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研究課題名（和文）動的なユビキチン鎖の構造特性と認識機構の研究

研究課題名（英文）Dynamic Character of the Ubiquitin System

研究代表者
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研究成果の概要（和文）：この2年間で本研究は順調に進んだ。得られた成果の多くは国際学術雑誌の査読付論文として発表した。K48、K63、M1結合型のユビキチン鎖の分子動力学計算とNMR実験では興味深い結果が得られ、X線結晶学等の実験結果と合致する内容であった。特に、K48結合型ジユビキチンといったユビキチン鎖は、結晶化条件の違いや結合タンパク質の有無で、異なる構造状態を取る。しかし本研究により、これらの異なる構造状態の全ては、分子動力学法で取得した位相空間の同じ軌跡上にあることが分かった。本研究成果は2021年のBiochemistry誌に論文として発表した。K48結合型以外の鎖に関する研究成果は今後論文発表する。

研究成果の学術的意義や社会的意義
長年多くの病理学者たちが抱いてきた不思議は、神経変性疾患で観察される異常タンパク質凝集体（封入体）のほとんどにユビキチンが含まれていることである。どのようにしてポリユビキチン鎖が正しく認識されなくなり、凝集体を形成するかを原子分解能で解析したものであり、得られた研究成果は封入体の形成機構の理解、つまり神経変性疾患の発症機構の理解の一助となると考える。また、得られた構造学的情報は、熱や流体力学的応力によるマルチドメインタンパク質の構造変化や分子間相互作用を理解する上で重要な知見となり得ると考える。

研究成果の概要（英文）：In these two years, our research has proceeded rather smoothly. We could publish several results of this project in international peer-reviewed journals. For example, molecular dynamics simulations and NMR experiments on K48-, K63-, and M1-linked ubiquitin chains produced intriguing results that well agree with observations by other researchers worldwide, e.g. in the field of X-ray crystallography. Specifically, various distinct ubiquitin chain conformations of the same molecule (e.g., a K48-linked diubiquitin molecule) have been reported by different researchers under different crystallization conditions or in the presence of different binding proteins. However, we found that all of these molecular states of diubiquitin lie on the same trajectory in phase-space as sampled by molecular dynamics. The main result of this project is summarized in our 2021 Biochemistry paper. However, several aspects of this study (especially for chains other than K48-linked) are yet to be published.

研究分野：生物物理学

キーワード：ubiquitin　ubiquitin binding　UBA domain　K48-linked chains　K63-linked chains　M1-linked chains　solution NMR　molecular dynamics

1. Research background at the time of the start of this project [研究開始当初の背景]

Polyubiquitin chains can be conjugated to almost all intracellular proteins. These chains function a signal tag to specify diverse cellular events such as proteolysis and immune responses. Such a polyubiquitin chain is formed by the covalent conjugation of individual molecules of the small 76-amino acid protein ubiquitin via eight distinct types of linkages. Seven of these occur via internal lysine residues of ubiquitin (K6, K11, K27, K29, K33, K48, K63), whereas one type uses the N-terminal α-amino group of M1. Because the tertiary structure of ubiquitin has been known for a long time to be physicochemically extremely stable and rigid, it seemed natural to assume that ubiquitin moieties in a polyubiquitin chain would be structurally virtually equivalent to one another. Another reason underpinning this assumption was that ubiquitin-binding proteins containing ubiquitin-associated (UBA) domains bind with almost equal affinity to each ubiquitin moiety in a polyubiquitin chain. In stark contrast, ubiquitin-binding proteins containing Npl4-like zinc-fingers or tandem repeat ubiquitin- interacting motifs recognize specific orientations and distances between the successive hydrophobic surfaces of the ubiquitin moieties. For this reason, these types of proteins are able to exhibit a strong polyubiquitin-linkage specificity. In addition, some UBPs such as deubiquitinating enzymes (DUBs) and antibodies directly associate with both an (iso-)peptide linkage and the hydrophobic surfaces of the ubiquitin moieties.

