Ubiquitin not only serves as a tag but also assists degradation by inducing protein unfolding

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Protein ubiquitination controls the cellular fate of numerous eukaryotic proteins. Despite its importance, many fundamental questions remain regarding its mechanism. One such question is how ubiquitination alters the biophysical properties of the modified protein and whether these alterations are significant in the cellular context. In this study, we investigate the effects of ubiquitination on the folding thermodynamics and mechanism of various substrates using computational tools and find that ubiquitination changes the thermal stability of modified proteins in a manner relevant to cellular processes. These changes depend on the substrate modification site and on the type of ubiquitination. Ubiquitination of the substrate Ubc7 at the residues that are modified in vivo prior to proteasomal degradation uniquely results in significant thermal destabilization and a local unwinding near the modification site, which indicates that ubiquitination possibly facilitates the unfolding process and improves substrate degradation efficiency. With respect to the substrate p19^{4inkd}, our results support a synergetic effect of ubiquitination and phosphorylation on the degradation process via enhanced thermal destabilization. Our study implies that, in addition to its known role as a recognition signal, the ubiquitin attachment may be directly involved in the cellular process it regulates by changing the biophysical properties of the substrate.

coarse-grained simulations | posttranslational modifications | protein degradation | protein folding | ubiquitination

Protein ubiquitination is a posttranslational modification that regulates many important cellular processes (1, 2). The covalent attachment of ubiquitin molecules to the substrate protein occurs via an isopeptide bond, usually on one of the substrate's lysine residues. The conjugated ubiquitin itself can be further connected, by one of its seven lysine residues, to other ubiquitin molecules, forming a polyubiquitin chain (1, 3). Chains created by using different lysine residues have different topologies and regulate different cellular processes. Lys-48 linked polyubiquitin chains (K48-pUb) usually target substrates for degradation in the 26S proteasome, whereas other types of linkage lead to nonproteolytic pathways. For example, Lys-63 linked chains (K63-pUb) are involved in DNA repair and trafficking, and single monoubiquitination (mUb) regulates diverse processes such as endocytosis and chromatin structure (2, 4, 5).

How can ubiquitination result in such a diversity of functional activities? Various posttranslational modifications can act either by directly modulating the biophysical properties of the substrate or by serving as a signal recognized by other proteins that execute the cellular task. Whereas phosphorylation (6) and glycosylation (7) were shown to function by using both mechanisms, the attached ubiquitin moiety is widely viewed as a recognition tag (4, 5). In this respect, it is established that each polyubiquitin topology, which is formed by a different lysine linkage (see Fig. 1), exposes a different surface that can be recognized by various ubiquitin-binding domains (8), and, indeed, there are several polyubiquitin receptors in the proteasome, as well as polyubiquitin-binding domains in the factors that mobilize substrates to it (9).

However, conjugating a protein with another protein, such as occurs during ubiquitination, may significantly alter the intrinsic properties of the conjugate. When proteins were covalently linked in vitro (10), their thermal stability was significantly altered. Studies of multidomain proteins show that the folding characteristics of their isolated constituent domains can be dramatically changed when they are connected to a neighboring domain (11–13). Furthermore, several studies speculated that ubiquitination may alter various biophysical properties, such as protein stability (14, 15), conformational dynamics (16), solubility, and refolding competence (17).

For example, one of the major roles of ubiquitin is to label proteins for proteasomal degradation (1, 9). In this process, the proteasome unfolds the protein prior to its degradation (18, 19), often by unraveling it from a local region in the vicinity of the ubiquitin attachment (19, 20). The proteasome is responsible for degrading a vast array of proteins; however, the degradation efficiency and success vary greatly from one substrate to another and depend on the substrate's properties (16, 19, 21). For example, it was suggested that the low stability observed for p53 is linked to its degradation (22). A number of studies on natural and artificial substrates have shown that the proteasome and its analogs are largely suited to the degradation of specific substrates depending on their ability to disrupt native structure and initiate the unfolding process (23). When these conditions are unmet, the substrate may elude capture (23), the proteasome may be unable to degrade the substrate (21), or the protein may be processed only partially, leaving some regions intact (24).

The substrate's dynamic and structural characteristics were shown to influence the degradation process. The addition of a ligand to dihydrofolate reductase prevents its degradation by enhancing its rigidity (21, 25). Similarly, low Ca^{2+} concentrations promote the proteolysis of calmodulin and other calcium-binding proteins by increasing their native-state dynamics (26). Various chemical modifications that damage the native structure can also enhance the degradation rate (26, 27). Similarly, the Rel homology domain's resistance to degradation is abolished when destabilizing mutations are introduced into its core (19). It was also observed that susceptibility to proteasomal degradation is affected by structural elements near the degradation sites (19). Additionally, the presence of an unstructured region can initiate the unfolding step and greatly facilitates the degradation process (28, 29).

