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Monomer/Oligomer Quasi-Racemic Protein Crystallography

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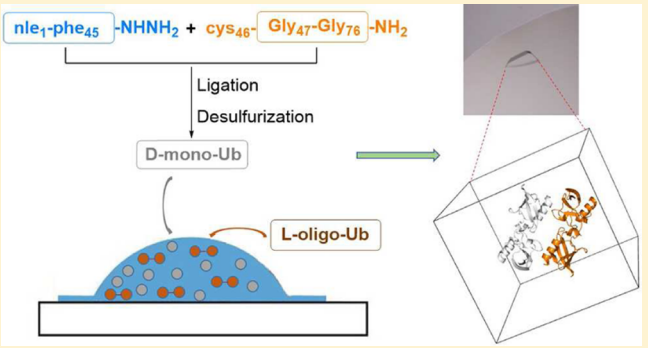
Supporting Information

ABSTRACT: Racemic or quasi-racemic crystallography recently emerges as a useful technology for solution of the crystal structures of biomacromolecules. It remains unclear to what extent the biomacromolecules of opposite handedness can differ from each other in racemic or quasi-racemic crystallography. Here we report a finding that monomeric D-ubiquitin (Ub) has propensity to cocrystallize with different dimers, trimers, and even a tetramer of L-Ub. In these cocrystals the unconnected monomeric D-Ubs can self-assemble to form pseudomirror images of different oligomers of L-Ub. This monomer/oligomer cocrystallization phenomenon expands the concept of racemic crystallography. Using the monomer/oligomer cocrystallization technology we obtained, for the first time the X-ray structures of linear M1-linked tri- and tetra-Ubs and a K11/K63-branched tri-Ub.



INTRODUCTION

The mixture of a chiral molecule and its opposite enantiomer often has tendency to form racemic crystals in which equimolar enantiomers are orderly present in the elementary cell.[1] Such racemic crystals are usually centro-symmetrical with only two possible phase angles (0 or π), offering advantages in phasing and structure determination.[2] Moreover, racemic mixtures can crystallize in achiral space groups that are not accessible to homochiral molecules so that crystals may be grown from racemic mixtures more readily, opening up new opportunities for the crystallogenesis of biomacromolecules.[3] Racemic crystallography has enabled the solution of the X-ray crystal structures of some difficult-to-crystallize proteins,[4−7] for instance, a therapeutically interesting potassium channel blocker protein BmBKTx1.[8] It has also been used to solve the structures of nucleic acids, for instance, Pribnow box consensus sequence involved in bacterial transcription regulation.[9−11]

Application of racemic crystallography to biomacromolecules requires total chemical synthesis of their unnatural mirror-image counterparts. To reduce the synthetic cost, an interesting and useful discovery was that molecules that are almost but not exactly mirror images of each other also have tendency to cocrystallize to form quasi-racemic crystals.[12−15] For instance, in a recent study it was found that protein crystals could be readily grown from the mixture of a chemokine (L)-Ser-CCL1 bearing a nonasaccharide glycan and nonglycosylated (D)-Ser-CCL1, while no crystal was obtained from glycosylated (L)-Ser-CCL1 alone.[16] Quasi-racemic crystallography is expected to find applications in the growing studies on proteins with modifications. It also evoked our curiosity regarding the extent to which the molecules of opposite handedness can differ from each other in quasi-racemic crystallography. In this context we now report an unexpected finding that monomeric D-ubiquitin (Ub) can readily cocrystallize with different dimers, trimers, and even a tetramer of L-Ub to form a new type of quasi-racemic protein crystals.

RESULTS AND DISCUSSION

Crystallogenesis of Linear Tri- and Tetra-Ubs. Our finding was made during the study toward obtaining the crystal structures of linear Ub chains, which are chain molecules formed via peptide bonds between the C-terminus of one Ub and the N-terminus of adjacent Ub.[17] Linear Ubs play crucial roles in many physiological processes, for example, they are recognized by the reader NF-κB essential modulator (NEMO) to control the tumor necrosis factor (TNF) pathway.[18] Structural studies on the linear Ubs are needed to elucidate their biochemistry, but only the crystal structure of linear di-Ub has so far been reported.[19−21] To obtain the X-ray crystal structures of linear tri- and tetra-Ubs, we expressed the two proteins in *E. coli*. They were screened under 574 different conditions (commercial screening kits from Hampton Research) for crystals, but no crystal was obtained after a few months.