Moreover, due to the high rigidity of the ubiquitin fold, the ubiquitin moieties in a polyubiquitin chain appear to be structurally and physically equivalent to each other. It was therefore unclear how a specific ubiquitin moiety in a chain may be preferentially recognized by some proteins, such as the kinase PINK1. In this project we have however shown that there is structural dynamic heterogeneity in the two ubiquitin moieties of K48-linked diubiquitin and the proof came overwhelmingly from NMR spectroscopic analyses. Our analyses captured subunit-asymmetric structural fluctuations that are not directly related to the closed-to-open transition of the two ubiquitin moieties in diubiquitin. Strikingly, these newly identified heterogeneous structural fluctuations may be linked to an increase in susceptibility to phosphorylation by PINK1. Coupled with the fact that there are almost no differences in static tertiary structure among ubiquitin moieties in a chain, the observed subunit-specific structural fluctuations may be an important factor that distinguishes individual ubiquitin moieties in a chain, thereby aiding both efficiency and specificity in post-translational modifications.

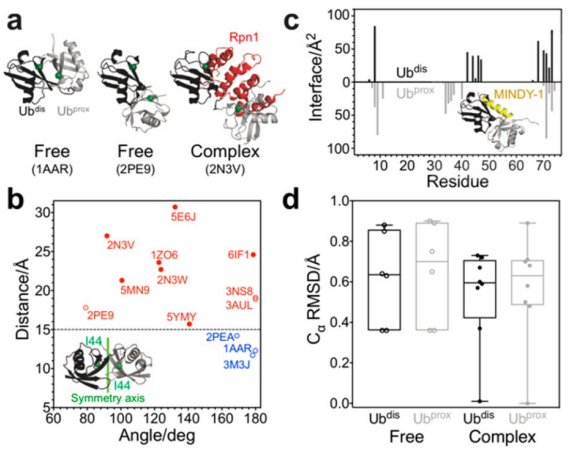

Fig. 1. High-degree of variability in possible structures formed by a single type of ubiquitin: K48-linked diubiquitin.

2. Research objectives [研究の目的]

Among the eight reported types of polyubiquitin chains (see point 1), polyubiquitin chains linked via

lysine residue 48 of one ubiquitin and the C-terminal carboxy-group of another ubiquitin (hereafter: K48-linked chains) are the most abundant in cells. In addition, both their structural properties and their physiological roles in ATP- dependent protein degradation have been extensively studied. The first published crystal structure of K48-linked Ub2 had captured the two ubiquitin moieties interacting with each other via their respective I44-centered hydrophobic patches, thereby forming a "closed" conformation (Figure 1a, left). In later studies, this closed conformation had also been confirmed to exist in aqueous solution and additionally, in a longer chain: K48-linked tetraubiquitin. However, this poses a problem when trying to reconcile structural biology with physiological events: in these closed structures, the I44-centered hydrophobic surfaces appear to be virtually inaccessible, although many physiological signaling processes strictly require their recognition (i.e., being non-covalently bound) by UBPs. A possible solution to this dilemma appeared to be that because ubiquitin moieties in a chain are connected by flexible linkers, K48-linked polyubiquitin chains are thought to adopt multiple conformations, much like "beads on a string". Indeed, more recent X-ray crystallography and NMR studies have reported various conformations of K48-linked Ub2 with different interdomain angles and distances; in some conformations, the I44-centered hydrophobic surfaces are more accessible (Figure 1a, middle; 1b) and in fact, K48-linked Ub2 is reported to be in equilibrium between the closed and open conformations in solution. We considered, therefore, that ubiquitin-binding proteins might recognize features of the dynamic structural properties of a K48-linked polyubiquitin chain to exert subunit preference (Figure 1a, right; 1c).

To test this hypothesis, in this study we set our major research objective to characterize the structural dynamics of K48-linked Ub2, as this molecule represents the minimal structural and recognition element of K48-linked polyubiquitin.

3. Research methods [研究の方法]

Main methods for this research were recombinant protein expression in bacteria and purification, NMR spectroscopy, and molecular dynamics simulations. Regarding protein sample preparation, human ubiquitin, and its mutants K48R and G75A/G76A were expressed in *Escherichia coli* strain BL21(DE3). 15N-selective isotope labeling of the distal or proximal subunit of Ub2 was achieved by expressing the respective ubiquitin subunits in either unlabeled LB media or 15N-labeled M9 minimum media containing 99% 15N-labeled ammonium chloride. Purification of ubiquitin was done as follows: first, ubiquitin was purified by ion exchange chromatography using a sample buffer consisting of 50 mM sodium acetate (pH 4.5) and an elution buffer consisting of 50 mM sodium acetate (pH 4.5) and 250 mM sodium chloride. Next, after cation exchange chromatography, the protein was further purified by size-exclusion chromatography. Diubiquitin samples with selective distal and proximal 15N-labeling were prepared by enzymatic reaction of unlabeled and 15N-labeled ubiquitin subunits as previously described. K48-linked Ub2 was enzymatically synthesized by the E1 (mouse UBA1) and E2 (human E2-25K) enzymes. To compare the dynamics between the samples, a control sample of wild-type monoubiquitin (hereafter, Ub1) was also prepared. The final NMR samples were either 1 mM Ub1 or 1 mM Ub2 in a buffer consisting of 20 mM potassium phosphate, pH 6.8, 1 mM EDTA, 20 mM potassium chloride, and 5% D_2O.