Because the proteasome handles many different substrates and its substrate's unfolding capabilities vary, the attachment of the ubiquitin moiety to the protein may assist the degradation process in some of these cases. Moreover, the site at which the ubiquitination occurs in vivo may be evolutionary selected for a feasible and efficient unfolding process.

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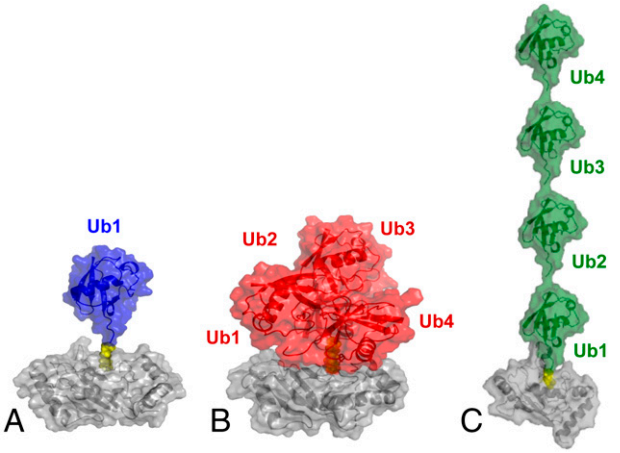

Fig. 1. Structure of ubiquitinated Ubc7. The structure of Ubc7 (PDB: 2ucz) following: (*A*) mUb at K94; (*B*) K48-pUb at K94; (*C*) K63-pUb at K94.

The above discussion suggests that attaching a ubiquitin moiety to a substrate may alter its biophysical properties. If so, it would be important to know whether the biophysical alternations promote the cellular process signaled by ubiquitination. These important possibilities have not been investigated to date, primarily due to difficulties in addressing them experimentally.

Here, we computationally study the effects of ubiquitination on the folding behavior of the natural substrates Ubc7 and $p19^{4inkd}$, as well as on the *src*-SH3 domain. Using various ubiquitin moieties (mUb, K48-pUb, and K63-pUb), we compare the effects of ubiquitination at the residues that are ubiquitinated in vivo and at other substrate locations. Our study examines the biophysical effects of ubiquitination and points to the role they may play in the cell. Our results suggest that ubiquitin not only serves as a recognition tag but also assists degradation by facilitating protein unfolding.

Results and Discussion

Ubiquitination of Ubc7, Which Mediates its Proteasomal Degradation, Leads to Thermal Destabilization and Local Structural Unwinding. The ubiquitin-conjugating enzymes (E2s) family can serve as a good system to examine our hypothesis, because the degradation of many of its members is characterized and mediated through a K48-pUb that occurs at a relatively conserved residue (30). This residue (Lys94) is located near the catalytic cysteine residue (Cys89). In the case of yeast Ubc7, which lacks this lysine (bearing a histidine in its place), degradation was shown to proceed via ubiquitination of the Cys89 catalytic residue instead (Fig. 2). Ubc7 in which His94 is mutated to Lys is degraded after ubiquitination at the new lysine site, as seen in other family members (30). Thus, Ubc7 can act as a good case study, as two residues (C89 and K94) serve as relatively conserved ubiquitination sites that lead to degradation, whereas other lysine residues are not ubiquitinated in vivo.

We ubiquitinated Ubc7 *in silico* at residues C89 and K94 by using K48-pUb. In a similar manner, we conjugated this chain topology to an additional seven of the nine surface-exposed Lys residues that were shown not to be ubiquitinated in vivo (3, 11, 18, 29, 62, 70, and 161). We chose these residues on the basis of the propensity of ubiquitination to occur mostly in loop regions and to a lesser extent on helices (31, 32). To compare the effects of different chain topologies on Ubc7 folding, we also ubiquitinated residues 18, 89, and 94 using mUb and a K63-pUb (Figs. 1 and 2*A*). We simulated the folding of the nonmodified Ubc7, as well as the selected ubiquitinated forms, and calculated their thermodynamic properties under equilibrium.

