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The Structure and Conformation of Lys63-Linked Tetraubiquitin

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Ubiquitination involves the covalent attachment of the ubiquitin (Ub) C-terminus to the lysine side chain of a substrate protein by an isopeptide bond. The modification can comprise a single Ub moiety or a chain of Ub molecules joined by isopeptide bonds between the C-terminus of one Ub with one of the seven lysine residues in the next Ub. Modification of substrate proteins with Lys63-linked poly-Ub plays a key nondegradative signaling role in many biological processes, including DNA repair and nuclear factor-κB activation, whereas substrates modified by Lys48-linked chains are targeted to the proteasome for degradation. The distinct signaling properties of alternatively linked Ub chains presumably stem from structural differences that can be distinguished by effector proteins. We have determined the crystal structure of Lys63 tetra-Ub at a resolution of 1.96 Å and performed small-angle X-ray scattering experiments and molecular dynamics simulations to probe the conformation of Lys63 tetra-Ub in solution. The chain adopts a highly extended conformation in the crystal, in contrast with the compact globular fold of Lys48 tetra-Ub. Small-angle X-ray scattering experiments show that the Lys63 tetra-Ub chain is dynamic in solution, adopting an ensemble of conformations that are more compact than the extended form in the crystal. The results of these studies provide a basis for understanding the differences in the behavior and recognition of Lys63 poly-Ub chains.

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Protein modification by covalent conjugation of one or more ubiquitin (Ub) molecules, referred to as ubiquitination (or ubiquitylation), has been shown to play important regulatory roles in many cellular processes, including protein degradation, transcription, cell-cycle progression, viral infection, and the immune response.[1,2] This conjugation involves a cascade of enzymatic steps that results in the covalent attachment of the C-terminal glycine residue of Ub to the ε-NH_2 of a lysine side chain in a substrate protein.[3] Ub itself can also be ubiquitinated at any of its seven lysine residues[4] or at its amino-terminus,[5] giving rise to different poly-Ub chains.

Poly-Ub chains of different linkage types, as distinguished by the lysine residue to which each successive Ub C-terminus is attached, have distinct biological functions. The best understood among these are the Lys48-linked Ub chains, which target proteins for degradation by the 26S proteasome.[6] Lys63-linked Ub chains, on the other hand, play nondegradative roles in different signaling pathways,[7] notably nuclear factor-κB transcription activation[8] and the DNA damage response.[9,10] Other types of chain linkages have also been identified in cells,[11] including branched and mixed-linkage chains.[12,13] Although the cellular roles of these "atypical" Ub chains are yet to be studied in detail, they have been implicated in a few biological processes.[14] For example, Lys33/Lys29 mixed chains have been shown to regulate AMPK-related kinase activation, while Lys29-linked poly-Ubs have been shown to play a role in lysosomal degradation of proteins.[15,16]

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Abbreviations used: Ub, ubiquitin; SAXS, small-angle X-ray scattering; MD, molecular dynamics; UBL, Ub-like.

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The distinct roles played by different poly-Ub chains indicate that effector molecules can distinguish among chains of differing linkage types. For example, the NZF domain of TAB2 binds specifically to Lys63-linked chains,[17] the ataxin-3 UIM repeat preferentially binds to Lys48-linked chains,[18] and the UBAN motif of NEMO recognizes linear head-to-tail Ub chains.[19] A thorough understanding of the molecular details of Ub-mediated signaling therefore requires elucidation of the structures of different poly-Ub chains and their complexes with cognate partners.

The crystal structure of Lys48-linked tetra-Ub determined at neutral pH showed that it adopts a compact globular conformation.[20] This conformation could not be adopted by Lys63-linked tetra-Ub as judged by the relation between the Lys63 side chains and the Ub C-termini. Indeed, NMR studies[21,22] and an X-ray crystal structure[17] of Lys63-linked di-Ub show an open structure with limited contacts between Ub monomers, as compared with the closed conformation of Lys48-linked di-Ub.