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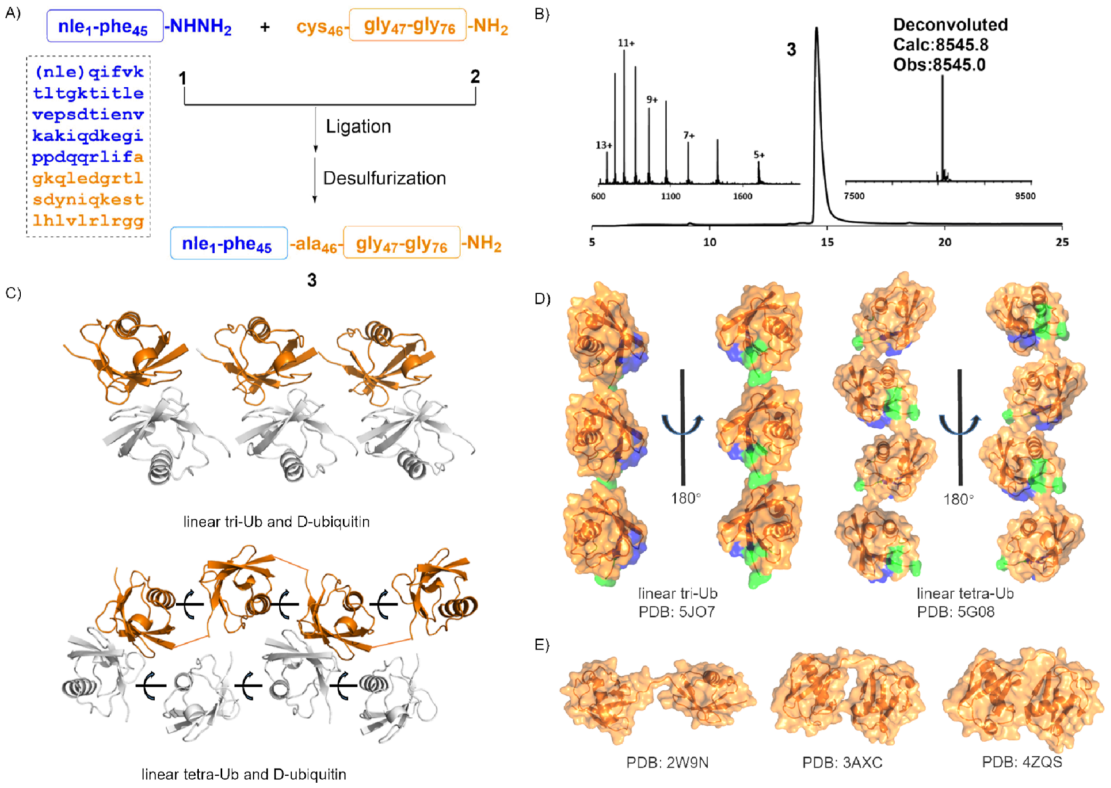

Figure 1. Cocrystallization of D-mono-Ub with linear M1-linked tri-Ub and tetra-Ub. (A) Synthesis route. (B) Analytical HPLC chromatogram of isolated D-mono-Ub and electrospray ionization mass spectrum (observed 8545.0 Da, calcd 8545.8 Da). (C) Crystal structures of quasi-racemates of linear tri-Ub (PDB ID: 5GO7) and tetra-Ub (PDB ID: 5GO8). Linear tri-Ub and tetra-Ub are colored in orange. D-Mono-Ub is colored in gray. (D) Views of linear tri-UB and tetra-Ub crystal structures (orange). Ile44 and Ile36 patches are colored in blue and green, respectively. (E) Structures of linear di-Ubs reported previously (PDB ID: 2W9N, 3AXC, and 4ZQS).

Inspired by our recent work on the quasi-racemic crystallography of K27-linked di- and tri-Ubs,[13] we planned to synthesize the D forms of linear tri- and tetra-Ubs to generate the racemic crystals. Before this costlier approach was carried out, we hit upon an idea to use D-mono-Ub. This molecule can be much more easily synthesized through Fmoc (9-fluorenylmethyloxy-carbonyl) solid-phase peptide synthesis (SPPS) and two-segment hydrazide-based native chemical ligation (Figure 1).[22−28] To avoid the oxidation of Met in the synthesis, we used Nle1 to replace Met1 in the sequence of synthetic D-mono-Ub. D-mono-Ub (1.5 mg/mL) was mixed with either linear tri-Ub (1.5 mg/mL) or tetra-Ub (1.5 mg/mL) and screened with 196 conditions. We were surprised to obtain 37 or 56 crystals for the mixtures of linear tri- or tetra-Ubs with D-mono-Ub from 196 conditions, while no crystal was grown from the control experiments for pure linear tri- or tetra-Ubs (3.0 mg/mL) under the same conditions.

X-ray structures were determined by molecular replacement and refined to the final R_{factor}/R_{free} of 22.6%/31.5% and 22.2%/32.2% (Supplementary Table 1). The crystal for linear tri-Ub (Protein Databank (PDB): 3GO7) formed in triclinic space group P1 and diffracted X-rays to 1.80 Å resolution. The crystal for linear tetra-Ub (PDB: 5GO8) formed monoclinic space group $P2_1$ and diffracted X-rays to 2.18 Å resolution. As shown in Figure 1, each asymmetric unit of the quasi-racemic linear tri-Ub crystal contains $^1/_3$ tri-Ub (i.e., one L-Ub unit) and one D-mono-Ub. Similarly, each asymmetric unit of the quasi-racemic linear tetra-Ub crystal contains $^1/_4$ tetra-Ub (i.e., one L-Ub unit) and one D-mono-Ub. All the Ub units in linear tri- or tetra-Ub adopts a native conformation of mono-Ub (PDB: 1UBQ) except for the flexible C-terminals.

Crystal structures represent snapshots of the possible conformations. In the structure of linear tri-Ub, the three Ub units are orderly arranged in the same orientations so that one Ub unit can overlap with its neighboring Ub after translation. By contrast, in the structure of linear tetra-Ub, the neighboring two Ub units are arranged in opposite orientations, i.e., one Ub can overlap with its neighbor after translation and rotation of 180°. Compared to the above two structures, the crystal structures of linear di-Ub show either opposite orientation[19,20] (PDB: 3AXC and 4ZQS) or even compact conformation[21] (PDB: 2W9N). These structures indicate that linear Ub chains have a high degree of flexibility, supporting a recent small-angle X-ray scattering study that also revealed the conformational diversity of linear Ub chains.[20]