Ubiquitination significantly affects the thermodynamic stability of Ubc7 (Fig. 2*B*). Whereas most systems are somewhat destabilized, some are greatly destabilized (with a free energy change of more than 3.5 kT higher than that of unmodified Ubc7), and one system is stabilized by ~2.3 kT. Because proteins are marginally stable, the outcome of these attachments can be significant in the cellular environment, because these changes

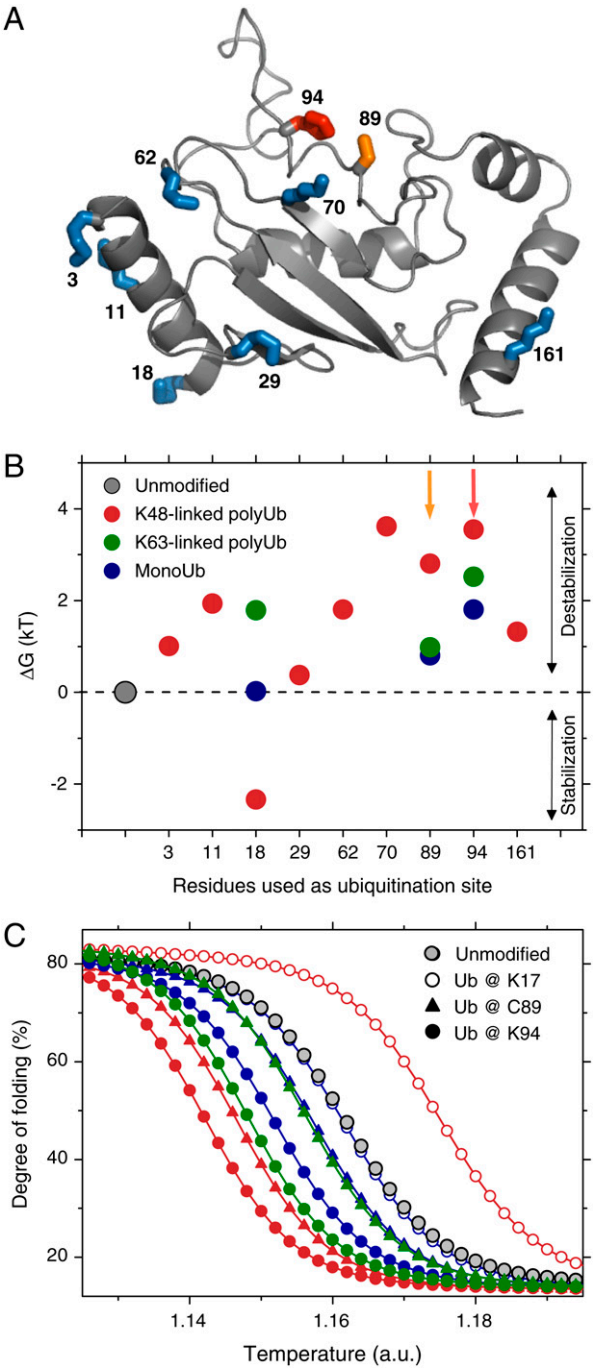

Fig. 2. Ubiquitination sites on Ubc7 and thermodynamics results. (*A*) Residues on Ubc7 that were ubiquitinated in this study. Residues ubiquitinated in vivo appear in red (His94) and in orange (Cys89). (*B*) The thermodynamic stability (ΔG) of various ubiquitinated Ubc7 systems at the T_F (folding temperature) of unmodified Ubc7. Maximal error: 0.3%. (*C*) Unfolding curves of various systems: degree of folding (percentages of native contacts) as a function of temperature. Different colors mark different ubiquitination types (as in Fig. 1).

correspond to a difference of a few kcal/mol. We found that attachment at each of the nine positions had a different thermodynamic consequence and that attachment of various types of ubiquitin molecules at the same residue produced different stabilities. We obtained similar results when we ubiquitinated the $p19^{4inkd}$ and *src*-SH3 domains, each at three different locations, and by using a variety of ubiquitin moieties. Overall, our results show the biophysical outcome to be highly dependent on the location of the modified residue (i.e., the ubiquitination site), the type of ubiquitination, and the substrate's structure (Fig. S1).

Interesting insights arise from examining the two sites at which ubiquitination can occur in vivo in Ubc7 compared to the seven other "nonnative" sites. Attachment of a K48-pUb at either of the two residues (C89 and K94) that signal cellular degradation significantly destabilizes the substrate. Other residues, which are not used as ubiquitination sites by the cellular machinery, give various results (strong stabilization at residue 18, no effect at residue 29, and varied degrees of destabilization at residues 3, 11, 62, 70, and 161). Other than polyubiquitination at residue K70, no other destabilization is as strong as those observed at the two locations at which ubiquitination takes place in vivo. Moreover, the other types of ubiquitination we studied—mUb and K63-pUb—do not lead to such a strong destabilization as K48-pUb.

