Effects of cyclization on conformational dynamics and binding properties of Lys48-linked di-ubiquitin

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Abstract

In solution, Lys48-linked di-ubiquitin exists in dynamic equilibrium between closed and open conformations. To understand the effect of interdomain motion in polyubiquitin chains on their ability to bind ligands, we cyclized di-ubiquitin by cross-linking the free C terminus of the proximal ubiquitin with the side chain of residue 48 in the distal ubiquitin, using a chemical cross-linker, 1,6-Hexane-bis-vinylsulfone. Our NMR studies confirm that the cyclization affects conformational dynamics in di-ubiquitin by restricting opening of the interface and shifting the conformational equilibrium toward closed conformations. The cyclization, however, did not rigidly lock di-ubiquitin in a single closed conformation: The chain undergoes slow exchange between at least two closed conformations, characterized by interdomain contacts involving the same hydrophobic patch residues (Leu8-Ile44-Val70) as in the uncyclized di-ubiquitin. Lowering the pH changes the relative populations of these conformations, but in contrast with the uncyclized di-ubiquitin, does not lead to opening of the interface. This restriction of domain motions inhibits direct access of protein molecules to the hydrophobic patch residues located at the very center of the interdomain interface in di-ubiquitin, although the residual motions are sufficient to allow access of small molecules to the interface. This renders di-ubiquitin unable to bind protein molecules (e.g., UBA2 domain) in the normal manner, and thus could interfere with Ub₂ recognition by various downstream effectors. These results emphasize the importance of the opening/closing domain motions for the recognition and function of di-ubiquitin and possibly longer polyubiquitin chains.

Keywords: di-ubiquitin; cyclization; Lys48-linked ubiquitin chain; interdomain dynamics; UBA domain

Supplemental material: see www.proteinscience.org

Ubiquitin (Ub) is a small, 76 amino acid polypeptide, present universally in eukaryotes (Redman and Rechsteiner 1988). Covalent attachment of ubiquitin to substrate proteins mediates a wide range of diverse cellular processes, including the proteasomal degradation of proteins (Gregori et al. 1990; Johnson et al. 1995),

DNA repair in the RAD6 pathway (Spence et al. 1995), kinase activation (Deng et al. 2000), endocytosis (Hicke and Dunn 2003), and translational regulation (Spence et al. 2000). Defects in Ub-mediated signaling pathways have been linked to neurodegenerative diseases (including Parkinson's disease), several forms of malignancy, the pathogenesis of several genetic diseases (including cystic fibrosis, Angelman's syndrome, and Liddle syndrome), and the pathology of muscle wasting (Schwartz and Ciechanover 1999). Ubiquitin often carries out its signaling functions in the form of polyubiquitin (polyUb) chains that are formed by an isopeptide bond between the C-terminal Gly-76 of one Ub and one of seven Lysines of the previous Ub unit in the chain. In the

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current model of Ub-mediated signaling, the fate of a polyubiquitinated protein depends on the length of the polyUb tag and on its conformation, determined by the particular isopeptide linkage between Ub units (Varadan et al. 2004).

Lys48-linked polyUb chains are the principal signal for targeting proteins to the proteasomal machinery for degradation (Chau et al. 1989; Finley et al. 1994). Lys48-linked Ub₄ chains (the minimal length required for proteolytic signal) (Thrower et al. 2000) have been crystallized in two different conformations, suggesting that the chains are inherently flexible and could adopt a range of conformations in solution. Although the chains are overall compact (Tenno et al. 2004), the relatively weak noncovalent interactions between the Ub units and the flexibility of the Ub–Ub linker (Varadan et al. 2002; Fushman et al. 2004), could allow the chains to adopt conformations with different patterns of Ub/Ub contacts. NMR data indicate that Ub/Ub contacts in Ub₂ and Ub₄ involve a specific Ub epitope comprising surface hydrophobic residues Leu8, Ile44, and Val70 (Varadan et al. 2002), and Ala replacements of these residues weaken the Ub/Ub contacts (Varadan et al. 2005). Consistent with the idea of the flexible nature of the chains, solution NMR studies of Lys48-linked Ub₂ (Lam et al. 1997; Varadan et al. 2002; Ryabov and Fushman 2006) have shown that this molecule exists in a dynamic equilibrium between at least two conformations: a “closed” conformation characterized by a well-defined interface between the two Ub units stabilized by van der Waals contacts between the Leu8-Ile44-Val70 hydrophobic patches, and one or more “open” conformations, with no definitive interface. The equilibrium populations of these states depend on pH, with >85% of the Ub₂ molecules adopting a closed conformation at pH 6.8 (Varadan et al. 2002; Ryabov and Fushman 2006). The likely reason for this pH dependence is the protonation of His68 (pK_a = 5.5) (Fujiwara et al. 2004), located at the Ub/Ub interface in the closed conformation of Ub₂ (Cook et al. 1992; van Dijk et al. 2005). The electrostatic repulsion between the charged histidines on the two Ub units will make this conformation energetically unfavorable at lower pH.