In order to gain insight into the structure and topology adopted by longer Lys63-linked poly-Ub chains, we determined the 1.96 Å resolution crystal structure of Lys63-linked tetra-Ub. The crystals contain one tetra-Ub chain per asymmetric unit and show a highly extended linear arrangement of Ub monomers. The extended arrangement showed no intersubunit interaction, in agreement with NMR results. The extended conformation of the Lys63 tetra-Ub in the crystal suggests that the chain would likely adopt other conformations in solution. To further explore this conformational flexibility of the Lys63-linked tetra-Ub chains in solution, we carried out small-angle X-ray scattering (SAXS) measurements of Lys63 tetra-Ub and used molecular dynamics (MD) simulations to deduce the ensemble of structures adopted by this tetra-Ub in solution. We found that Lys63 tetra-Ub adopts conformations in solution that are more compact than the extended form seen in the crystal yet do not exhibit any specific intersubunit interactions. These studies provide a detailed understanding of the structure and dynamics of Lys63 tetra-Ub and of how these chains could be recognized in the cell.

Experimental details

Lys63-linked tetra-Ub chain was synthesized using two Ub mutants (K48R–K63R and Ub-D77) following Pickart and Raasi.[23] All the enzymes required for this synthesis, viz., E1 (human), Ubc13/MMS2 (yeast), and Yuh1 (yeast), were also expressed and purified as recombinant proteins in *Escherichia coli*. After the chain of required length was synthesized, it was subjected to a final step of purification using size-exclusion chromatography in Superdex 75 prep-grade 26/60 column. The protein was then dialyzed in 10 mM Tris–HCl buffer, pH 7.6, containing 25 mM NaCl overnight at 4 °C. It was then concentrated using Vivaspin concentrators and stored at −80 °C in small aliquots.

Crystals of Lys63 tetra-Ub screen were initially obtained at 20 °C in hanging-drop setups from sparse matrix screening with buffer 1 from Wizard II (Emerald Biosciences). The condition was then optimized to get fewer and better crystals. The best crystals were obtained using 4%–6% PEG (polyethylene glycol) 3000, 100 mM Na-acetate, pH 4.6–5.0, and 100 μM Zn acetate. However, all the crystals were found to diffract poorly (best, 3.5 Å) and showed very high mosaicity levels and *B*-factor values after data processing. The diffraction improved substantially upon dehydration of the crystals by stepwise increase of PEG 3000 to 25% over a period of 4–5 days, and a 1.96 Å data set was collected from frozen crystals at 100 K at Advanced Photon Source (Argonne, IL) at the fixed-wavelength beamline 14-BMC.

The data were processed in space group $P2_13$ using the program HKL2000.[24] The structure was solved by molecular replacement using the program MOLREP[25] from CCP4 Suite.[26] The initial model was subjected to iterative building and refinement cycles using COOT[27] and REFMAC5.[28] All the side chains were built first, followed by the completion of the chain. Isopeptide bonds were created toward the end with addition of water molecules.

SAXS data were collected at the SIBYLS beamline (beamline 12.3.1) at the Advanced Light Source of the Lawrence Berkeley National Laboratory. Data were collected from the sample at concentrations of 3, 6, and 9 mg/ml, and no concentration dependence was observed. The sample buffer contained 10 mM Tris–HCl, pH 7.6, 50 mM NaCl, and 0.5 mM ethylenediaminetetraacetic acid at 10 °C. Two exposures (at 5 s and 0.5 s) were merged to create a final scattering profile. $P(r)$ functions were determined from the scattering profiles using GNOM.[29] Scattering profiles were calculated from crystal structures using CRYSOL.[30] The MES (Minimal Ensemble Search)

Table 1. Data collection and refinement statistics of K63 Ub_4 crystals

Data collection statistics	
Space group	$P2_13$
Cell dimensions	$a=b=c=106.95$ Å
Resolution (Å)	50–1.96 (2.01–1.96)
R_{merge} (%)	8.1 (90)
Completeness (%)	99.6 (100)
Redundancy	42.9 (43.1)
$I/\sigma(I)$	54 (6)
Refinement	
R/R_{free} (%)	19/24
No. of atoms	
Protein	2538
Zn^{2+}	6
Water	209
r.m.s.d. values	
Bond (Å)	0.023
Angles (°)	1.885

Values in the parentheses denote the highest-resolution shell.

program was run as described previously,[31] with three independent runs per radius of gyration ranging from 20 to 60 Å at 1 Å steps.

Crystal structure of Lys63 tetra-Ub

Crystals of Lys63 tetra-Ub formed in space group $P2_13$ and contain one chain per asymmetric unit. Crystals obtained after initial rounds of optimization typically diffracted to 3.5–4.0 Å resolution. However, the diffraction improved significantly upon gradual dehydration of the crystals with stepwise increases of the precipitant concentration, yielding crystals diffracting to 1.96 Å resolution. The structure was solved by molecular replacement using the structure of monomeric Ub[32] as the search model. Data collection and refinement statistics are presented in Table 1.