Because the unfolding process in the proteasome often starts near the ubiquitination site, and because its success and efficiency are largely dependent on the resistance of the local structure to being unfolded, we focused on the effects of ubiquitination on the dynamics of the folded substrate structure. K48-pUb at K94 (Fig. 3*A*) or C89 (Fig. S2) results in significant unwinding of the local structure in the vicinity of the attachment. However, this effect is weaker if a K63-pUb or a mUb is attached at these spots (Figs. 3*C* and S2). For other ubiquitination sites and types (Figs. 3*E* and S2), the strong unwinding effect varies greatly in its strength or is completely absent.

Thus, the attachment of a K48-pUb to the residues naturally used by the cell to direct the substrate to the proteasome is followed by distinctive behavior: uniquely significant destabilization and local unwinding. This striking behavior suggests that, in the case of Ubc7, ubiquitination facilitates the proteasomal unfolding process. The outcome of significant destabilization is that a larger fraction of substrate molecules would reach the proteasome already unfolded. As illustrated in Fig. 2*C*, at a wide range of temperatures, K48-pUb at K94 results in a relatively high percentage of unfolded substrates, relative to the unmodified system and to other ubiquitinated systems (such as at K18). Moreover, the local unwinding of the folded substrate may greatly enhance the unfolding that starts in this region, because the native structure becomes distorted and is more prone to unfolding.

Thermodynamic Analysis of Ubiquitinated Ubc7 Variants. To study the origins of the thermodynamics results, we first decomposed the folding free energy values into their enthalpic and entropic contributions. We then compared the differences in the folding entropy and enthalpy of each ubiquitinated system to the differences observed in the unmodified system. For all ubiquitinated systems, both $\Delta\Delta H$ ($=\Delta H^{ubiquitinated} - \Delta H^{unmodified}$) and $\Delta\Delta S$ ($=\Delta S^{ubiquitinated} - \Delta S^{unmodified}$) have negative values. Negative $\Delta\Delta H$ contributes to overall stabilization, whereas negative $\Delta\Delta S$ contributes to destabilization. Therefore, because $\Delta\Delta S$ and $\Delta\Delta H$ have opposing effects, the balance between them dictates whether ubiquitination results in stabilization or destabilization. Plotting $\Delta\Delta S$ against $\Delta\Delta H$ clearly indicates that the entropic effect dominates for most systems, and this phenomenon is more pronounced as the system becomes more destabilized (i.e., more positive $\Delta\Delta G$) (Fig. 4*A*). Accordingly, in spite of the higher enthalpic gain during the folding reaction that contributes to stabilization, most ubiquitinated systems are overwhelmed by the larger entropic loss, which destabilizes the system. In

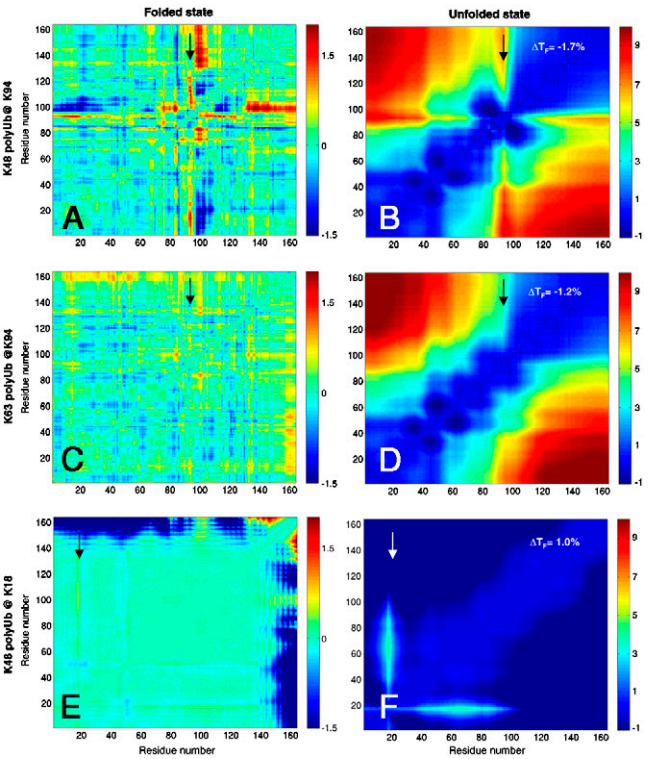

Fig. 3. Differences in dynamics between ubiquitinated and unmodified Ubc7. The differences in the dynamics of the folded and unfolded state of Ubc7 following (*A–B*) K48-pUb at K94; (*C–D*) K63-pUb at K94; (*E–F*) K48-pUb at K18. The difference distance matrices were calculated by using the average distance between each pair of substrate residues in the ubiquitinated system compared to unmodified Ubc7. Matrices were calculated at the folding temperature T_F (the temperature at which 50% of the molecules are folded) of the unmodified Ubc7. Color indicates difference in Å; an arrow indicates the location of the ubiquitination site.

the following paragraphs we analyze the cause of the varying degrees of destabilization that are observed in the majority of the modified systems. We then discuss the effects that occur in the polyubiquitination of K18 that results in a strong stabilization.