The Leu8-Ile44-Val70 hydrophobic patch on Ub is not only directly involved in Ub/Ub interaction stabilizing the “closed” conformation in Lys48-linked Ub₂ (Cook et al. 1992; Varadan et al. 2002; van Dijk et al. 2005), it is also the site through which monoUb and Ub₂ bind various ligands, including the CUE and UBA domains, Vps27 and S5a UIMs, and ubistatin (Walters et al. 2002; Prag et al. 2003; Ryu et al. 2003; Swanson et al. 2003; Mueller et al. 2004; Verma et al. 2004; Raasi et al. 2005; Varadan et al. 2005; Wang et al. 2005). Furthermore, Lys63-linked Ub₂, which has no definitive Ub/Ub interface, binds one hHR23A UBA2 domain on each of the two Ub units as if they were two Ub monomers acting independently from

each other (Varadan et al. 2004). In contrast, UBA2 binding to Lys48-linked Ub₂ occurs at the Ub/Ub interface, i.e., involves interface opening and wrapping of both Ub units around a single UBA2 domain (Varadan et al. 2005). A similar mode of binding was proposed for Ub₂ interaction with the Mud1 UBA domain (Trempe et al. 2005).

This leads to the model in which the binding of certain substrates to Lys48-linked Ub₂ is preceded by and depends upon an opening of the Ub₂ interface, allowing the buried hydrophobic patch residues to be exposed and recognized. Therefore, we hypothesized that hindering the opening-closing dynamics of Ub₂ would alter ligand binding properties of this chain. Using two ubiquitin mutants, K48C and G76C, we synthesized a cyclized Ub₂ construct (cyc–Ub₂), which contains the “native” isopeptide bond between Gly76 of the distal Ub and Lys48 of the proximal Ub, and a reciprocal Cys76(proximal)–Cys48(distal) linkage, via a cross-linker, that thus cyclized the chain (see Fig. 1). Given the close location

Figure 1. Schematics of the cyclization reaction (A). Also shown are (B) the three-dimensional structure of Ub₂ (PDB code 1AAR) (Cook et al. 1992), with the site of the cyclization indicated, and (C) the chemical structure of the cross-linker HBVS. The distal and proximal Ub units are labeled.

of residue 48 of the distal Ub to the free and flexible C terminus of the proximal Ub (Fig. 1B), this modification is not expected to alter significantly the closed conformation of Ub₂. Our results presented below show that the cyclization restricted the opening and closing events of Ub₂ and resulted in decreased solvent accessibility to regions at the Ub/Ub interface. This interferes with the strength of binding of small molecules by Ub₂, and affects the mode and strength of binding of larger molecules, like UBA domains. These findings provide direct experimental evidence that opening of the Ub/Ub interface is required for Lys48-linked Ub₂ to bind hHR23A UBA2 domain in a high-affinity Ub–UBA–Ub sandwich-like complex observed in Varadan et al. (2005).

Results and Discussion

The Lys48-linked Ub₂ chain, Ub(K48C)–Ub(G76C), was cross-linked at Cys residues using HBVS as described in Materials and Methods and schematically shown in Figure 1. The presence of cross-linkage and its exact location were verified by several experiments, described below.

Verification of Ub₂ cyclization

Gel mobility shift assays

In order to confirm that cross-linking occurred, we assayed gel mobility shifts in Ub₂ after the cyclization reaction. Figure 2 shows different migration patterns for monoUb, Ub₂ and cyc-Ub₂ on a native polyacrylamide gel, confirming that HBVS did modify Ub₂. The NMR data presented below provide clear evidence that this

Figure 2. Experimental verification of Ub₂ cyclization. (A) Native gel showing the mobility of the monomer, regular Ub₂, and cyc-Ub₂ (first six lanes). (B) Native gel for IsoT cleavage: Ub₂ (Ub[K48R]–Ub[D77]) before and after addition of IsoT, cyc-Ub₂ (Ub[K48C]–Ub[G76C]+HBVS) before and after addition of IsoT. Ub₂ is completely cleaved into monomer by IsoT, while cyc-Ub₂ is not. A trace of monoUb seen in the rightmost lane is likely due to a minor fraction of the sample that was not cyclized. These assays used nondenaturing, nonreducing polyacrylamide gels, loaded with ~1–4 nm of the protein. IsoT cleavage of the uncyclized Ub₂ constructs, Ub(K48R)–Ub(D77) and Ub(K48C)–Ub(G76C) is shown in Supplemental Figure 1.

modification resulted in the cyclization of Ub₂. To rule out the possibility of intermolecular cross-linking (which could result in Ub₄ and longer chains), the cyc-Ub₂ and Ub₂ samples were compared using SDS-PAGE (not shown), in which they both migrated like Ub₂.