Interesting behaviors were observed for the arrangement of D-mono-Ub in the quasi-racemic crystals. In the linear tri-Ub system, although there is no covalent bond between two D-mono-Ubs, the C terminus of one D-mono-Ub is positioned very close to the N-terminus of its neighboring D-mono-Ub. Moreover, one D-mono-Ub can perfectly overlap with its neighboring D-mono-Ub after translation. As a result, three continuous D-mono-Ub molecules formed a pseudomirror image of one linear tri-Ub. The same phenomenon was also observed for the linear tetra-Ub case, where the C terminus of one D-mono-Ub is positioned close to the N terminus of its neighboring D-mono-Ub and the two D-mono-Ubs can overlap after translation and rotation of 180°. Through both static light scattering (SLS) and analytical ultracentrifugation (AUC)

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experiments (Supporting Information), we concluded that there is not any interaction between linear tri-Ub and D-mono-Ub in the solution. Thus, the organized arrangement of D-mono-Ub in the quasi-racemic crystals is a result of crystal packing.

Cocrystallization with Isopeptide-Linked Di-Ubs. The cocrystallization of D-mono-Ub with linear tri- and tetra-Ubs invoked our curiosity as to whether D-mono-Ub can help the crystallization of other oligo-Ubs carrying isopeptide bonds. These Ub chains linked through different Lys residues control many types of intracellular signalings such as proteasome-dependent protein degradation and DNA repair.[29−31] For the purpose of our study, we chemically synthesized K6-, K11-, K27-, K29-, K33-, K48-, and K63-linked di-Ubs again through Fmoc SPPS and hydrazide-based native chemical ligation.[32−37] With seven different di-Ubs in hand, we screened 196 crystallization conditions for 14 protein solutions, namely, 7 L-forms of di-Ubs (3.0 mg/mL each) and 7 mixtures of L-di-Ub (1.5 mg/mL) with D-mono-Ub (1.5 mg/mL). It was striking to see that crystals were rapidly grown from 7 to 73 conditions in 24 h for the quasi-racemic mixtures, while no crystal was obtained for L-di-Ubs under the same conditions until 7 days.

X-ray structures of the seven di-Ubs were solved by molecular replacement (Table 1 and Figure 2). It is interesting to find that the structures of K6-, K11-, K29-, K33-, K48-, and K63-linked di-Ubs all adopt open conformation with no mutual interactions at the interfaces between the two Ub units. These observations challenge the previous opinions that K6-, K11-, and K33-linked di-Ubs may only exhibit compact structures, echoing the viewpoint that compact and open conformations of Ub chains may exist in fast equilibrium in the solution.[29] Nonetheless, K27-linked di-Ub still adopts a compact conformation consistent with our previous work.[13] Furthermore, unlike the isopeptide bonds of K6-, K11-, K29-, K33-, K48-, K63-, and M1-linked di-Ub, another unique feature of K27-linked di-Ub is that its isopeptide bond is entirely inner-buried and not exposed to the solvent. Regarding to the arrangement of D-mono-Ubs in the seven quasi-racemic crystals, the C-terminus of one D-mono-Ub is always positioned close to the ε-amino group of the other D-mono-Ub. In this manner the two unconnected D-mono-Ubs adopt a conformation that forms a pseudomirror image of the corresponding L-di-Ub bearing a covalent isopeptide linkage between the two L-Ub units.

To gain some insights into why D-mono-Ub can readily cocrystallize with Ub chains, we examined the space groups of the above nine quasi-racemic crystals. It was found that except for linear tetra-Ub ($P2_1$), K29-linked di-Ub ($P2$), and K63-linked di-Ub ($P2_12_12_1$), all the other crystals formed space group $P1$. This observation is consistent with the previous theory of Wukovitz and Yeats that $P\bar{1}$ (or $P1$ in the present study due to the mutation of Met1 to Nle1 in D-mono-Ub) should be the space group that is most favorable for crystallization.[3] Moreover, it is interesting to observe that the quasi-racemic di-Ub crystals have a lower solvent content (24−38%) compared to that of the previous di-Ub crystals (39−56%). This phenomenon may indicate more compact packing of the L- and D-Ub units in the quasi-racemic crystals.

X-ray Crystal Structure of a Branched Tri-Ub. To demonstrate the practical usefulness of the monomer/oligomer quasi-racemic crystallography method, we examined its application to solve the structure of a branched Ub oligomer. Branched Ubs are recently discovered chain molecules that may play pivotal roles in several cellular pathways. For instance, K11/K48-branched Ubs have been proposed to enhance protein degradation,[48] while K11/K63-ubiquitin chains may control major histocompatibility complex class I (MHC1) endocytosis.[49] Biochemical and structural studies of branched Ubs are needed to investigate these interesting biological

Table 1. Resolution, Space Group, and Solvent Content (%) of the Crystals of Di-Ubs

di-Ub	resolution (A)	space group	solvent quasi-racemic (%)	solvent reported (%)
K6-linked	1.15	$P1$	24	56[38]
K11-linked	1.73	$P1$	27	64,[39] 39[40]
K27-linked	1.15	$P1$	34	null
K29-linked	1.98	$P2$	25	39[41]
K33-linked	1.95	$P1$	35	50[42]
K48-linked	1.59	$P1$	24	33,[43] 42,[44] 34,[45] 34[46]
K63-linked	1.55	$P2_12_12$	27	56,[21] 57[47]

