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ROLE OF METHIONINE-1 IN UBIQUITIN CONFORMATION AND ACTIVITY*

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Methionine-1 of ubiquitin was oxidized to the sulfone without significant effect on biological activity or conformation at neutral pH. However, at low pH, the oxidized protein expanded to a more open conformation, similar in gel sieving properties to denatured ubiquitin but similar in secondary structure to native ubiquitin. This conformational transition was absent in the native protein. Interpretation of these results in the light of X-ray data suggests that ubiquitin contains two independently folded domains that are held together in part by a hydrogen bond between Met-1 and Lys-63 and which can be separated when this bond is broken. It is suggested that separation of these domains may occur upon ubiquitin conjugation.

Ubiquitin (Ub)[1] has the most highly conserved sequence of any known protein (1). This conservation presumably reflects the fact that it must be recognized by a variety of proteins during the sequence of events associated with Ub-dependent proteolysis (2) and Ub conjugation to chromatin (3) and cell surface receptors (4). The structure of crystalline Ub has recently been reported at the 2.8 Å level of resolution (5). Nonetheless, with the exception of the demonstrated role of Ub's carboxyl-terminal glycine, the site of conjugation to protein amino groups (2,3), the functional role of individual segments of the sequence is unknown.

Ub conformation has been considered highly resistant to unfolding. As monitored by NMR, using either native Ub or its des-Gly-Gly derivative[2], no significant unfolding occurs over the pH range 1-11, below 80°C or at

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[1] Abbreviations used: Ub, ubiquitin; CD, circular dichroism; NMR, nuclear magnetic resonance; SDS, sodium dodecyl sulfate

[2] Des-Gly-Gly ubiquitin is Ub from which the carboxyl-terminal two glycines have been lost. We (unpublished results) and others (18) have found no evidence of conformational differences between native Ub and its des-Gly-Gly derivative as monitored by CD.

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concentrations of guanidine.HCl below 4 M (6,7). On the other hand, oxidation of des-Gly-Gly Ub by chloramine T leads to a decrease in electrophoretic mobility on acetic acid gels suggestive of a partially unfolded state (8). The oxidized residues responsible for the conformational change have not been identified, but a role in this effect for Ub's single methionine (Met-1) was tentatively excluded (8). We have probed the effects of performic acid oxidation on Ub structure and its function in proteolysis. Ub has no cysteine or cystine and, under controlled conditions, Met-1 can be specifically oxidized to the sulfone. Our results demonstrate that oxidation of Met-1 facilitates a major low pH conformational transition that is associated with only limited changes in secondary structure and which suggests an expandable two domain structure for Ub of potential biological significance.

Methods

Preparation of native and modified ubiquitin: Ubiquitin was prepared from human erythrocytes as described (9) and oxidized with performic acid using the procedure of Hirs (10). Amino acid analysis of the final product indicated essentially quantitative conversion of the single Met to the sulfone as the sole amino acid modification. CNBr treatment of Ub was carried out in 0.1 N HCl at room temperature for 20 hrs using the procedure of Schroeder et al. (11).

Studies of ubiquitin function in proteolysis: Rabbit reticulocyte lysate Fraction II was prepared as described (2,9,12). The ability of modified ubiquitin derivatives to substitute for native Ub in the ATP- and Ub-dependent degradation of ^{125}I-casein was monitored using Fraction II (which does not contain Ub) by comparing the increase in ATP-dependent casein degradation induced by the presence of graded concentrations of native and oxidized Ub. Assay procedures were those described elsewhere (9,12). The ability of oxidized Ub to substitute for native Ub in protein conjugation reactions was monitored by incubation of ^{125}I-labelled Ub or modified Ub with lysate Fraction II in the presence of ATP, followed by SDS gel electrophoresis and autoradiography as described (9).