Isopeptidase-T cleavage

Isopeptidases are members of a large family of C-terminal ubiquitin hydrolases. One particular isopeptidase, Isopeptidase T (IsoT), recognizes the C terminus of the proximal Ub in a Lys48-linked polyUb chain and trims the proximal Ub from the chain (Mayer and Wilkinson 1989; Stein et al. 1995; Wilkinson et al. 1995; Reyes-Turcu et al. 2006). Cyclization of Ub₂ was further verified by using IsoT to cleave the Gly76–Lys48 isopeptide bond between the Ub units in Ub₂ and checking if the two are still linked. The uncyclized Ub₂ migrated on a polyacrylamide gel as a Ub-dimer before the addition of IsoT, and as a monomeric Ub after the reaction with IsoT, indicating cleavage of the isopeptide bond (Fig. 2B; see also Suppl. Fig. 1). However, IsoT had negligible effect on cyc-Ub₂ as very little monoUb was detected on the gel, indicating that the two Ub units still remained connected. In addition, the cyc-Ub₂ band appeared to have the same exact mobility before and after the incubation with IsoT. To exclude the possibility that IsoT cleaves the isopeptide linkage in cyc-Ub₂ and the resulting Ub–HBVS–Ub product migrates similarly to the cyc-Ub₂, we synthesized a Ub–HBVS–Ub construct, by cross-linking two monoUb molecules, Ub(K48C) and Ub(G76C) using HBVS, and tested its mobility on a native gel. As evident from Supplemental Figure 2, this (uncyclized) Ub–HBVS–Ub construct migrated on a gel even slower than the uncyclized Ub₂, thus suggesting that HBVS does crosslink uncyclized Ub₂, and that the resulting cyc-Ub₂ is resistant to cleavage by IsoT.

As shown previously (Wilkinson et al. 1995), efficient IsoT cleavage requires a free C-terminal carboxyl group on the proximal end of the chain; moreover, the cleavage rates are reduced when the C-terminal Arg-Gly-Gly motif is modified. Interestingly, our data (Fig. 2B; Suppl. Fig. 1) show that neither the replacement of Gly76 with a cysteine nor the Asp77 extension of the C terminus hinders IsoT cleavage of the uncyclized Ub₂. Therefore, the negligible effect of IsoT on the cross-linked Ub₂ provides further evidence that the C terminus of this Ub₂ chain is modified by HBVS. Note that a modification of the side chain of C-terminal Cys on the proximal unit in Ub₂, Ub(K48R)–Ub(G76C), with an alternative maleimide crosslinker, 1,4-Bis-maleimidobutane, significantly hindered the cleavage reaction (Suppl. Fig. 3). Thus, there is still a possibility that hindered IsoT cleavage of cyc-Ub₂ could be due to HBVS modification of the C-terminal Cys in Ub(K48C)–Ub(G76C) rather than cyclization. However, combined

with all other data presented above and the NMR data (below) clearly showing the inability of cyc-Ub₂ to adopt an open conformation at acidic pH, this indicates that the cross-linked Ub₂ is, in fact, cyclized.

Chemical shift perturbations induced by cross-linking

The effects of cyclization were also monitored by NMR. Figure 3 shows chemical shift perturbations (CSPs) observed in backbone amides in the Ub(K48C)–Ub(G76C) construct (¹⁵N-labeled on the distal K48C Ub) at pH6.8 as a result of cyclization with HBVS. Significant CSPs are seen around Cys48 where the chemical modification occurred. In addition, CSPs were observed in several amides located at the Ub₂ interface and in the “native” (isopeptide) Ub–Ub linker. In particular, Leu8, His68, Val70, Leu71, Leu73, and Gly76 showed significant perturbations upon cyclization. These perturbations reflect altered interactions at the Ub/Ub interface as a result of the cyclization, which holds Ub₂ in a more closed conformation (see below).

Several amides showed doubling of resonances in NMR spectra after cyclization. These include residues Val5, Lys6,

Thr7, Leu8, Thr9, Gly10, Lys11, Leu13, Leu15, Asp32, Lys33, Glu34, Gln40, Gln41, Ile44, Asp52, Leu67, His68, and Gly75. In these cases, neither of the two peaks corresponded to mono-Ub or uncyclized Ub₂, thus eliminating the possibility of the presence of contaminating mono-Ub or uncyclized Ub₂ in the sample. (Note that no mono-Ub was detected in the cyclized Ub₂ sample by SDS-PAGE.) Therefore, it is likely that these duplicate resonances are indicative of a slow exchange between at least two conformations of cyc-Ub₂. Most of the residues having duplicate peaks are either involved in the Ub/Ub interface or located close to it or to the HBVS attachment site, with the exception of Gly75, adjacent to the native isopeptide Ub–Ub linker, and residues 32–34 in the C-terminal turn in the α -helix. These latter sites face the β 2-strand (Lys11, Leu13, Leu15); thus, the duplicate peaks could reflect some rearrangement between the α -helix and this strand. Several signals are significantly broadened, potentially due to an intermediate exchange, which also suggests that cyc-Ub₂ can adopt multiple conformations. The NMR signals from the proximal Ub in cyc-Ub₂ (¹⁵N-labeled on the proximal, G76C Ub) were broadened so severely that individual peaks were indiscernible. All these data suggest that cyclizing Ub₂ with HBVS does not create a uniquely locked interface between the two Ub units, but instead allows the chain to form at least two conformations that interconvert in the slow or intermediate exchange regime on the NMR chemical shift time scale (see below). The ratio of intensities of the duplicate peaks at pH6.8 is on average 2.6 (range 1.1–3.7, standard deviation 0.8); thus, we estimate the occupation probabilities of the two conformations as 73% and 27%. Although these numbers are similar to the occupation probabilities (85% and 15%, respectively) for the closed and open conformations of uncyclized Ub₂ at this pH (Varadan et al. 2002), they cannot be compared directly, as the interconverting structural states of cyc-Ub₂ are not exactly the same as for the uncyclized construct.