When looking at the structural differences caused by ubiquitination at various locations, several aspects of the altered thermodynamic characteristics are revealed. These differences, at the level of the distance between any pair of residues, are demonstrated in the delta-distance matrices of the folded state and the unfolded state of each system (Figs. 3 and S2). In the folded state, ubiquitination results in the expansion of some regions relative to the unmodified system. These local unwinding events vary in magnitude (up to ~2 Å) and are more localized in some cases (e.g., K48-pUb at K94, Fig. 3*A*) than in others (e.g., K63-pUb at K94, Fig. 3*C*). When the disruption of the folded states is more pronounced, the system destabilizes and shifts toward the unfolded state. In the unfolded state, the distortion is larger than in the folded state (some regions are drawn away from one another by more than 10 Å, whereas others seem to come closer, although to a much smaller extent). These phenomena seem to stem from the point of attachment, and a cross centered on the attachment point is often observed.

Because the region near the attachment point seems to experience the largest effects and to affect the rest of the structure, we correlated the structural properties of the region around the ubiquitination site, in each ubiquitinated system, to its folding temperature T_F (the temperature at which 50% of the molecules are folded, which highly correlates with the free energy of the system and is indicative of its stability). There is a strong negative

correlation between the number of contacts each of the residues has in the folded state and the thermal stability of the modified system that is ubiquitinated at this residue (Fig. 4*B*). Negative correlations are also observed between the thermal stability and other measures of the degree to which the vicinity of the attachment is structured. These findings indicate a strong linkage between the local properties of the substrate and the thermodynamic outcome of attaching a ubiquitin moiety to it.

Our results are strongly complemented by results from an analogous system that was studied in vitro. As noted above, due to technical difficulties, it is difficult to study the effects of ubiquitination on substrate folding behavior experimentally. However, in a series of studies made on a different but equivalent system, phenomena consistent with our findings were observed (10). In these studies, a substrate protein, staphylococcal nuclease, was engineered to form a cross-linked dimer via a chemically modified cysteine introduced at various locations (Fig. S3). These dimers were often less stable than their monomeric forms, with the magnitude of destabilization differing from one location to another. We analyzed the local structural density of each location and the stability observed after cross-linking at this residue. The negative correlation observed is again very strong (Fig. 4*B*).

The fact that significant correlations are observed in both the Ubc7 and nuclease systems, despite the use of different substrates and conjugates, not only validates our results but points to an important fundamental observation. When linking a protein with another protein moiety, the outcome of this attachment is largely dependent on the local properties in the region of the conjugation site. The more densely structured this region is, the greater the likelihood that the system will destabilize.

Taking together the results of the thermodynamics decomposition (Fig. 4*A*), the structural dynamics observations (Figs. 3 and S2), and the correlation between the destabilization and the local region's structural density properties (Fig. 4*B*), we can relate the system's structural characteristics to the thermodynamic outcome. When a substrate is modified at a region characterized by high-structural density, it is more sensitive to the ubiquitination effects. The folded state is more easily distorted, and the unfolded state becomes more unwound and less structured due to the attachment., which is illustrated in the distribution of the radius of gyration R_g of various modified systems (Fig. S4*a*). Whereas there is little difference between the R_g values of the folded states of ubiquitinated compared to unmodified proteins (the distortion is limited to a few regions), a larger effect is observed in the unfolded state, and, when the local region near the modification site is more densely structured, the unfolded state tends to include more extended configurations.