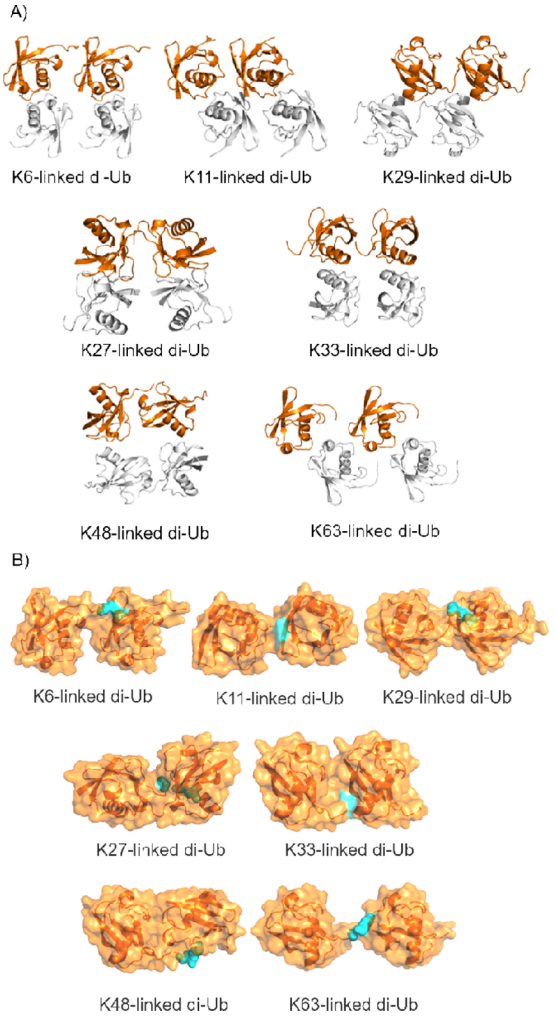

Figure 2. Cocrystallization of D-mono-Ub with seven di-Ubs. (A) Crystal structures of quasi-racemates of seven di-Ubs (PDB ID: 5GOB, 5GOC, 5GOD, 5GOG, 5GOH, 5GOI, and 5GOJ). Di-Ubs are colored in orange. D-mono-Ub is colored in gray. (B) Views of seven di-Ub crystal structures (orange). Isopeptide bonds are colored in cyan.

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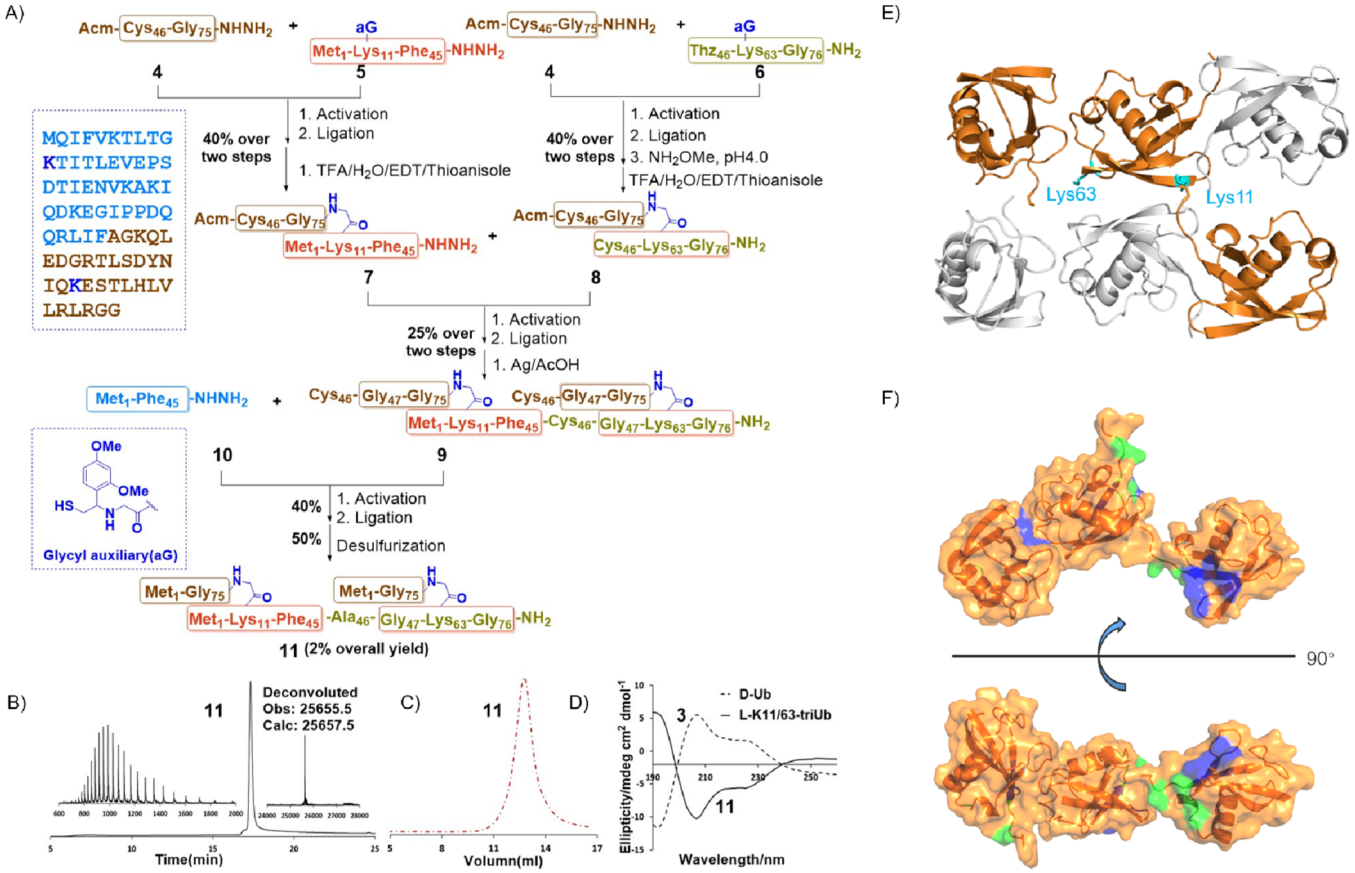