It is worth pointing out here several conclusions that follow from these data. First, the relatively small magnitudes of the observed CSPs (versus both mono-Ub, Fig. 4, and uncyclized Ub₂, Fig. 3) indicate that the intradomain structure/fold of the individual Ub units in Ub₂ remains unaltered upon the cyclization. Moreover, the small magnitude of the chemical shift differences between the duplicate resonances (<0.2 ppm, except for Ile13, where it is 0.38 ppm) also suggests that the corresponding states of cyc-Ub₂ are similar to each other in terms of the backbone structure of Ub domains. Second, the data indicate that, whatever the conformations of cyc-Ub₂ are, they are similar to the closed conformation of the uncyclized Ub₂, in the sense that the interdomain contacts involve the same surface hydrophobic patches on both Ub units. This conclusion follows from the observation that the strongest CSPs and the duplicate resonances in cyc-Ub₂ are present primarily in

Figure 3. Amide chemical shift perturbations in the distal domain in Ub₂ as a result of the cross-linking reaction with HBVS, compared with uncyclized Ub₂ (A). These data correspond to pH6.8. In the case of duplicate resonances (residues indicated by the asterisks), the data shown here represent the biggest of the two observed CSPs. Shown in B and C are representative fragments of the ¹H-¹⁵N TROSY spectrum illustrating multiple peaks. Crosses indicate the position of the corresponding signals (labeled with the subscript “u”) in the uncyclized Ub₂.

Figure 4. The effect of pH on the Ub/Ub interface equilibrium in the cyclized and uncyclized Ub₂. Shown are amide chemical shift differences between mono-Ub and the distal Ub in Ub₂ for the uncyclized (*left* panels) and cyclized (*right* panels) chains observed at pH values of 8.0 (A,B), 6.8 (C,D), and 4.5 (E,F). The asterisks indicate residues showing signal attenuation (>50%) in Ub₂ compared to mono-Ub. A definitive interdomain interface present in the uncyclized Ub₂ under neutral and alkaline conditions (pH6.8 and 8.0) disappears as pH is lowered to 4.5, which indicates a shift toward a predominantly open conformation at these conditions. A similar interface is seen at all pH values in cyc-Ub₂, thus demonstrating that the cyclization precludes a complete opening of Ub₂. Stronger CSPs observed in cyc-Ub₂ (B,D vs. A,C) likely reflect a closer contact between Ub units in this chain. In the case of duplicate resonances, the data shown here represent the biggest of the two CSPs.

the same residues that form the Ub/Ub interface in the uncyclized Ub₂. The fact that the resonance frequencies of both duplicate signals are different from those in mono-Ub further corroborates this conclusion. Thus, although the NMR data indicate conformational heterogeneity in cyc-Ub₂, the observed conformations of this chain are all similar in terms of the epitopes involved and might differ in specific residue–residue contacts at the interface.

pH-Dependent changes in cyclized Ub₂

As mentioned above, the uncyclized Ub₂ is in fast (on the NMR time scale) dynamic equilibrium between a closed and one or more open conformations, and the relative populations of these states are controlled by pH (Varadan et al. 2002; Ryabov and Fushman 2006). Since the cyclization restricts the opening of Ub₂, one might expect that it will affect the pH-dependent equilibrium between these states. Indeed, the NMR data for cyc-Ub₂ at acidic pH are in striking contrast with those for the uncyclized Ub₂ (Fig. 4). While there was no indication of an interface between the two Ubs in the uncyclized Ub₂ at pH4.5 (Varadan et al. 2002), the CSPs and signal attenuations (Fig. 4F) observed in the cyclized construct

clearly show the presence of a Ub/Ub interface at these conditions. The finding that cyc-Ub₂ does not fully open even at acidic conditions thus indicates substantial restriction in domain motions, and therefore serves as direct evidence that this construct is indeed cyclized.