Other methods: Routine electrophoresis of Ub and its derivatives was performed using 15% polyacrylamide gels polymerized in 0.9 M acetic acid and a running buffer of 0.9 M acetic acid according to the method of Panyim and Chalkley (13). Gels typically contained a final urea concentration of 2.5 M (13), but the urea concentration was also varied (Results). Electrophoresis in 15% gels using other buffers was also performed (Results). SDS gel electrophoresis under reducing conditions was performed on 15% gels as described (9). CD studies were performed using a Jobin-Yvon Mark 5 CD spectrometer. Amino acid analyses were performed as described (14).

Results

Behavior of native and performic acid-oxidized ubiquitin on acetic acid-urea gels: Fig. 1 shows the behavior of native and oxidized Ub on "standard"

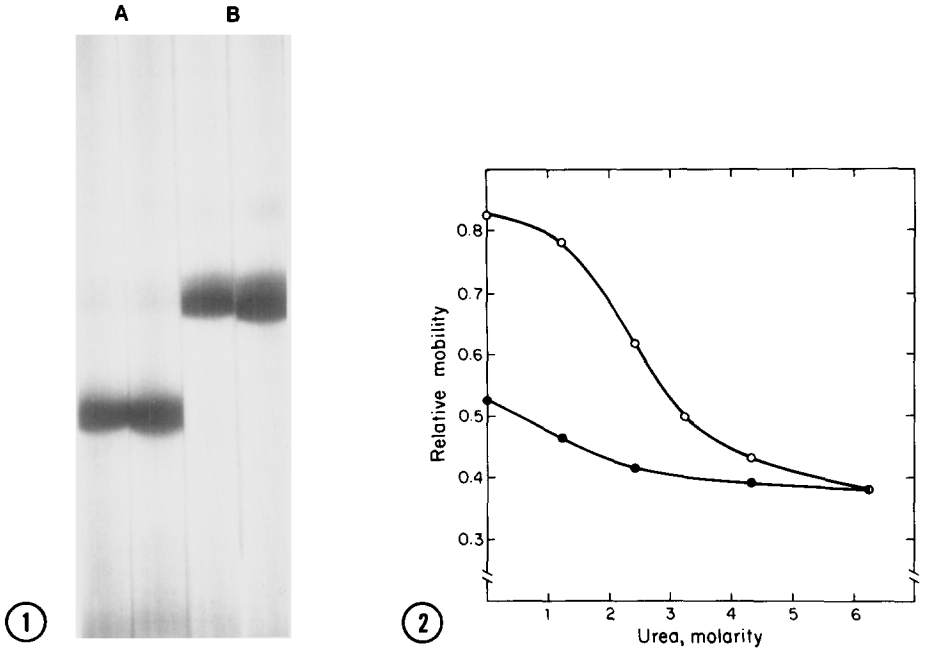

Figure 1. Electrophoretic patterns of native ubiquitin (A) and performic acid-oxidized ubiquitin (B) on acid-urea gels containing 2.5 M urea. Direction of migration is from top to bottom. Gels were stained with Amido black.

Figure 2. Electrophoretic mobilities of native (o) and performic acid-oxidized (•) ubiquitin in 0.9 M acetic acid containing urea at the final concentrations shown. Mobilities are calculated relative to the dye front.

acid-urea gels (2.5 M urea) and indicates a markedly lower mobility for the oxidized protein. Cleavage of Met-1 by CNBr (Methods) produces the same mobility decrease as performic acid oxidation (data not shown) confirming that the effect originates from Met-1. Fig. 2 compares the relative mobilities of native and performic acid-oxidized Ub on acetic acid gels as a function of urea concentration. Differences in mobility of the two proteins are maximal in the absence of urea and decline abruptly between 1.25 and 4 M urea concentration, where the native protein undergoes a major mobility decrease that is absent or diminished in the modified protein. This behavior is analogous to that seen for the products of chloramine T oxidation (8).

The low mobility of oxidized Ub at low urea concentrations is not due to covalent crosslinking since native and oxidized Ub have the same mobilities on SDS gel electrophoresis (data not shown). Additionally, mobility differences between native and oxidized Ub in the absence of urea are pH-dependent.