Figure 3. Cocrystallization of D-mono-Ub with K11/K63-branched tri-Ub. (A) Total chemical synthesis of K11/K63-branched tri-Ub. (B) Analytical HPLC chromatogram of isolated K11/K63-branched tri-Ub and electrospray ionization mass spectrum (observed 25655.5 Da, calcd 25657.5 Da). (C) Gel filtration chromatography (Superdex 75 column) of K11/K63-branched tri-Ub. (D) Circular dichroism spectra of D-mono-Ub and K11/K63-branched tri-Ub. (E) Crystal structure of quasi-racemate of D-mono-Ub (colored in gray) and K11/K63-branched tri-Ub (colored in orange). (F) Views of K11/K63-branched tri-Ub crystal structure (orange). Ile 44 and Ile 36 patches are colored in blue and green, respectively.

processes. However, no crystal structure has ever been reported for any branched Ub chain.

In this work we prepared K11/K63-branched tri-Ub through total chemical synthesis[50] (Figure 3). The target protein containing 228 amino acids was divided into six peptide segments, namely, two copies of **4**, one copy of **5**, one copy of **6**, and two copies of **10**. Ala46 of each Ub unit was temporarily mutated to Cys as the ligation site. The isopeptide bond was made by using a trifluoroacetic acid (TFA)-labile 1-(2,4-dimethoxyphenyl)-2-mercaptoethyl auxiliary. Acetamidomethyl (Acm) and thiazolidine (Thz) groups were used to protect the N-terminal Cys of segment **4** or **6** to avoid oligomerization or self-cyclization. The synthesis started with ligation of **4** and **5** and subsequent removal of the auxiliary to produce **7**. At the same time, **4** and **6** were ligated leading to **8** after Thz deprotection and auxiliary removal. The condensation between **7** and **8**, followed by Acm removal, generated **9** bearing two isopeptide bonds on one Ub unit. Final ligation of **9** with two copies of **10** led to the full length peptide. After desulfurization was conducted to convert all the Cys residues back to the native Ala residues, target protein **11** was obtained with an overall isolated yield of 2%. After characterization with RP-HPLC (reversed-phase high-performance liquid chromatography) and ESI-MS (electrospray ionization mass spectrometry), **11** was subjected to urea-gradient dialysis to afford folded K11/K63-branched tri-Ub with good homogeneity on size exclusion chromatography.

D-Mono-Ub (1.0 mg/mL) was added into K11/K63-branched tri-Ub (1.0 mg/mL), and the mixture was subjected to monomer/oligomer cocrystallization with the sitting drop method. Diffraction-quality crystals were readily obtained from only 48 commercial screening conditions. Crystals grown from 0.2 M $MgSO_4$, 20% PEG3350, and 4 mM $CdCl_2$, pH 6.0, formed in an orthorhombic space group $P2_12_12_1$ and diffracted X-rays to 1.84 Å resolution. Its structure was solved by molecular replacement and refined to the final statistics (Supplementary Table 1). As shown in Figure 3E, K11/K63-branched tri-Ub (PDB: 5GOK) displays a "V" shape, in which the two distal Ubs are separated away from each to bear a fully open conformation. The Ile 44 patches (blue color in Figure 3F) of both the proximal Ub and the K11-linked distal Ub are solvent-exposed. Such arrangement of hydrophobic patches may provide unique handles for the interaction of K11/K63-branched Ubs with special ubiquitin binding proteins and deubiquitinases. Finally, it is important to notice that three unconnected D-mono-Ubs self-assembled into a unique conformation that forms a pseudomirror image of K11/K63-branched tri-Ub bearing two covalent isopeptide linkages (Figure 3E).

SUMMARY

We report a new type of racemic crystallization phenomenon that can be named as monomer/oligomer quasi-racemic protein crystallography. This finding expands the concept of racemic

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crystallography, which challenges the notion how quasi-racemic molecules could be almost, but not exactly, mirror images of each other. The monomer/oligomer quasi-racemic crystallography method also has practical value as shown by its application to obtaining the first X-ray structures of linear M1-linked tri- and tetra-Ubs and a K11/K63-branched tri-Ub. It is thought-provoking to see that detached fragments of a molecule can noncovalently self-assemble into a pseudomirror image of its full-sized enantiomer to facilitate cocrystallization. The next question is whether or not this phenomenon could be extended to proteins consisting of more than one types of domains.

EXPERIMENTAL SECTION

Expression and Purification of Linear Tri-Ub and Tetra-Ub. cDNA fragments of linear tri-Ub and tetra-Ub were synthesized and cloned into pet22b vector by Genescript. Plasmids were transformed into *E. coli* BL21 (DE3) competent cells, and cells were cultured with LB medium (containing ampicillin antibiotic) at 37 °C. Protein expression was induced by 200 μM IPTG at 16 °C for 10 h. Cells were lysed by sonication in deionized distilled water, and the lysate was centrifuged at 14 000 rpm for 30 min. Then, 1% $HClO_4$ was added to precipitate other proteins, and the supernatant was centrifuged at 14 000 rpm for 30 min. Proteins were dialyzed in deionized distilled water (containing 0.1% TFA) to remove $HClO_4$ and then purified by anion exchange chromatography (MonoQ, GE Healthcare) using a NaCl gradient from 0 to 600 mM. Linear tri- and tetra-Ub were finally obtained for crystallization following gel filtration chromatography (Superdex 75, GE Healthcare) using a buffer of 20 mM Tris 7.5, 50 mM NaCl.