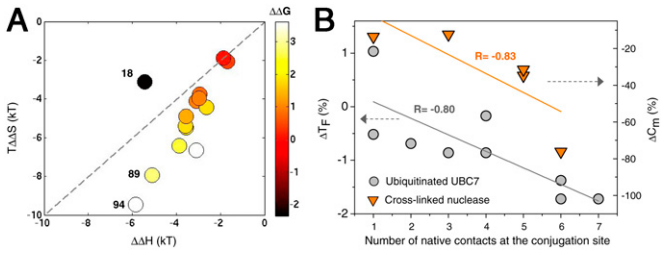

Fig. 4. Thermodynamic analysis of ubiquitinated Ubc7 systems. (*A*) Decomposition of the free energy of various ubiquitinated Ubc7 systems. The free-energy difference between the ubiquitinated Ubc7 and the unmodified Ubc7 (ΔΔG) was separated to its enthalpic (ΔΔH) and entropic (ΔΔS) components. $T\Delta\Delta S$ is plotted against ΔΔH, where T is constant and equal to T_F of the unmodified Ubc7. The values of ΔΔG appear in color. (*B*) Correlation between the structural properties of the ubiquitination site and the stability of the K48-linked polyubiquitinated Ubc7 (in gray). The stability change is indicated by $\Delta T_F = (T_F^{\text{ubiquitinated}} - T_F^{\text{unmodified}})/T_F^{\text{unmodified}}$. The number of contacts in the native state is used as a measure of structural density in the ubiquitination site's vicinity. Similarly, in studies of in vitro cross-linking of nuclease, a correlation is observed between the structural properties of the conjugation site and the stability of the cross-linked nuclease (in orange). Stability is indicated by the change in C_m (the denaturant concentration at the midpoint) relative to the nonconjugated nuclease monomer ($\Delta C_m = (C_m^{\text{dimer}} - C_m^{\text{monomer}})/C_m^{\text{monomer}}$).

These observations are thermodynamically mirrored in both the enthalpy and the entropy components. The more extended the unfolded state becomes due to ubiquitination, the larger its entropy becomes, which stabilizes the unfolded state and so increases the entropic loss associated with the overall folding reaction (Fig. 4*A*). Thus, the entropic stabilization of the unfolded state leads to destabilization of the substrate. On the other hand, because the unfolded state becomes more extended, it retains less residual structure, and the enthalpic gain upon folding becomes greater (Fig. 4*A*). These two factors oppose each other, but the entropy in this case is superior, and the outcome is destabilization.

The net result of these opposing factors can be diverse, depending on the substrate's size and topology and the ubiquitination type. For example, when ubiquitinating the *src*-SH3 domain with various ubiquitin moieties, the systems are usually stabilized (Fig. S1). Although a more extended unfolded state is observed (Fig. S4*b*), the enthalpic gain is higher than the entropic loss, and therefore the overall effect is stabilization (Fig. S5). The differences between the two systems are related to their different sizes and topologies. The unfolded state of unmodified Ubc7 has more residual structures than the *src*-SH3 domain, due to its greater helical content. Additionally, Ubc7 is 3 times larger than the *src*-SH3 domain, making the ratios between the substrate and the ubiquitin moiety very different and the entropic loss much greater.

Despite the importance of the local density of the surroundings of the ubiquitination position, other effects may play a role as well. The size of the interface formed between the substrate and the ubiquitin moiety can also affect the substrate thermodynamics (Fig. S6). Its effect is seen when comparing the outcome of attaching different ubiquitination types to the same residue (Fig. 3*B*). Their effects are different, because a different surface is exposed to the substrate and because the ubiquitin moiety's dynamics is different (Fig. S6).

We now turn to discuss the case in which factors other than the local properties of the modified residue clearly affect folding. When modifying K18 with a K48-pUb, a strong stabilization occurs. A very weak unwinding process is seen in the folded and unfolded states (Fig. 3*E–F*), and thus the substrate is relatively undisturbed near the point of modification. The low number of contacts of this residue provides a possible explanation for the observed stabilization, because this region is relatively flexible and can accommodate the modification. However, other residues with few contacts show minor destabilization, and the attachment of a mUb or K63-pUb to K18 does not result in significant stabilization. So why does such strong stabilization occur when K18 is ubiquitinated with a K48-pUb? Here, a stabilizing confinement effect takes place, due to the relative locations of the substrate and the ubiquitin chain. This effect is seen in the structural analysis (Fig. 3*E–F*), where parts of the substrate become more compact due to the attachment. In the distribution of R_g (Fig. S4*a*), the unfolded state is constricted to more contracted conformations, which is a hallmark of confinement (33, 34). The loss of entropy in the unfolded state results in an overall stabilization. Stabilization by confinement is also seen in one case of polyubiquitination of the *src*-SH3 domain (Figs. S4*b* and S5).