To test our hypothesis that the duplicate signals from residues at the interface correspond to conformations of cyc-Ub₂ which are in equilibrium with each other (and not to different species or nonexchanging conformations), we analyzed pH dependence of the relative intensities of the duplicate resonances. Signal ratio for duplicate resonances represents, to a good approximation, relative populations of the interconverting conformations. As a control, similar experiments were also performed for uncyclized Ub₂. Both uncyclized and cyc-Ub₂ showed negligible changes in peak positions when the pH increased from 6.8 to 8.0, but in cyc-Ub₂, there were clear changes in the intensity ratios of the duplicate signals, indicating changes in the relative populations of the interconverting conformations. Specifically, 14 out of 16 amides show a reduction in the intensity ratio compared to that at pH6.8, suggesting a shift in the equilibrium between the cyc-Ub₂ conformations at higher

pH. The average population ratio at pH8.0 is 1.9 (std = 0.7) compared to 2.6 at pH6.8. Signals from several residues located at the Ub/Ub interface (Thr9, Gly10, Val70) or in the linker region (Gly75, Gly76) are so broadened that they could not be reliably detected, again likely due to conformational exchange.

The ratios of the duplicate peaks also changed at lower pH conditions (pH 4.5), where the average peak ratio is 1.75 (std = 0.64), again indicating a shift in the equilibrium between cyc-Ub₂ conformations. In addition, lowering the pH caused 14 out of the 19 duplicate resonances (observed at pH 6.8) to collapse into a single peak at a shifted position that did not always correspond to either of the two original peaks in slow exchange. This behavior likely indicates a transition from slow into the intermediate or fast exchange regime in these amides, suggesting that at acidic pH, the interconversion becomes faster than at neutral/alkaline conditions. Thus, all these data indicate that cyc-Ub₂ undergoes a pH-dependent exchange between two or more conformations.

Because the HBVS linker is 14 Å in length, it is conceivable that the cyclization did not completely abolish the dynamic nature of the Ub/Ub interface, and still allows Ub₂ sufficient conformational freedom to adopt more than one conformation. The fact that these resonances are in slow exchange on the NMR time scale (compared to fast exchange between open and closed states in the uncyclized Ub₂) (Varadan et al. 2002) suggests that the duplicate resonances represent relatively long-lived states (high activation barriers). In the slow exchange regime on the chemical shift time scale the exchange rate $k_{ex} \ll \Delta\omega$, where $\Delta\omega$ is the frequency difference between the exchanging peaks. Therefore, we estimated the upper bound on the exchange rate k_{ex} as approximately one-eighth of the smallest $\Delta\omega$ for duplicate peaks. At pH 6.8, the smallest frequency difference between the observed duplicate signals is $\Delta\omega/2\pi \sim 24$ Hz in ¹⁵N and 28 Hz in ¹H. Thus, the exchange rate is estimated as $k_{ex} \leq 20 \text{ sec}^{-1}$, which is at least about 100-fold slower than the 70–500-μsec conformational exchange in the uncyclized Ub₂ (Fushman et al. 2004). Therefore, we conclude that the cyclization drastically slowed down the opening/closing dynamics at the interface, in addition to restricting the amplitudes of these motions such that cyc-Ub₂ cannot open completely.

Probing solvent accessibility

Hydrogen–deuterium exchange

To probe the solvent accessibility of residues at the interface in cyc-Ub₂, we performed H-D exchange NMR experiments. As control, a set of similar experiments was performed on mono-Ub and uncyclized Ub₂. All the amides that exchanged within minutes in cyc-Ub₂ have shown similar behavior in the control samples. Because

of the fast exchange at pH6.8, it was not possible to tell the difference in the exchange rates for these peaks between the uncyclized and cyc-Ub₂. Of the remaining, slower exchanging signals, residues located away from the interdomain interface, such as Ile30 and Leu56, showed similar exchange rates in mono-Ub, uncyclized, and cyclized Ub₂. However, several residues at the Ub₂ interface, including Ile44, Phe45, Gln49, Leu67, His68, and Val70 displayed markedly slower exchange rates in cyc-Ub₂. For example, amide resonances from Phe45, Gln49, and Val70 in mono-Ub and in uncyclized Ub₂ disappeared in <45 min, but exchanged markedly slower in cyc-Ub₂. Figure 5 illustrates the difference in the H-D exchange kinetics for His68. These data indicate that cyclization of Ub₂ resulted in a decrease in solvent accessibility of the interface residues.

HyTEMPO studies

We also probed if the interior of the interface in the cyc-Ub₂ is accessible to small molecules, using for this purpose a nitroxyl radical HyTEMPO. In the uncyclized Ub₂, the opening–closing dynamics at pH 6.8 allow HyTEMPO to enter into Ub/Ub interface, as inferred from strong signal attenuations observed for the interface residues (Fig. 6B; see also Varadan et al. 2002). Moreover, the extent of the signal attenuations covering essentially all residues at the Ub/Ub interface indicates an extended residence time for HyTEMPO, thus suggesting the sequestration of this molecule at the interface in uncyclized Ub₂ at this pH (Varadan et al. 2002). In contrast, essentially identical signal attenuations were observed in mono-Ub and uncyclized Ub₂ at pH 4.5 (Varadan et al. 2002); thus, the predominantly open Ub₂

Figure 5. Amide H-D exchange monitored by the decay in peak intensities as a function of time in mono-Ub (squares), Ub₂ (circles), and cyc-Ub₂ (triangles) samples for His68. The horizontal bars represent the corresponding levels of experimental noise in signal intensities.