General Protocol of Hydrazide Resin. Commercially available 2-chlorotrityl chloride resin (2 g, substitution: 0.56 mmol/g) was swelled in DCM for 15 min. Fmoc-$NHNH_2$ (4 equiv) and DIEA (10 equiv) in DMF (20 mL) were added into the resin at 0 °C. The reaction mixture was gradually warmed to ambient temperature and stirred overnight. After completion, methanol was added, and the solution was stirred for 10 min to block out the activated chlorine group. Finally, the resin was filtered and washed with DMF, methanol, DCM, and diethyl ether.

Peptides Preparation. All the peptides were synthesized using standard Fmoc method under microwave conditions. Resins were chosen based on peptide segments during peptide synthesis. Resin (500 mg) was swelled in DMF for 5−10 min. Piperidine (20% v/v) and 0.1 M oxyma in DMF were added and the reaction was stirred for 10 min at room temperature to remove the Fmoc protecting group. The NMP solution of protected amino acids (4 equiv), oxyma (4 equiv), and DIC (4 equiv) was poured into the resin. Coupling time was 10 min. After completion, DMF and DCM were used to wash the resin. The peptide chain elongation proceeded until the last amino acid deprotection. Finally, a cleavage cocktail (TFA/H_2O/thioanisole/EDT 87.5/5/5/2.5, v/v/v/v) was added to cleave peptide from resin (cultured for 3 h). Cold ether was used to precipitate and wash the crude peptide. Then, the crude peptide was dissolved in water (containing 0.1% TFA) mixed with acetonitrile (containing 0.1% TFA) and prepared for HPLC analysis and purification.

Fmoc-lys(Alloc)-COOH was introduced, and Alloc (0.25 mmol) was removed using phenylsilane (600 μL) and tetrakis (triphenylphosphine) palladium (60 mg) in 5 mL of DCM for 3 h.[51] Glycyl 1-(2,4-dimethoxyphenyl)-2-mercaptoethyl auxiliary was introduced to react with the free amino group. Glycyl auxiliary was subsequently removed using cleavage reagent as mentioned above.

Native Chemical Ligation of Peptides Hydrazides. Ligation buffer (6 M Gn·HCl, 0.1 M Na_2HPO_4, pH ∼3) and 1 M $NaNO_2$ aqueous solution were prepared in advance. Hydrazide peptide was dissolved in ligation buffer.[52] $NaNO_2$ (10 equiv) was added into reaction buffer at −10 to −20 °C. After 30 min, MPAA (40 equiv) was added into reaction buffer, and the pH value was adjusted to 5.0 with 2 M aqueous NaOH. Cysteine peptide was dissolved into reaction buffer next and pH adjusted to 6.5 with 2 M aqueous NaOH. The reaction was monitored by HPLC.

Removal of ACM Group and Desulfurization Reaction. Lyophilized peptides were dissolved into acetic acid/water (1:1, v/v), and the concentration was 0.6 mM. Silver acetate (50 equiv) was added, and the peptide mixture was stirred overnight at room temperature; 6 M GnHCl containing 1 M DTT was added. Semipreparative HPLC was used to purify the product.

Peptides were dissolved in PBS buffer (6 M Gn·HCl, 0.1 M Na_2HPO_4, pH 7.4). TCEP (500 mM, pH 6.9) was added into peptide solution, and the peptide concentration was 0.6 mM. tBuSH (106 μL/μmol peptide) and VA-044 (34 mg/μmol peptide) were added, and the mixture was stirred overnight at 37 °C.

Circular Dichroism (CD) Experiments. Oligo-Ub chains were dissolved in water containing 20 mM tris 7.5 and 50 mM NaCl. CD spectra were recorded three times with 1 mm path length quartz cell quartz cell from 190 to 260 nm using Applied Photo physics Pistar π-180 CD spectrometer.

Protein Crystallization and Data Acquisition. Crystallization screening was performed in 48-well plates (XtalQuest) using the sitting drop vapor diffusion method at 16 °C. Each poly-Ub mixture (1 μL) was mixed with crystallization solution (1 μL). The following conditions were used to obtained crystals: linear tri-Ub (0.2 M $MgSO_4$, 20% PEG 3350, 4 mM $CdCl_2$, pH 6.0), linear tetra-Ub (0.2 M sodium acetate trihydrate, 20% PEG 3350, pH 8.0), K6-linked di-Ub (0.2 M magnesium chloride hexahydrate, 20% PEG 3350, pH 5.9), K11-linked di-Ub (0.2 M Li_2SO_4, 0.1 M tris 8.5, 30% PEG 4000), K27-linked di-Ub (0.2 M magnesium acetate tetrahydrate, 0.1 M sodium cacodylate trihydrate, pH 6.5, 20% PEG 8000), K29-linked di-Ub (0.2 M potassium sulfate, 20% PEG3350), K33-linked di-Ub (0.2 M magnesium sulfate heptahydrate, 20% PEG 3350), K48-linked di-Ub (0.1 M sodium citrate tribasic dehydrate, pH 5.6, 20% 2-propanol, 20% PEG 4000), K63-linked di-Ub (0.1 M TRIS hydrochloride, pH 8.5, 2.0 M ammonium phosphate monobasic), and K11/K63-branched tri-Ub (0.2 M $MgSO_4$, 20% PEG 3350, 4 mM $CdCl_2$, pH 6.0). X-ray diffraction data were collected on the RIGAKU MICROMAX system and at the SSRF beamlines BL18U1 and BL19U1.