Ubiquitination and Phosphorylation May Act in Synergy to Destabilize $p19^{4inkd}$ Prior to its Degradation. $p19^{4inkd}$ is degraded after modification by K48-pU at residue K62 (35), which is not as conserved as the modified residues in the E2 family (36, 37). We chose to study the effect of ubiquitination on $p19^{4inkd}$ because it has a

unique ankyrin repeat topology (Fig. 5*A*) and because its folding was studied experimentally (38). We ubiquitinated its three lysine residues (K43, 62, and 91) with mUb or K48- or K63-pUb and studied their folding thermodynamics and mechanism in comparison to the unmodified substrate.

The thermodynamics of the ubiquitinated $p19^{4inkd}$ shows relatively smaller changes in comparison to ubiquitinated Ubc7. The T_F was reduced by less than 1% (Fig. S1), and the destabilization that is caused by attaching ubiquitin chains to the in vivo ubiquitination residue (K62) is smaller than for the other two residues. The mild destabilization may be related to the fact that the modified residues are not located in a well-structured region. It can also originate in the substrate's structure, which has a topology consisting of small repeating units with local structure.

The relatively minor destabilization observed upon ubiquitination can also be related to the fact that phosphorylation is also involved in the process. Two residues in the vicinity of the ubiquitination site were shown to undergo phosphorylation (S66 and S76) (39). The phosphorylation process destabilizes the substrate, and the region near the modification unfolds (40). In addition, the phosphorylated system showed an increase in in vitro ubiquitination, implying that the two processes are related.

To examine whether these two modifications (i.e., ubiquitination and phosphorylation) enhance each other's effect, we introduced nonspecific electrostatic interactions on top of the native-topology-based potential (see *Methods*). By using this enriched model, we studied the unmodified $p19^{4inkd}$ system, the phosphorylated system (at S66 and S76), the ubiquitinated system (at K62), and the phosphorylated ubiquitinated system. The thermodynamic results show that each of the modifications destabilizes the substrate separately (Fig. 5*B*). In accordance with experimental observations, phosphorylation results in larger and relatively localized motions in the folded state (Fig. S7*a*). Ubiquitination results in destabilization as well. However, after this modification, the folded state is affected in regions located more distally from the modification point (Fig. S7*c*), which implies that the two modifications may act together to enhance destabilization. Indeed, combined ubiquitination and phosphorylation of the substrate further destabilizes the system (Fig. 5*B*). When looking at the unfolded state of the various systems, large effects are seen as well (Fig. S7). Thus, whereas the folded state is distorted, effects in the unfolded state may also enhance the overall destabilization. Therefore, the diverse effects of phosphorylation and ubiquitination affect both the folded and unfolded states of $p19^{4inkd}$ and synergistically destabilize it.

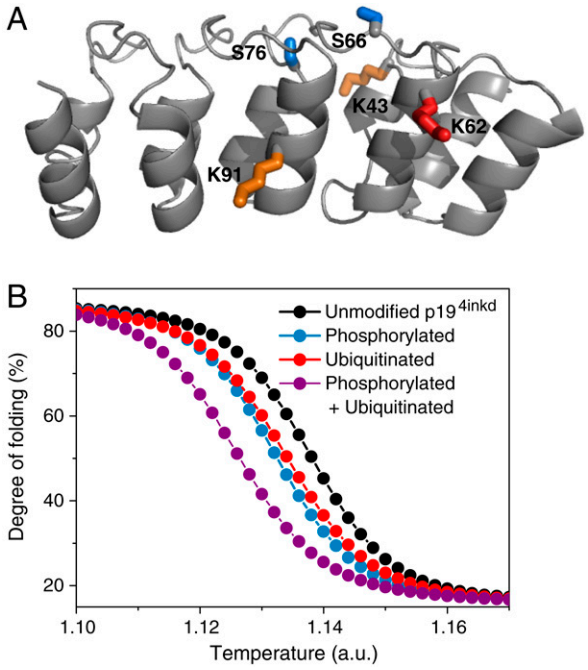

Fig. 5. Modification sites used on $p19^{4inkd}$ and thermodynamics results. (*A*) The structure of $p19^{4inkd}$ and the residues that served as ubiquitination or phosphorylation sites in this study: Red represents K62, which is the in vivo ubiquitination site; orange represents the other Lys residues (K43 and K91) we *in silico* ubiquitinated; and cyan represents S66 and S76, which are the in vivo phosphorylation sites. (*B*) Unfolding curves of variants of $p19^{4inkd}$ ubiquitinated at K62 and phosphorylated at both S66 and S76.