The crystal structure shows a linear arrangement of the Lys63-linked Ub monomers (Fig. 1a and b) that is starkly different from the compact globular fold adopted by Lys48-linked tetra-Ub (Fig. 1c). Instead, the monomers in the Lys63-linked chain are joined by well-ordered linkages but do not contact one another. The absence of interactions between the individual Ub monomers of the Lys63 tetra-Ub agrees with the previously reported NMR results, where no stable intersubunit interaction could be observed in Lys63-linked di- and tetra-Ub chains,[21,22] and with the extended structure of crystallized di-Ub.[17] The extended conformation of the tetra-Ub chain and the absence of intra-chain contacts that could stabilize the observed conformation suggested that Ly63-linked tetra-Ub in solution would likely be flexible and adopt other conformations in addition to that seen in the crystal.

We also note that the globular regions of each of the four Ub monomers in the Lys63 tetra-Ub chain alternate between two conformations that differ in the loop region comprising residues 6 through 12. Inclusion of this region during superposition of the globular region of the Ub monomers (residues 1 through 70) increases the r.m.s.d. from ~0.4 Å to ~0.85 Å. The reason for this difference remains unclear and could not be attributed to the differences in crystal packing environments of each monomer.

Recognition surface of Lys63-linked Ub

Ub has a conserved hydrophobic patch comprising residues Leu8, Ile44, and Val70 that has been shown to be important for function.[33] These residues mediate the interaction of Ub with E2 enzymes[34] and with Ub binding domains (reviewed by Hicke *et al.*[35]). The importance of these hydrophobic residues in the Lys48-linked Ub chain