Figure 6. Comparison of the solvent accessibility for the cyclized (solid bars) and uncyclized (open bars) Ub₂ at pH6.8 (A,B) and pH4.5 (C,D), monitored via signal attenuation (in %) in the presence of paramagnetic relaxation agent HyTEMPO. The data presented in D correspond to mono-Ub; the attenuations in the uncyclized Ub₂ at this pH are essentially identical (Varadan et al. 2002). Also shown is the chemical structure of HyTEMPO (E).

conformation at this pH allows HyTEMPO access to the hydrophobic patch residues in Ub units but does not promote its sequestration. As shown in Figure 6A, residues around Leu8, Ile44, and Val70, showed strong signal attenuation, indicating that a small molecule, like HyTEMPO, can access the interface in cyc-Ub₂. This further supports the conclusion that the cyclization did not rigidly constrain Ub₂ in a completely closed conformation, and some residual opening/closing dynamics are still present at the Ub₂ interface. Similar attenuations were observed at pH 4.5 (Fig. 6C) and pH 8.0 (not shown).

Interestingly, although HyTEMPO can access the interface residues, the effect of HyTEMPO on cyc-Ub₂ at pH 6.8 is less than on the uncyclized Ub₂ at these conditions (Fig. 6A). This is likely due to limited access to the interface as a result of restricted amplitudes of interdomain motions compared to uncyclized Ub₂ (also supported by the H-D exchange data presented above). The smaller extent of HyTEMPO-induced attenuations in the interface residues in cyc-Ub₂ also suggests a lesser HyTEMPO residence time at the interface, apparently due to the presence of the cross-linker, although the actual mechanism of this remains to be understood.

UBA2 binding assay

We recently showed (Varadan et al. 2005) that the conformation of Lys48-linked Ub₂ is important for the linkage-specific binding of a UBA2 domain (C-terminal UBA domain of hHR23A, human homolog of yeast Rad23) to these chains. UBA2 binds Ub₂ in a sandwich-like manner that allows UBA2 to simultaneously interact with hydrophobic surfaces on both Ub units. Therefore, as Ub₂ cyclization restricts the opening of the interface, it could render Ub₂ unable to bind UBA2 in this highly specific mode. In order to test if this was indeed the case, twofold molar excess of UBA2 was added to 0.6 mM cyc-Ub₂ ¹⁵N-labeled at the distal Ub.

We used changes in the NMR signals from the backbone amides in Ub₂ to monitor UBA2 binding. The addition of UBA2 resulted in small shifts and attenuations in the resonances belonging to several residues in Ub₂ (Fig. 7). However, the CSPs observed in cyc-Ub₂ are markedly smaller (≤ 0.1 ppm, mean = 0.029 ppm and std = 0.030 ppm, some

Figure 7. Chemical shift perturbations in (A) cyclized and (B) uncyclized Ub₂ upon addition of UBA2 at the 2:1 UBA2:Ub₂ molar ratio. The asterisks indicate residues showing signal attenuation (>50%) characteristic of intermediate exchange. The biggest CSP is shown in the case of duplicate peaks. The small magnitude of CSPs in the cyclized Ub₂ and their nonspecific distribution along the Ub sequence clearly indicate the absence of specific binding of UBA2 to the Ub/Ub interface. Panel (C) shows CSPs in HBVS-loaded monoUb (K48C-HBVS) in the presence of a twofold molar excess of UBA2. The data were obtained at the following concentrations of the proteins: [Ub₂] = 0.6 mM and [UBA2] = 1.2 mM (A and B) and [monoUb] = 0.8 mM, [UBA2] = 1.6 mM in (C).