As for NMR spectroscopic experiments, we conducted all our experiments on an Avance II 700 MHz instrument (Bruker BioSpin Co.). This instrument was equipped with a 5 mm 15N/13C/1H

z-gradient triple resonance cryogenic probe. After measurement, the acquired NMR data were then processed by using the program NMRPipe. Chemical shift referencing using the methyl proton signal of 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) was performed for 1H. Subsequently, 15N chemical shifts were referenced indirectly with respect to DSS. Further analysis was conducted in CcpNmr Analysis and NMRView. All backbone amide HSQC cross-peaks of each type of Ub2 were assigned to the individual amino acid residues from 15N-edited TOCSY and NOESY spectra based on signal assignments of wild-type Ub1 in the Biological Magnetic Resonance Bank (BMRB) database (entry 6457).

Main experiments for in-depth NMR spectroscopic analysis were (1) differentiation between linear and nonlinear amide proton chemical shift temperature dependence, (2) thermodynamic analysis of curvature in chemical shift temperature dependence, (3) relaxation dispersion; molecular dynamics simulations of ubiquitin in GROMACS under the AMBER99sb-ILDN forcefield were carried out to supplement these experimental data with canonical parameters of temperature, ion concentration, MD timestep, etc..

4. Research results [研究成果]

To elucidate the dynamic characteristics of K48-linked Ub2, we first classified its experimentally observed conformations based on the relative orientation and distance of the two ubiquitin moieties (Figure 1b). As would be expected, in the reported closed state of K48-linked Ub2, there is little variety in orientation and distance due to the interaction between the two ubiquitin moieties (Figure 1b, blue circles). By contrast, Ub2 adopts diverse conformations in the extended (open) state, and the I44 Cα atoms of the two ubiquitin moieties are spaced more than 15 Å apart (Figure 1b, red circles). Notably, in the bound form, the orientation and distance differ depending on the UBP (Figure 1b, filled circles), indicating that each UBP selects a specific conformation of Ub2 for binding. In addition, a per-residue analysis of the interface between Ub2 and each UBP indicated that both the interface area and the residues contributing to the interface differ between the distal and proximal subunits (hereafter, Ubdis and Ubprox, respectively) in most Ub2−UBP complexes (Figure 1c). On the one hand, this observation suggested that UBPs recognize a specific relative position of the two successive hydrophobic surfaces of K48-linked Ub2 (conformational-selection mechanism); on the other hand, these data imply that each UBP may also induce conformational rearrangement of K48-linked Ub2 to fit into the unique binding surface of the UBP (induced-fit mechanism).

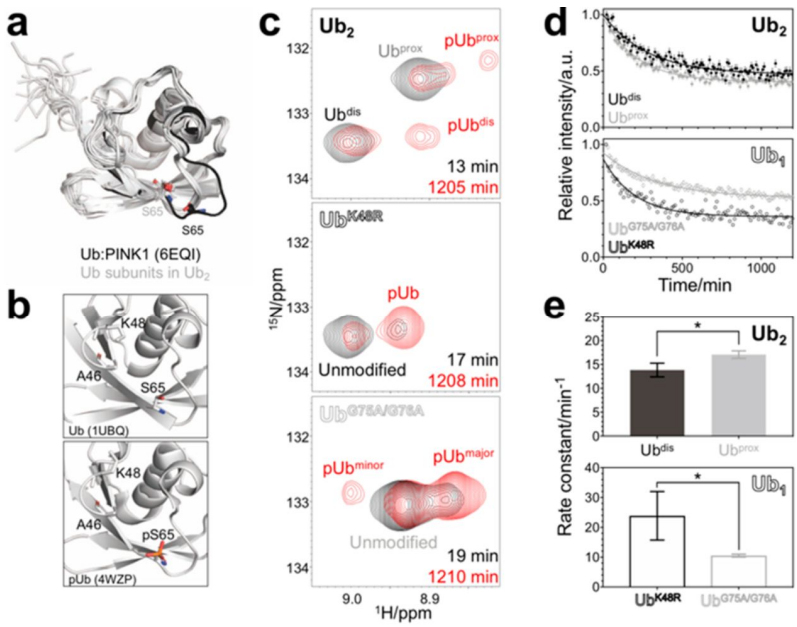

Fig. 2. Altered phosphorylation susceptibility in K48-linked diubiquitin.