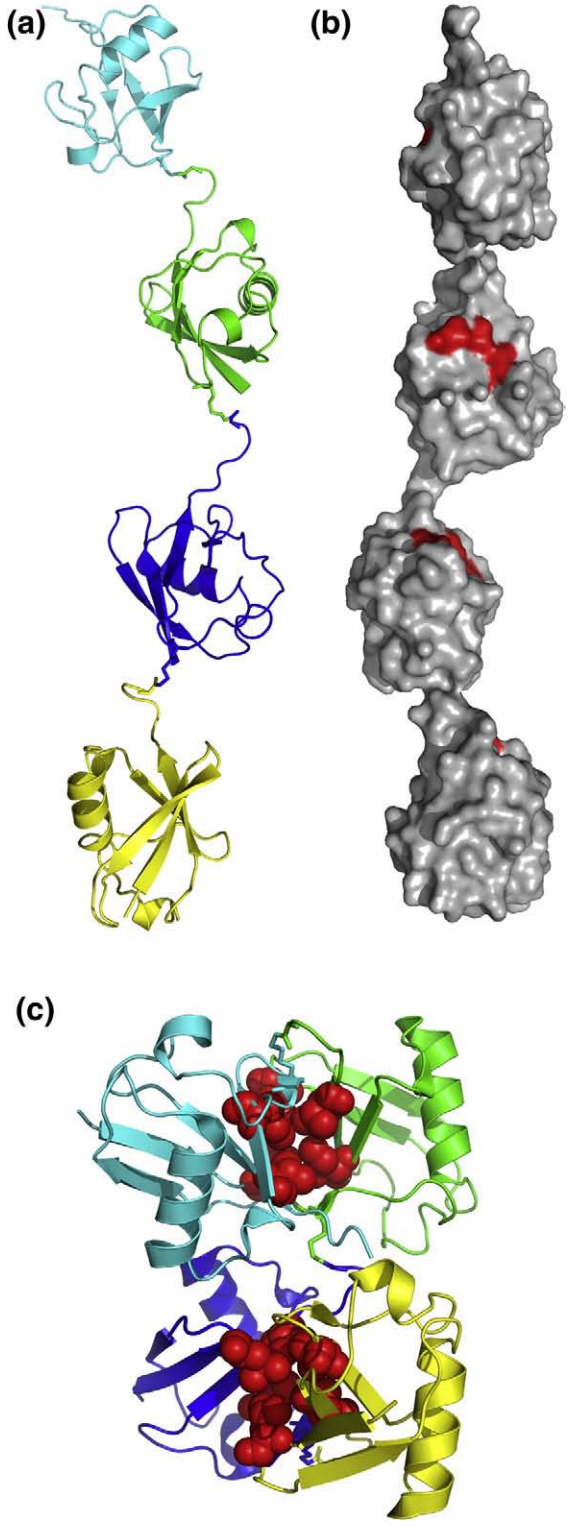


Fig. 1. Crystal structure of Lys63-linked tetra-Ub. (a) Ribbon diagram and (b) surface representation of Lys63 tetra-Ub crystal structure showing the highly extended chain conformation. The proximal Ub is shown in cyan, while the distal one is shown in yellow. In (b), the hydrophobic patches of individual Ub monomers consisting of residues Leu8, Ile44, and Val70, which are important for interacting with cognate proteins, are shown in red. (c) Crystal structure of Lys48 tetra-Ub (Protein Data Bank ID 2OOV).[20] The structure of Lys48 is highly compact and globular. The hydrophobic patches from Ub monomer can be seen to interact with one another, partially burying the interaction surface. The coloring scheme has been kept identical with that for Lys63 tetra-Ub.

recognition has been studied in detail.[36–38] In addition, the hydrophobic patch has also been implicated in Lys63-linked chain interactions[39] and is shown to participate in the linear di-Ub recognition by NEMO.[40] In contrast with Lys48 tetra-Ub, in which the hydrophobic patches from two adjacent monomers coalesce to form a single surface (Fig. 1c), the hydrophobic patches in the crystal structure of Lys63 tetra-Ub are exposed in all four Ub monomers (Fig. 1b). We also note that the hydrophobic patches of Lys63 monomers do not lie on the same surface of the molecule but are rotated roughly 90° relative to one another in successive monomers. This particular arrangement is probably favored in the crystal, where the molecules are conformationally "locked" in one particular orientation due to crystal packing. However, we note that one of the solution structures presented by Varadan *et al.*[22] also shows this orthogonal positioning of the Ub hydrophobic surface in successive monomers along with alternate arrangements. Given the presumed flexibility in the linkages that join the Ub moieties in the Lys63 chain, the individual monomers would probably be free to undergo rotational movements that would change the relative orientation of these patches.

Despite the strong tendency of Ub to associate via the hydrophobic patches, we were surprised to find that these patches do not take part in crystal packing of the Lys63 tetra-Ub chains. Instead, the crystal contacts are formed by the interaction of a Ub monomer in one chain with the isopeptide linkage of another chain in the crystal (Fig. 2). This crystal packing interaction could potentially reflect a mode of interaction between poly-Ub chains or Ub-like (UBL) domains and Lys63-linked chains. Several proteins, including IKKα and IKKβ, have been found to contain UBL domains. These UBL domains could possibly interact with Lys63 chains in a manner similar to that seen in the crystal. However, to our knowledge, no such interaction has been reported to exist in the literature.

SAXS studies of Lys63 tetra-Ub in solution

Since the extended conformation of the Lys63 tetra-Ub is clearly influenced by lattice contacts, we used SAXS combined with MD simulations to explore the structure of this tetra-Ub in solution. SAXS is a powerful tool to study the conformation of macromolecules in solution, particularly when a high-resolution crystal structure is available.[41] We measured the X-ray scattering profile of Lys63 tetra-Ub in solution (Fig. 3a) and derived the pair distribution function, $P(r)$, from the scattering profile to determine the distribution of interatomic vectors in the tetra-Ub chain (Fig. 3b). As shown in Fig. 3a, the measured X-ray scattering profile does not fit the theoretically calculated profiles from the crystal structures of either Lys63 tetra-Ub (this study) or Lys48 tetra-Ub.[20] This indicates that Lys63 tetra-Ub adopts a distinct conformation in solution that is more compact than that seen in the crystal.

