Abridged version of Doctoral Thesis

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Characterization of conformational dynamics of Lys48-linked ubiquitin chains as design frameworks for creating allosterically controllable multidomain proteins

Sophisticated protein functions are, in many cases, mediated through the cooperative interplay between two or more domains. Proteins with a modular architecture of multiple domains connected by linkers often exhibit diversity in relative positions of the individual domains organized through weak and even transient inter-domain interactions. Moreover, it is suggested that the motion, orientation, and interaction of these domains could be regulated and affected by various environmental factors in cell, including pH, temperature, oxidative stress, and molecular crowding. Therefore, in order to extend our understanding of the working mechanisms of multidomain proteins in living systems, the quantitative characterization of their conformational interchanges in solution is necessary.

Polymeric ubiquitin (Ub) chains, in which several Ub proteins are connected through specific isopeptide bonds, are known to play regulatory roles in various cellular processes, including cell cycle progression, DNA repair, transcriptional regulation, and apoptosis. The Ub chains conjugated by different linkages carry distinct biological information in the form of a “Ub code” that is read out by specific Ub-interacting proteins. The Lys48-linked Ub chain serves as a tag for protein degradation by the 26S proteasome and interacts with the related proteasomal proteins through a hydrophobic surface. According to the crystal structures, Lys48-linked Ub chains often exhibit closed conformations, in which the hydrophobic patches are shielded due to Ub-Ub interactions in chains. On the other hand, our previous NMR study enabled the characterization of the conformational interchange of the native form of Lys48-linked diUb (n-diUb) between the open and closed conformations, based on conventional chemical shift data.

In this study, I thus extended our previous work by characterizing conformational interconversions of the native forms of Lys48-linked triUb (n-triUb) and tetraUb (n-tetraUb) chains in solution. Firstly, I successfully optimized the protocol of ubiquitylation reaction *in vitro* and prepared a series of Lys48-linked Ub chains with uniform isotope labeling in native and cyclic forms, and carried out NMR studies for characterizing their conformational dynamics. In contrast to n-diUb, I found that n-triUb and n-tetraUb exhibited multiple peaks for many residues, suggesting differences in the local environment among the Ub units. I performed the spectral assignments using a series of n-triUb and n-tetraUb analogs, in which a specific Ub unit was isotopically labeled, and thereby found that each Ub unit in these Ub chains experienced a dynamic

transition in the moderately fast exchange between the open and closed states on the relevant NMR timescale. Furthermore, under this condition, the comparative NMR analyses using monomeric Ub and cyclic diUb as reference molecules enabled the quantification of populations of the open and closed states for each Ub unit of the native Ub chains. My NMR data indicated that the most distal Ub unit in the Ub chains is the most apt to expose its hydrophobic surface, suggesting its preferential involvement in interactions with the Ub-recognizing proteins.

To explain the higher open-state propensity of the distal Ub units in n-triUb and n-tetraUb, I considered the possible end effects attributed to the distal and proximal end of the Ub chain. I found that the amino acid substitutions at position 48 in the distal Ub of the Ub chain remotely affected the solvent exposure of the hydrophobic surfaces of the other Ub units through the competitive sharing of the hydrophobic surfaces among the Ub units. Thus, the mutational effect at the most distal Ub is allosterically transmitted to the remaining Ub units in a chain-reaction manner.

These results suggested that the Lys48-linked Ub chains may offer unique design frameworks for creating allosterically controllable multidomain proteins. For proof of this concept, I attempted to design artificial multidomain proteins based on the Lys48-linked diUb. I could construct environmentally responsive biosensing probes, in which Förster resonance energy transfer is enhanced in the closed state of Lys48-linked diUb. Furthermore, I designed cyclic multidomain proteins in which diUb is conjugated with another multidomain protein for controlling their domain rearrangements in environment-responsive manners.

This study provided a quantitative view of conformational interconversions of the Lys48-linked Ub chains in solution, offering new strategies for probing and manipulating the conformational dynamics of multidomain proteins.