of them almost at the level of spectral resolution) compared to those (up to 0.4 ppm) in the uncyclized Ub₂ at similar molar concentrations of UBA2 and Ub₂ (Fig. 7). Moreover, the CSPs in cyc-Ub₂ are spread throughout the backbone, in contrast to the uncyclized Ub₂ where the perturbations are clearly clustered in and around the interface sites. Most notably, no significant CSP or attenuation was observed in residues 13 and 69–72. Strong perturbations in these residues can be regarded as a hallmark of a direct UBA2 binding to the distal Ub in both Lys48- and Lys63-linked Ub₂s (Varadan et al. 2004, 2005) as well as to monoUb (Mueller et al. 2004; Varadan et al. 2005). In particular, Val70 is one of the few residues in the distal Ub of the uncyclized Ub₂ whose signal gets strongly attenuated at the early steps of UBA2 titration. This residue is a key contributor to the extended hydrophobic pocket in Lys48-linked Ub₂, and a V70A mutation in the distal Ub significantly weakens UBA2 binding to Lys48-linked Ub₂ (Varadan et al. 2005). A similar effect of V70A mutation was observed for UBA2 binding to monoUb (Varadan et al. 2005). The absence of such perturbations in cyc-Ub₂ suggests that UBA2 does not interact fully with the hydrophobic patch on the distal Ub. Indeed, many of the perturbed sites in Figure 7A are located at the periphery of the Ub/Ub interface in the Ub₂ structure (Fig. 8). In particular, the methyl groups of Leu8 and Thr9, positioned at the edge of the Ub/Ub interface, are oriented such that they form a hydrophobic spot on the surface of Ub₂. This is consistent with cyclization allowing partial opening of the Ub₂ interface, while still restricting access to sites (e.g., residues 69–71) of the distal Ub that are located deep in the interior of the interface. It is worth mentioning here that in the Ub₂/UBA complex the cross-linking sites are far away from each other and cannot theoretically be linked by HBVS. For example, the shortest distance between the C_α atoms of Lys48 of the distal Ub and Gly76 of the proximal Ub observed in the ensemble of 10 Ub₂/UBA structures is 23.1 Å, which is significantly larger than the length of HBVS (14 Å). This excludes the possibility for the cross-linked Ub₂ to accommodate a UBA domain in the same sandwich-like fashion as in the case of the uncyclized Ub₂.

The absence of a clear specific perturbation pattern in cyc-Ub₂ suggests that UBA2 interacts with cyc-Ub₂ either non-specifically or in a different mode (e.g., involving hydrophobic contacts with the Ub₂ surface and/or with the cross-linker), which does not directly involve the hydrophobic patch residues on the distal Ub. It is important to emphasize that HBVS acts here as a molecular “staple” that restricts the interface opening but does not modify the hydrophobic-patch residues involved in Ub binding to UBA2. In a control experiment, the addition of UBA2 to HBVS-loaded monomeric Ub (K48C-HBVS) resulted in a typical for Ub pattern of perturbations (Fig. 7C), thus confirming that HBVS modification of Cys48 did not abolish UBA-binding

Figure 8. (A) Surface map of the sites in the distal Ub that show perturbations (CSPs and/or signal attenuation upon addition of UBA2) in both cyclized and uncyclized Ub₂ (red) or only in the uncyclized Ub₂ (blue). (B) Surface map of those residues in the distal Ub that exhibit different (slower) H-D exchange rates in cyc-Ub₂ compared to the uncyclized construct. Those residues not affected by UBA2 (in cyc-Ub₂) and those that show slower H-D exchange (hence decreased accessibility to D₂O) both are located in or close to the center of the interface. Perturbations in some interface residues (e.g., Ile44, Gln49) could be an indirect effect of some rearrangement in interdomain contacts rather than direct UBA binding. The surface corresponds to the crystal structure of Ub₂ (PDB code 1AAR) (Cook et al. 1992), the distal and proximal Ub units are colored gold and gray, respectively; the drawings on the left represent the distal Ub rotated by 60°.

properties of Ub. We therefore conclude that the UBA2 binding is significantly weakened because of the restriction on domain mobility imposed by the cross-linker which makes cyc-Ub₂ unable to adopt the appropriate conformation allowing UBA2 full access to the interface sites necessary for the high-affinity, sandwich-like mode of binding observed in the uncyclized Ub₂.

Conclusions

To verify the importance of interdomain dynamics for the binding properties of Lys48-linked polyUb, we cyclized di-ubiquitin chain by cross-linking the flexible C-terminus of the proximal Ub with the side chain of residue 48 in the distal Ub. The data presented in this paper indicate that the cyclization of Lys48-linked Ub₂ by HBVS affects conformational dynamics in Ub₂ by restricting opening of the interface and shifting the conformational equilibrium of

Ub₂ toward closed conformations. Presumably, the length of the linker used in this study still allows some interdomain dynamics such that cyclized Ub₂ exists in at least two conformations which are in slow exchange with each other. These are all closed conformations, i.e., characterized by a well-defined interface involving the same hydrophobic patch residues as in uncyclized Ub₂. Lowering the pH changes the relative populations of these conformations, but in contrast with the uncyclized Ub₂, does not lead to opening of the interface. The residual motions in Ub₂ turn out sufficient to allow access of small molecules like HyTEMPO to the Ub/Ub interface, although larger ligands like UBA2 cannot bind to the interface sites directly.

These results indicate that the cyclization affects the binding properties of Ub₂ by restricting its opening/closing interdomain dynamics. This restriction of domain motions does not allow cyc-Ub₂ to adopt the appropriate conformation with the Ub/Ub interface being open. This then inhibits direct access of protein molecules to the hydrophobic patch residues (Leu8-Ile44-Val70) located at the very center of the interface between two Ub units in Ub₂. This renders Ub₂ unable to bind other protein molecules (e.g., UBA2) in the normal manner, and thus could interfere with Ub₂ recognition by various downstream effector molecules. These results emphasize the importance of the chain's ability to adopt opened conformations for the recognition and function of di-ubiquitin and possibly longer polyUb chains.