The interatomic vectors calculated in the pair distribution function (Fig. 3b) show that the maximum dimension (D_{max}) is 118±2 Å and the radius of gyration (R_g) is 32.3 Å in Lys63 tetra-Ub in solution, which are considerably smaller than the D_{max} (154 Å) and R_g (44 Å) values calculated from the crystal structure. These apparent reductions of D_{max} and radius of gyration indicate that Lys63-linked tetra-Ub is more compact in solution than it is in the crystal. At the same time, the peaks in the experimental $P(r)$ also show that Lys63 tetra-Ub adopts a much more extended structure than the crystal structure of Lys48 tetra-Ub (Fig. 3a and b). Interestingly, the second maximum in $P(r)$ of ~41 Å

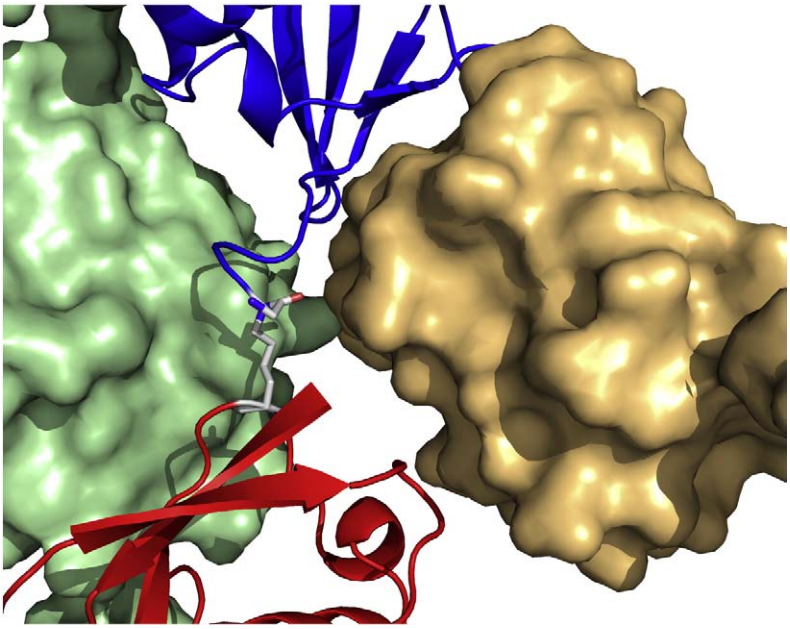


Fig. 2. Crystal packing interactions of Lys63-linked tetra-Ub showing two Ub monomers from two chains (surface representation in green and yellow) interacting at the isopeptide linkage of a third chain (cartoon representation). The isopeptide bonds between Lys63 of proximal Ub (in red) and Gly76 of distal Ub (in blue) are represented with sticks.

