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Polyubiquitin conjugates with lysine-specific linkage: Synthesis and structural characterization using high resolution mass spectrometry

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Introduction

Ubiquitin is a 76aa polypeptide that can be covalently attached to substrate proteins [1]. Ubiquitin conjugation is mediated by an enzymatic cascade consisting of three different classes of enzymes: a ubiquitin activating enzyme E1, ubiquitin-conjugating enzymes E2, and ubiquitin ligases E3, which are responsible for the specific recognition of substrates [2]. Substrates can be modified with single ubiquitin moieties (mono-ubiquitination) or with so-called ubiquitin chains (poly-ubiquitination), whereby the conjugated ubiquitin itself serves as a substrate for the next ubiquitin conjugation step. Ubiquitin contains seven lysine residues (K6, K11, K27, K29, K33, K48, K63) and each of these can serve as an acceptor for ubiquitin chain formation [3]. Whereas K48 ubiquitin chains usually serve as a signal for proteolysis, K63 ubiquitin chains have non-proteolytic roles and serve as regulatory modifications in endocytosis, DNA damage or ribosomal protein synthesis. At present, only the functions of K48 and K63 ubiquitin chains are well characterized, while very little is known about the function of the other types of ubiquitin chains. The ***aim*** of this interdisciplinary project is, therefore, to learn more about the function of protein modification by different ubiquitin chains. In order to synthesize such conjugates specific chemical strategies for the build-up of polyubiquitin chains are pursued that use thioether ligation of C-terminal ubiquitin-thiol residues with N-(chloroacetyl)-side chain protected Lysine residues [4]. This procedure provided specific and original lysine residues at each distinct branching site. High resolution FT-ICR mass spectrometry (FTICR-MS) and was predominantly used for the characterization of the chemically synthesized polyubiquitin chains, and affinity proteomics methods are especially suitable for identifying the reconjugation structures of polyubiquitin.

Results and Discussion

The synthetic concept pursued makes use of ubiquitin derivatives, in which the specific lysine residue of an "acceptor" ubiquitin to be conjugated is activated by chloroacetylation, whereas in a "donor" ubiquitin the C-terminal glycine is substituted with a cysteine residue. Ubiquitin partial peptide building blocks were first synthesized using conventional orthogonal solid phase peptide synthesis (SPPS) that contain the specific ε-amino-chloroacetylated lysine residue in the "acceptor" peptide sequence. The side chain conjugation to the "donor" ubiquitin sequence was performed by S-alkylation to a C-terminal cysteine residue introduced in addition to and/or in place of the terminal glycine-53 of ubiquitin, GSSHHHHHHSSGLVPRGSH-Ubiquitin(1-52)-Cys, [Figure 1].

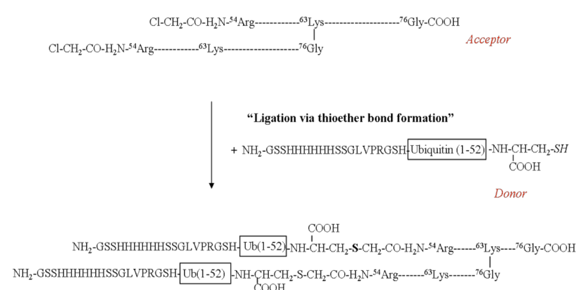

Figure 1. Outline of the synthesis of K-63 linked di-Ubiquitin conjugates by chemoselective ligation (Thioether Linkage)

In a first variant recombinant "donor" ubiquitin containing a C-terminal cysteine was prepared by bacterial expression according to established protocols, and conjugated to yield a diubiquitin. Initial studies showed that reaction of the *N*-terminal chloroacetylated peptides with C-terminal thiol-peptides was relatively slow under slightly basic conditions, and required long reaction times, increasing the risk of side reactions. Therefore, we investigated the increase of reactivity by substituting the chloroacetyl peptides with the corresponding - bromo or –iodo derivatives. The conjugation reactions were performed in solution between a N-terminal of chloroacetylated di-ubiquitin peptide$[54\text{-}76]_2$ and each elongated by a cysteine residue at the C-terminus, by treatment with a 6M Guanidine hydrochloride solution containing 1M sodium carbonate, under alkaline conditions (pH 9). The conjugation reaction was monitored by analytical RP-HPLC on a C4 column. The resulting conjugation product of chloroacetylated ubiquitin $(54\text{-}76)_2$ was characterized by MALDI-TOF-MS.(Figure 2).

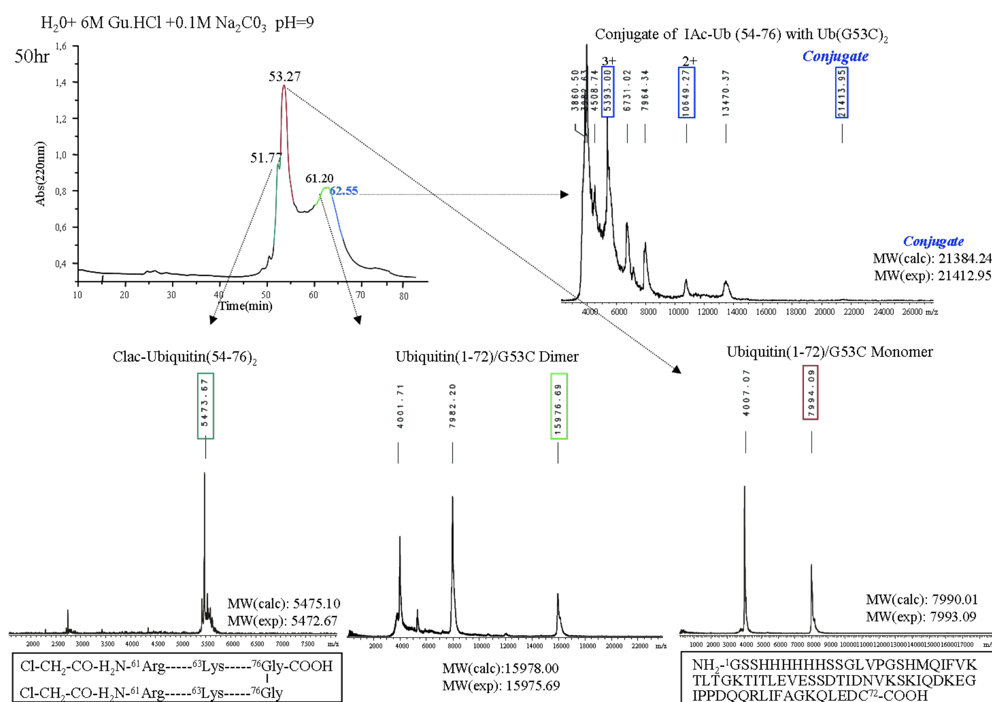

Figure 2. Conjugation of Ubiquitin(G53C) with branched Chloroacetylated Ubiquitin(54-76)2 followed by analytical RP-HPLC and mass spectrometry

Acknowledgments

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