Materials and methods

Materials

Biochemicals were from Sigma. E1 and Isopeptidase T (IsoT) were from BostonBiochem Inc. 1, 6-Hexane-bis-vinylsulfone (HBVS) and 1,4-Bis-maleimidobutane (BMB) were from Pierce. 4-Hydroxy-2,2,6,6-tetramethylpiperidinyl-1-oxy (HyTEMPO, Aldrich) was used as a paramagnetic relaxation agent. Plasmids for Ub constructs and GST-E2 were generously provided by Dr. Cecile Pickart (Johns Hopkins University). C170S-E2_{25K}, Ub(K48C), Ub(K48R), Ub(D77), and Ub(G76C) were expressed and purified as described (Haldeman et al. 1997). For ¹⁵N-labeled Ub (K48C), *Escherichia coli* cells were grown in minimal media with ¹⁵NH₄Cl as the sole source of nitrogen.

Purification

After a particular Ub₂ sample was synthesized through the E1/E2 reaction (as described elsewhere) (Varadan et al. 2002), the solution was incubated at 37°C in about 30 mM DTT overnight to break any intermolecular or intramolecular disulfide bonds that may have formed. The sample was then separated using a HiLoad 16/60 Superdex 75 prep grade gel filtration column using a 50 mM ammonium acetate, 150 mM NaCl, 5 mM DTT, 1mM EDTA pH 4.5 buffer. This yields pure Ub₂, free of monoUb and the reaction enzymes, as verified by SDS-PAGE.

Cyclization of Lys48-linked Ub₂

Once a pure Ub₂ sample was obtained, the solution was adjusted to pH 8.0 to favor the closed conformation (Varadan et al. 2002). The solution was then diluted to about 2.5 mg/mL in order to promote intramolecular cross-linking over an intermolecular reaction. HBVS is a non-cleavable, homo-bifunctional cross-linking agent that is selectively reactive toward sulfhydryl groups through two vinylsulfone reactive groups (Fig. 1C). It couples two sulfhydryl groups without stereoisomer formation via Michael addition. Approximately 5 mg of HBVS were dissolved in DMF and immediately added in 20 µl aliquots to the protein sample, to provide approximately a 30:1 molar ratio of HBVS to Ub₂. The solution was allowed to react overnight at 35°C. No cross-linking was seen between two or more Ub₂s as verified by SDS-PAGE, indicating that the intramolecular cross-linkage was highly preferred under our reaction conditions.

Isopeptidase-T reaction

IsoT reactions were performed following the work of Wilkinson et al. (1995) and used a reaction mixture containing 0.1 µM DTT, 1.5 µM IsoT and ~0.6 mM Ub₂. The mixture was incubated overnight or longer before being monitored by gel electrophoresis.

NMR studies

All NMR studies were performed on a Bruker Avance 600 spectrometer at 24°C. ¹H-¹⁵N HSQC or TROSY spectra and 2D planes for ¹⁵N T₁ relaxation experiments were acquired with spectral widths of 7.2 kHz and 2 kHz in the ¹H and ¹⁵N dimensions, respectively. Typically, 64 or 128 t₁ increments, each consisting of 1024 complex points, were collected for each 2D plane. Combined amide chemical shift perturbations (CSPs) were calculated using the following equation: $\Delta\delta = [(\Delta\delta_H)^2 + (\Delta\delta_N/5)^2]^{1/2}$, where $\Delta\delta_H$ and $\Delta\delta_N$ are shifts in ¹H and ¹⁵N signals, respectively, observed upon cyclization or addition of UBA2. In the case of duplicate peaks, $\Delta\delta$ values were calculated for each of the two signals separately, and the biggest CSP is reported in Figures 3, 4, and 7.

For H-D exchange studies, the protein sample (Ub₂ or Ub) was lyophilized, re-dissolved in D₂O, and the exchange of amide protons was monitored by comparing intensities of the amide signals in a series of ¹H-¹⁵N HSQC spectra recorded at various time-intervals up to about five days.

Solvent accessibility studies were also performed using a paramagnetic relaxation reagent, 4-hydroxy-2,2,6,6-tetramethylpiperidinyl-1-oxy (HyTEMPO), as detailed in (Varadan et al. 2002). The paramagnetic relaxation enhancement caused by HyTEMPO was monitored by comparing signal intensities in ¹H-¹⁵N HSQC spectra recorded in the absence and in the presence of 20 mM HyTEMPO.

Electronic supplemental material

Supplemental material includes three gels of control reactions for IsoT cleavage assays. These include (1) demonstration of successful IsoT cleavage of the Ub₂ constructs containing amino acid residue modifications of the free C terminus (proximal Ub), a replacement of Gly76 with a cysteine and an extension by Asp77; (2) a native gel showing mobility of the Ub-HBVS-Ub construct (no isopeptide linkage); and (3) a demonstration that a

covalent modification of the side chain of Cys76 (proximal Ub) and of both Gly76 (proximal Ub) and Cys48 (distal Ub) hinders the IsoT cleavage of Ub₂.

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