Table I. Electrophoretic mobilities of native and oxidized ubiquitin in different buffer systems*

pH	Running Buffer	Relative Mobilities		
		Native/Dye	Oxidized/Dye	Oxidized/Native
2.4	0.9 M acetic acid	0.86	0.54	0.63
2.3	1.8 M propionic acid	0.90	0.73	0.83
2.5	0.9 M propionic acid	1.05	0.95	0.91
2.8	0.9 M acetic acid, 0.01 M Na acetate	1.0	1.0	1.0
5.5	0.046 M pyridine, 0.016 M acetic acid	----	----	1.0

* The gel system used was the 15% polyacrylamide system of Panyim and Chalkley (13) modified to use the running buffers indicated by polymerizing the gels to contain the final concentration of ions present in the corresponding running buffer. Mobilities relative to the dye front were obtained using pyronin Y as a tracking dye. The presence or absence of dye was shown not to alter relative mobilities. In the pyridine-acetate gel system, both native and oxidized ubiquitin had relatively low mobilities and no tracking dye was used. Instead, samples were electrophoresed until cytochrome c, in a separate gel, had reached the end of the tube. At this point, ubiquitin had migrated a distance of approximately 30% the gel length.

Using different buffer systems, we observed that the mobilities of the two proteins are identical above pH 3, but that, upon lowering the pH below 2.8, the mobility of oxidized Ub diminishes while that of native Ub is essentially unaltered (Table I); specific buffer effects also appear to be present. These results indicate a low pH conformational expansion of oxidized Ub that is absent in the native protein. Within this context, the different effects of urea on native and oxidized Ub can be explained by the assumption that both achieve a similar unfolded conformation in the presence of 6 M urea, but that the low pH conformation of the native protein in the absence of urea is more compact than that of the oxidized protein.

Circular dichroism studies of native and oxidized ubiquitin: Conformational changes induced by lowering the pH and by the addition of urea at low pH were further probed by CD, using conditions identical to those used for gel electrophoresis. These studies focused on the region above 210 nm where acetic acid and urea offer less interference with light transmission than at

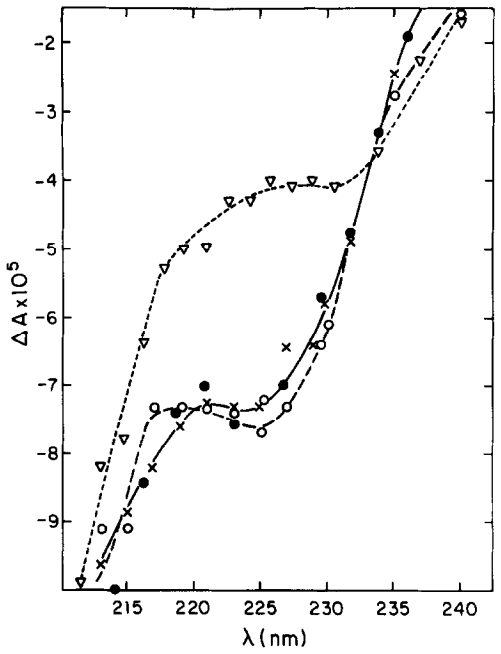

Figure 3. Circular dichroism spectra of native and performic acid-oxidized ubiquitin in 0.2 mm path cells and 0.5 mg/ml concentration. Native (•) and oxidized (o) ubiquitin in 10 mM KCl; x, oxidized ubiquitin in 0.9 M acetic acid; ∇, native ubiquitin in 0.9 M acetic acid, 4 M urea. Spectra of oxidized ubiquitin were obtained on samples diluted from the same stock solution to insure identical concentrations.

lower wavelengths. Native Ub has an α-helix content of ∿16% (5) manifest in part (15) by a distinct shoulder near 225 nm in the CD spectrum (Fig. 3). In accord with earlier studies of Ub stability (Introduction), this shoulder is essentially unchanged in 0.9 M acetic acid (the electrophoresis buffer in Fig. 1 and 2) and is completely lost in 6 M guanidine.HCl (data not shown). In 4 M urea, 0.9 M acetic acid, the intensity of the shoulder is reduced by 40% (Fig. 3), while in 3 M urea (data not shown), the reduction is 20%. The unfolding responsible for the decrease in Ub mobility in 4 M urea at low pH (Fig. 2) is therefore accompanied by a significant loss of α-helix content.