Structure Determination. Crystal structures were solved by the molecular replacement method using the program Phaser,[53] and the models were manually adjusted in coot[54] and refined in Phenix.[55] Full data collection and processing statistics are shown in Supplementary Table 1.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b09545.

Experimental details and compound characterizations (PDF)

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Yeates, T. O.; Kent, S. B. H. *Annu. Rev. Biophys.* **2012**, *41*, 41.
(2) Mackay, A. L. *Nature* **1989**, *342*, 133.
(3) Wukovitz, S. W.; Yeates, T. O. *Nat. Struct. Biol.* **1995**, *2*, 1062.
(4) Bunker, R. D.; Mandal, K.; Bashiri, G.; Chaston, J. J.; Pentelute, B. L.; Lott, J. S.; Kent, S. B. H.; Baker, E. N. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, 4310.
(5) Avital-Shmilovici, M.; Mandal, K.; Gates, Z. P.; Phillips, N. B.; Weiss, M. A.; Kent, S. B. H. *J. Am. Chem. Soc.* **2013**, *135*, 3173.
(6) Mandal, K.; Uppalapati, M.; Ault-Riche, D.; Kenney, J.; Lowitz, J.; Sidhu, S. S.; Kent, S. B. H. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 14779.
(7) Yeung, H.; Squire, C. J.; Yosaatmadja, Y.; Panjikar, S.; López, G.; Molina, A.; Baker, E. N.; Harris, P. W. R.; Brimble, M. A. *Angew. Chem., Int. Ed.* **2016**, *55*, 7930.
(8) Mandal, K.; Pentelute, B. L.; Tereshko, V.; Kossiakoff, A. A.; Kent, S. B. H. *J. Am. Chem. Soc.* **2009**, *131*, 1362.
(9) Mandal, P. K.; Collie, G. W.; Kauffmann, B.; Huc, I. *Angew. Chem., Int. Ed.* **2014**, *53*, 14424.
(10) Mandal, P. K.; Collie, G. W.; Srivastava, S. C.; Kauffmann, B.; Huc, I. *Nucleic Acids Res.* **2016**, *44*, 5936.
(11) Rypniewski, W.; Vallazza, M.; Perbandt, M.; Klussmann, S.; Delucas, L. J.; Betzel, C.; Erdmann, V. A. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2006**, *62*, 659.
(12) Kreitler, D. F.; Mortenson, D. E.; Forest, K. T.; Gellman, S. H. *J. Am. Chem. Soc.* **2016**, *138*, 6498.
(13) Pan, M.; Gao, S.; Zheng, Y.; Tan, X.; Lan, H.; Tan, X.; Sun, D.; Lu, L.; Wang, T.; Zheng, Q.; Huang, Y.; Wang, J.; Liu, L. *J. Am. Chem. Soc.* **2016**, *138*, 7429.
(14) Hayouka, Z.; Thomas, N. C.; Mortenson, D. E.; Satyshur, K. A.; Weisblum, B.; Forest, K. T.; Gellman, S. H. *J. Am. Chem. Soc.* **2015**, *137*, 11884.
(15) Pentelute, B. L.; Gates, Z. P.; Tereshko, V.; Dashnau, J. L.; Vanderkooi, J. M.; Kossiakoff, A. A.; Kent, S. B. H. *J. Am. Chem. Soc.* **2008**, *130*, 9695.
(16) Okamoto, R.; Mandal, K.; Sawaya, M. R.; Kajihara, Y.; Yeates, T. O.; Kent, S. B. H. *Angew. Chem., Int. Ed.* **2014**, *53*, 5194.
(17) Iwai, K.; Tokunaga, F. *EMBO Rep.* **2009**, *10*, 706.
(18) Shimizu, Y.; Taraborrelli, L.; Walczak, H. *Immunol. Rev.* **2015**, *266*, 190.
(19) Rohaim, A.; Kawasaki, M.; Kato, R.; Dikic, I.; Wakatsuki, S. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2012**, *68*, 102.
(20) Thach, T. T.; Shin, D.; Han, S.; Lee, S. *Acta Crystal. D* **2016**, *72*, 524.
(21) Komander, D.; Reyes-Turcu, F.; Licchesi, J.; Odenwaelder, P.; Wilkinson, K. D.; Barford, D. *EMBO Rep.* **2009**, *10*, 466.
(22) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776.
(23) Kent, S. B. H. *Chem. Soc. Rev.* **2009**, *38*, 338.
(24) Fang, G.; Li, Y.; Shen, F.; Huang, Y.; Li, J.; Lin, Y.; Cui, H.; Liu, L. *Angew. Chem., Int. Ed.* **2011**, *50*, 7645−7649.
(25) Zheng, J.; Tang, S.; Qi, Y.; Wang, Z.; Liu, L. *Nat. Protoc.* **2013**, *8*, 2483.
(26) Pedersen, S. L.; Tofteng, A. P.; Malik, L.; Jensen, K. J. *Chem. Soc. Rev.* **2012**, *41*, 1826.
(27) Collins, J. M.; Porter, K. A.; Singh, S. K.; Vanier, G. S. *Org. Lett.* **2014**, *16*, 940−943.
