Kenner (1922-1978).

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