Conclusions

In this work, we studied the effects of ubiquitination on the folding, stability, and dynamics of various substrates. The results are in line with previous conjectures (14–16) that the attachment of ubiquitin may directly affect the substrate's biophysics in addition to its signaling role. We showed that ubiquitination strongly affects thermodynamic stability, with the outcome depending on the type of ubiquitin moiety and the position of the ubiquitination site on the substrate. The outcome's dependence on the location of the modification is reminiscent of the effects of protein glycosylation (7), cross-linking (10), and surface tethering (41).

Several observations regarding the effect of ubiquitination on the substrate's function may be interpreted in light of our thermodynamics results. The activity of several transcription factors has been shown to be inhibited or amplified after ubiquitination (42), and protein synthesis is influenced by ubiquitination of ribosomal proteins (43). By changing the substrate's stability, as observed here, its function can be modulated in a scenario whose outcome depends on the substrate's characteristics and the ubiquitination type and location.

The proteasome successfully unfolds and degrades a vast variety of substrates in a robust and efficient manner. However, the efficiency of the process depends on substrate properties such as rigidity and dynamics. Our results suggest a mechanism by which the proteasome is able to achieve its task. Previous studies have highlighted the importance of the characteristics of the structure that is first encountered by the proteasome to the unfolding process. This region, which is in the vicinity of the ubiquitin tag, must be sufficiently dynamic to enable the proteasome to exploit its fluctuations and to start unraveling the substrate. Several studies have indicated that an unstructured region (28, 29) is needed to initiate the degradation process, and indeed these regions were observed in many substrates (32, 44). However, many proteins that are degraded by the proteasome lack unstructured regions (31, 45). Here, we showed that attachment of a ubiquitin moiety, a process that occurs in most substrates processed by the proteasome, can facilitate the unfolding process. Our results can also be related to studies in which the rates of ubiquitination were shown to vary between different substrates (46). It is possible that, in some substrates that are more resistant to degradation, the ubiquitin moiety is larger and remains attached to the substrate for a longer time in order to facilitate their degradation.

In Ubc7, K48-pUb at the in vivo ubiquitination sites results in significant thermal destabilization and a strong local unwinding effect. The consequence of these two effects may present to the proteasome a configuration that is significantly less structured. The fact that these effects were observed uniquely when using a K48-pUb on the residues that are in vivo modified supports this hypothesis. Observed differences in the outcome of the attachment on different residues imply that the selection of the ubiquitination site is influenced by its biophysical result. Moreover, the fact that a K48-pUb affects the substrate in this specific manner suggests that it may have evolved for this function as well.

Our results regarding $p19^{4inkd}$ suggest cross-talk between phosphorylation and ubiquitination. Here, the unwinding process starts when the substrate is phosphorylated and may be augmen-

ted by its ubiquitination. Because many ubiquitination substrates are regulated by other modifications, it would be interesting to see how various combinations of posttranslational modifications modulate the biophysics of the modified proteins.

Our study suggests that ubiquitination can alter the folding properties of the modified substrates and thus may serve not solely as a signaling tag. It is unclear how universal this mechanism is and to what extent nature exploits it in facilitating protein degradation. Beyond degradation, this mechanism could be relevant to various cellular processes that are regulated by ubiquitination. Further studies should be conducted to elucidate the biophysical consequences of ubiquitination of nonproteolytic processes, as well as to investigate the evolutionary process of selecting ubiquitination sites in various substrates in light of these results.

Methods

There are few protein systems for which the structures are solved and the ubiquitination sites are identified. We used Ubc7 and $p19^{4inkd}$, for which the ubiquitination sites that lead to degradation are well characterized. In addition, we studied the ubiquitination of the *src*-SH3 domain, which is characterized in terms of its folding behavior (Fig. S1). These proteins were ubiquitinated *in silico* at various locations with various ubiquitin moieties: a single ubiquitin or ubiquitin-chain tetramers, internally linked at either K48 or K63. Selected proteins were studied by using molecular dynamics. A thorough investigation of the folding behavior was achieved by using a simple native-topology-based model in which each residue was represented by its C^{α} atom. This model has reproduced various experimental results. It was also used to study protein tethering (47) and protein glycosylation (7). In most cases, interactions between the substrates and the ubiquitin moiety were purely repulsive. However, when studying the effects of nonspecific interactions between the substrate and the ubiquitin moiety, such as in the case of $p19^{4inkd}$, electrostatic interactions were added to the force field as an additional term. These interactions included any pair of charged residues (and phosphorylated residues) and were calculated by using the Debye–Hückel equation as described elsewhere (48). Because ubiquitin is thermally stable, it was not allowed to undergo unfolding and was treated as a rigid body. Details of the simulation model can be found in *SI Text*.

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