(28) Bondalapati, S.; Jbara, M.; Brik, A. *Nat. Chem.* **2016**, *8*, 407.
(29) Komander, D.; Rape, M. *Annu. Rev. Biochem.* **2012**, *81*, 203.
(30) Alfano, C.; Faggiano, S.; Pastore, A. *Trends Biochem. Sci.* **2016**, *41*, 371.
(31) Akutsu, M.; Dikic, I.; Bremm, A. *J. Cell Sci.* **2016**, *129*, 875.
(32) Kumar, K. S.; Spasser, L.; Erlich, L. A.; Bavikar, S. N.; Brik, A. *Angew. Chem., Int. Ed.* **2010**, *49*, 9126.
(33) El Oualid, F.; Merkx, R.; Ekkebus, R.; Hameed, D. S.; Smit, J. J.; de Jong, A.; Hilkmann, H.; Sixma, T. K.; Ovaa, H. *Angew. Chem., Int. Ed.* **2010**, *49*, 10149.
(34) Huang, Y. C.; Fang, G. M.; Liu, L. *Nat. Sci. Rev.* **2016**, *3*, 107.
(35) Huang, Y. C.; Liu, L. *Sci. China: Chem.* **2015**, *58*, 1779.
(36) Bondalapati, S.; Mansour, W.; Nakasone, M. A.; Maity, S. K.; Glickman, M. H.; Brik, A. *Chem. - Eur. J.* **2015**, *21*, 7360.
(37) Hemantha, H. P.; Bavikar, S. N.; Herman-bachinsky, Y.; Haj-Yahya, N.; Bondalapati, S.; Ciechanover, A.; Brik, A. *J. Am. Chem. Soc.* **2014**, *136*, 2665.
(38) Virdee, S.; Ye, Y.; Nguyen, D. P.; Komander, D.; Chin, J. W. *Nat. Chem. Biol.* **2010**, *6*, 750.
(39) Bremm, A.; Freund, S. M. V.; Komander, D. *Nat. Struct. Mol. Biol.* **2010**, *17*, 939.
(40) Matsumoto, M. L.; Wickliffe, K. E.; Dong, K. C.; Yu, C.; Bosanac, I.; Bustos, D.; Phu, L.; Kirkpatrick, D. S.; Hymowitz, S. G.; Rape, M.; Kelley, R. F.; Dixit, V. M. *Mol. Cell* **2010**, *39*, 477.
(41) Kristariyanto, Y. A.; Abdul Rehman, S. A.; Campbell, D. G.; Morrice, N. A.; Johnson, C.; Toth, R.; Kulathu, Y. *Mol. Cell* **2015**, *58*, 83.
(42) Michel, M. A.; Elliott, P. R.; Swatek, K. N.; Simicek, M.; Pruneda, J. N.; Wagstaff, J. L.; Freund, S. M. V.; Komander, D. *Mol. Cell* **2015**, *58*, 95.
(43) Hirano, T.; Serve, O.; Yagi-Utsumi, M.; Takemoto, E.; Hiromoto, T.; Satoh, T.; Mizushima, T.; Kato, K. *J. Biol. Chem.* **2011**, *286*, 37496.
(44) Lai, M. Y.; Zhang, D.; LaRonde-LeBlanc, N.; Fushman, D. *Biochim. Biophys. Acta, Mol. Cell Res.* **2012**, *1823*, 2046.
(45) Trempe, J.; Brown, N. R.; Noble, M. E. M.; Endicott, J. A. *Acta Crystallogr., Sect. F: Struct. Biol. Cryst. Commun.* **2010**, *66*, 994.
(46) Cook, W. J.; Jeffrey, L. C.; Carson, M.; Chen, Z.; Pickart, C. M. *J. Biol. Chem.* **1992**, *267*, 16467.
(47) Weeks, S. D.; Grasty, K. C.; Hernandez-Cuebas, L.; Loll, P. *Proteins: Struct., Funct., Genet.* **2009**, *77*, 753.
(48) Meyer, H. J.; Rape, M. *Cell* **2014**, *157*, 910.
(49) Boname, J. M.; Thomas, M.; Stagg, H. R.; Xu, P.; Peng, J.; Lehner, P. J. *Traffic* **2010**, *11*, 210.
(50) Dixon, E. K.; Castañeda, C. A.; Kashyap, T. R.; Wang, Y.; Fushman, D. *Bioorg. Med. Chem.* **2013**, *21*, 3421.
(51) Huang, Y.-C.; Li, Y.-M.; Chen, Y.; Pan, M.; Li, Y.-T.; Yu, L.; Guo, Q.-X.; Liu, L. *Angew. Chem., Int. Ed.* **2013**, *52*, 4858.
(52) Fang, G.-M.; Wang, J.-X.; Liu, L. *Angew. Chem., Int. Ed.* **2012**, *51*, 10347.
(53) McCoy, A. J.; Grosse-Kunstleve, R. W.; Adams, P. D.; Winn, M. D.; Storoni, L. C.; Read, R. J. *J. Appl. Crystallogr.* **2007**, *40*, 658.
(54) Emsley, P.; Lohkamp, B.; Scott, W. G.; Cowtan, K. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2010**, *66*, 486.
(55) Adams, P. D.; Afonine, P. V.; Bunkóczi, G.; Chen, V. B.; Davis, I. W.; Echols, N.; Headd, J. J.; Hung, L. W.; Kapral, G. J.; Grosse-Kunstleve, R. W.; McCoy, A. J.; Moriarty, N. W.; Oeffner, R.; Read, R. J.; Richardson, D. C.; Richardson, J. S.; Terwilliger, T. C.; Zwart, P. H. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2010**, *66*, 213.

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