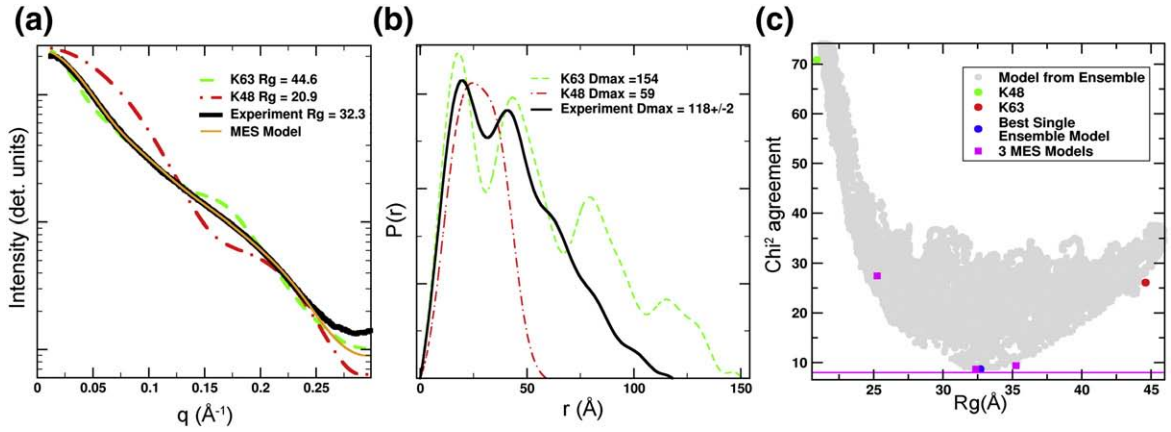


Fig. 3. SAXS of Lys63 tetra-Ub in solution. (a) A comparison of the experimental scattering profile (black) with theoretically calculated profiles from the crystal structures of Lys63 tetra-Ub (green), Lys48 tetra-Ub (red), and the minimal ensemble (orange). The minimal ensemble (orange) of Lys63 tetra-Ub was identified using the MES program, which best fits the experimental data (black). (b) The experimental electron pair distribution function [$P(r)$] (in black). The curve reveals a long maximum dimension with smeared third and fourth Ub–Ub peaks, suggesting an ensemble of distances on this length scale. In contrast, the theoretically calculated $P(r)$ for Lys48 tetramer (red) shows one broad peak, indicating that all Ub–Ub distances are similar, while the $P(r)$ calculated Lys63 structure (in green) shows four major peaks that correspond to all major Ub–Ub distances. (c) Searching for the minimal ensemble using the MES program.[31] The radius of gyration (R_g) of each model is plotted against the χ^2 agreement to the experimental data. All the conformations (13,200) from which MES models were extracted are shown as gray circles. A single best model was identified (cyan), while the three conformations making up the MES model (orange squares) improve the χ^2 agreement by 11% (orange line *versus* minimum of envelope formed by single conformations). The parameters for the two crystal structures are also shown (green and red circles).

corresponding to the average distance between any two consecutive Ub monomers within the Lys63 poly-Ub chain closely matches the calculated value from the crystal structure (Fig. 3b). However, the third maximum, which corresponds to the distance between first and third Ub monomers or that between the second and fourth monomers, is considerably shorter in the observed profile (63 Å) as compared with the one calculated from the crystal structure. The combination of a large D_{max} but minor third and fourth maxima suggested that the Lys63 Ub chains most likely exist as an ensemble of conformations at larger-length scales.

Further analysis of the solution conformation of Lys63 tetra-Ub was done using the program MES.[31] This program finely samples the conformational space of the macromolecule using MD simulations at high temperatures, where only covalent bonds and van der Waals interactions are taken into consideration. A genetic algorithm then identifies the minimal ensemble from all these conformations that best fits the data. The result shows that no single conformation from the MD simulations adequately explains the data. Instead, an optimal fit could be obtained with an ensemble of a minimum of three conformations (Figs. 3c and 4). Inclusion of more conformations in the ensemble did not lead to any significant improvement in the fit, as measured by χ^2 values (data not shown). The first two dominant conformations (represented by 69% and 17% in the ensemble) (Fig. 4a and b) preserve the overall linearity observed in the crystal structure but are notably more compact than the conformation in the crystal. The ensemble also contains a minor population (13% in the ensemble) (Fig. 4c) that is highly compact as compared with the crystal structure.

Although the resolution of SAXS data cannot be used to determine the intersubunit orientations accurately, we note that, in all of these MES conformations, the conserved hydrophobic interaction surfaces (colored red) of the Ub monomers do not interact with one another and are either completely or partially exposed. This probably suggests that Lys63 chains are likely to be found in solution conformations that leave the hydrophobic patches exposed and would therefore be readily accessible for interaction with cognate proteins.

In summary, our results show that Lys63-linked poly-Ub forms a relatively open conformation, in agreement with the existing biochemical and biophysical data. The elongated structures of the Lys63 tetra-Ub, stabilized by a crisscrossed arrangement of chains within the crystal lattice, do not belong to any closed symmetry group. Thus, addition of more Ub monomers to either the proximal end or the distal end of the chain can occur without requiring any rearrangement of the structure. Although the highly extended crystal structure of Lys63 tetra-Ub agrees with predicted models based on the di-Ub structure,[17] the longer chain is notably more collapsed in solution. Nevertheless, none of the representative solution conformations or the crystal structure buries the conserved hydrophobic patch of any of the Ub monomers. This indicates that these patches remain available for interactions with other proteins.

The requirement of an ensemble instead of a single conformation to explain the SAXS data points toward the fact that Lys63 Ub chains with four or