Nevertheless, it remained unclear how UBPs such as PINK1 preferentially associate with a specific ubiquitin moiety in Ub2 without using the subunit-selective recognition. To investigate whether there are variations in the static structure of the ubiquitin moieties in Ub2 and Ub1, we examined differences in the reported tertiary structures of the two ubiquitin moieties in K48-linked Ub2. Structural alignment showed that the root-mean-square deviation (RMSD) in the main chain (Cα atoms) of the ubiquitin moieties was less than 1 Å for Ub1 and Ubdis and for Ub1 and Ubprox (Figure 1d). This result indicated the absence of distinct differences in static tertiary structures between the two ubiquitin moieties in Ub2, suggesting that any subunit-specific reactions catalyzed by enzymes such as PINK1 must arise from other structural characteristics of the two Ub2 moieties.

To determine whether the subunit-asymmetric conformational fluctuations play a role in subunit-specific recognition of K48-linked Ub2 by UBPs, we investigated the subunit preference of PINK1 for phosphorylation of K48-linked Ub2. Although phosphorylation of K48-linked diubiquitin and tetraubiquitin was previously reported, the possibility of a subunit preference of PINK1 for K48-linked polyubiquitin had not been examined in detail. Based on the structure of Ub1 in complex with PINK1, the loop between the 3_{10}-helix and the β5 strand of Ub1 needs to be relatively exposed to solvent to allow phosphorylation at S65 (Figure 2a). Notably, one of the clusters of residues for which we could demonstrate Ubprox-specific structural fluctuations was located on this loop; therefore, we hypothesized that the fluctuations might be related to the phosphorylation susceptibility of Ub2.

To compare the phosphorylation susceptibility between Ubdis and Ubprox in K48-linked Ub2, we monitored the phosphorylation of the two subunits simultaneously by real-time NMR. Although the $^{1}H-^{15}N$ cross-peaks of almost all residues overlap perfectly between Ubdis and Ubprox, the respective cross-peaks of A46, which is spatially close to S65 (approximately 8 Å; Figure 2b), are isolated from one other by using two ubiquitin mutants: UbK48R and UbG75A/G76A (Figure 2b). Note that these mutants were used to prepare K48-linked Ub2 and that did not influence the structure of ubiquitin based on the observed $^{1}H-^{15}N$ cross-peaks. The amide cross-peak of A46 undergoes a large shift upon phosphorylation at S65 (Figure 2c), enabling us to individually trace the phosphorylation of Ubdis and Ubprox in fully ^{15}N-labeled K48-linked Ub2 in the same experiment by measuring the signal decays of A46 of the unphosphorylated ubiquitin moieties (Figure 2d, upper). Although it is also possible to analyze the increasing signals of phosphorylated Ub2, phosphorylated Ub1 is reported to exist in a two-state equilibrium; therefore, we considered that the real-time profiles of generated phosphorylated Ub2 would be more complicated to analyze and interpret quantitatively.

Notably, the real-time NMR analysis showed that Ubprox undergoes phosphorylation faster than Ubdis (Figure 2d, upper, and Figure 2e, upper). As a control experiment, we analyzed phosphorylation of the Ub1 components of our Ub2 sample. UbK48R underwent phosphorylation faster than UbG75A/G76A (Figure 2d, lower, and Figure 2e, lower). This might be a possible side effect of the point mutations because K48 of monoubiquitin was previously shown to be one of the residues participating in the interaction with PINK1. Taken together, these data suggest that the Ubprox-specific structural fluctuations identified on the loop between the 3_{10}-helix and the β5 strand significantly enhance its phosphorylation susceptibility (relative to Ubdis) because the phosphorylation susceptibility of the individual Ub1 components was inverted in Ub2. Such a phenomenon is consistent with the requirement that the side chain of S65 must be exposed to the solvent and to fit into the active site of PINK1 for phosphorylation.

５．主な発表論文等

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掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.bbagen.2019.06.006	査読の有無 無
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3．雑誌名 Biochemistry	6．最初と最後の頁 573～583
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〔学会発表〕　計0件

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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6．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

7．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関