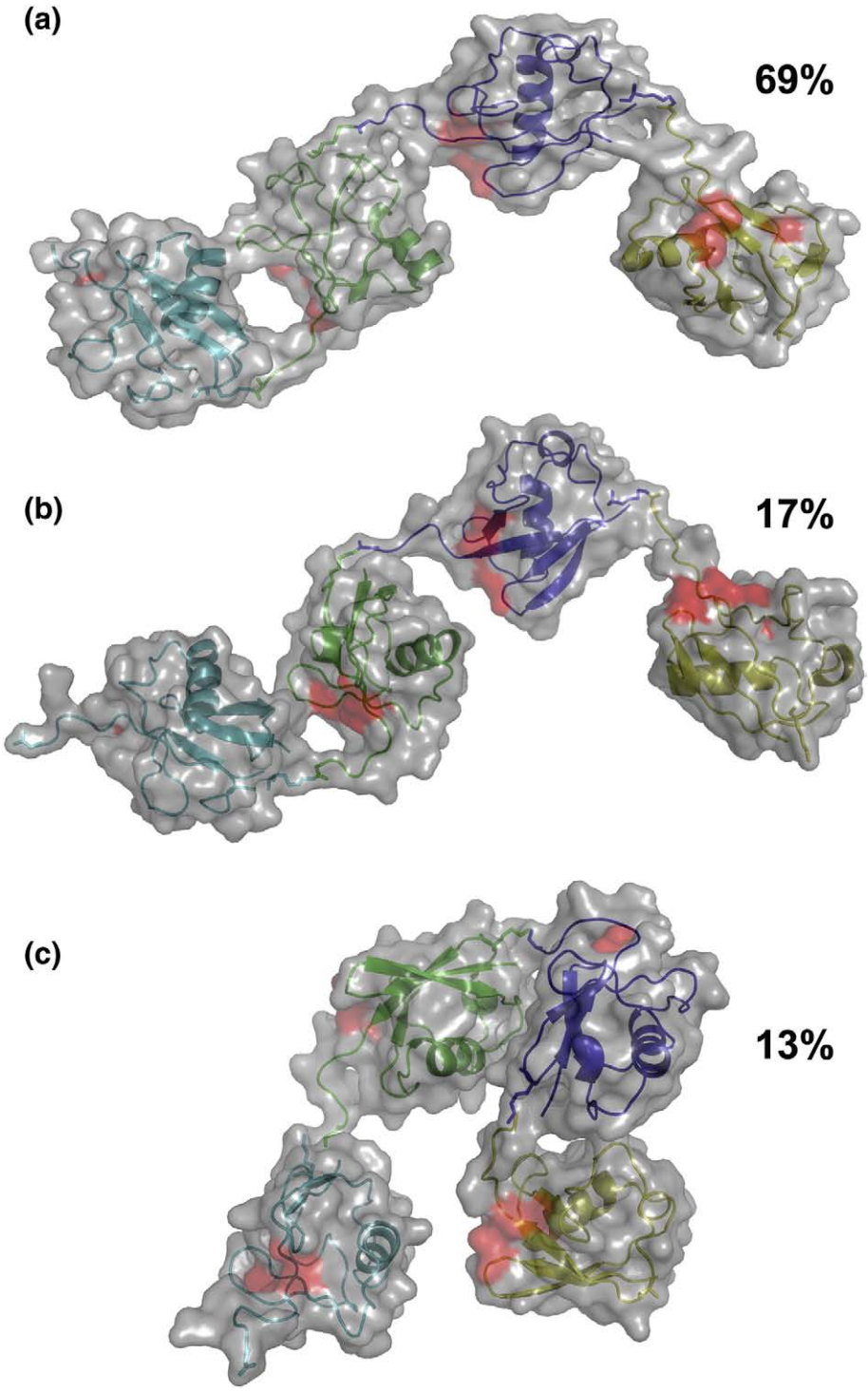


Fig. 4. Ensemble conformations of Lys63 tetra-Ub in solution obtained with MES. Three conformations of Lys63 tetra-Ub (a–c) that make up the MES model are shown along with their respective percentages. Three hydrophobic residues (L8, I44, and V70) on the surface of the Ubs, those that are responsible for their interactions with cognate proteins, are depicted in red.

more monomers do not form a single well-defined structure in solution. Instead, tetra-Ub and presumably longer Lys63 chains exist in a dynamic equilibrium of many conformations. Upon binding to a cognate protein, these chains would probably adopt one specific topology depending on the binding partner. This fluidity in the structure of Lys63 poly-Ub may be important for its biology, where these chains are recognized by proteins with potentially different structures in different signaling pathways. Moreover, the conformational space sampled by the free Lys63 poly-Ub can be restricted upon attachment to substrate proteins, each of which could have a unique influence on the Lys63 poly-Ub chain

behavior. All these considered together point toward a highly plastic nature of Lys63-linked poly-Ub-mediated signaling mechanisms, which could account for the diverse roles played by this type of poly-Ub modification in multiple signaling pathways.

Accession number

Coordinates and structure factors have been deposited in the Protein Data Bank with accession number 3HM3.

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