In contrast to the above effects of urea denaturation, the unfolding responsible for the low pH mobility decrease of oxidized Ub in the absence of urea is not associated with significant changes in α-helix content. Thus, in 10 mM KCl, pH 6 (Fig. 3), ellipticities of native and oxidized Ub in the 225 nm region show only subtle differences. Moreover, in 0.9 M acetic acid

(Fig. 3) CD data indicate that the 225 nm region of the oxidized protein is insignificantly reduced in intensity from its normal value or from values for the native protein. As with native Ub, addition of urea to oxidized Ub in acetic acid reduces the intensity of the 225 nm region (data not shown).

The pH of 0.9 M acetic acid is 2.4. We have also compared the CD spectra of native and oxidized Ub at pH 2.0 in dilute HCl in order to obtain better data below 210 nm. Again, no significant differences were observed in the 225 nm region, although subtle differences, not yielding to direct interpretation, were evident below 210 nm. These results therefore demonstrate that, at low pH, performic acid-oxidized Ub undergoes a major molecular expansion in the absence of a significant change in α-helix content. Ub also has a significant content of β-sheet (5) which should contribute to the negative ellipticity above 210 nm (16). Thus, the data also do not suggest any large decrease in β-structure associated with the conformational transition.

Activity of performic acid-oxidized ubiquitin in ubiquitin-dependent proteolysis: The ability of oxidized Ub to participate in Ub-dependent proteolysis was monitored using fraction II of reticulocyte lysates (Methods). Under conditions where the rate of proteolysis was dependent on ubiquitin concentration, addition of oxidized Ub to fraction II led to 96% of the increase in ATP-dependent proteolysis observed with the same concentrations of native Ub. Additionally, both ^{125}I-native Ub and oxidized Ub were equally effective in conjugation to endogenous proteins of fraction II in the presence of ATP and the distribution of both labelled proteins among the different conjugates (9) was similar (data not shown).

Discussion

These results demonstrate that the side-chain of Met-1 is not critical to Ub function. However, at low pH, oxidation of Met-1 to the sulfone facilitates a transition to a more expanded conformation that has less than 70% the electrophoretic mobility of the native protein on polyacrylamide gels. This effect is similar to that induced by chloramine T oxidation (8). In the latter studies, oxidation of Met-1 was excluded as a source of the conformational

change because it was not reversed by mercaptans (8). However, oxidation to the sulfone would not have been reversed under these conditions and may indeed have been contributory.[3]

The most significant feature of the low pH transition is that it differs from the unfolding induced here by urea or by 6 M guanidine.HCl in that it is not associated with the same obvious changes in secondary structure. Thus, oxidized Ub appears capable of major expansion without significant net loss of α-helix or β-structure. These results suggest that Ub contains independently folded domains which can be separated from each other under appropriate conditions without significant unwinding of the individual domains. Interestingly, based on very different considerations, Wilkinson has also suggested the potential for a two domain structure of Ub (17).

The reason that oxidation of Met-1 reduces conformational stability is explained by the X-ray structure of Ub (5). This indicates a hydrogen bond from the backbone -NH of Lys-63 to the Met-1 sulfur. Clearly, oxidation of Met-1 will break or otherwise alter this bond. This effect appears insufficient to open up the conformation at neutral pH, but destabilizes it to facilitate a conformational expansion at low pH. The nature of the low pH expansion and the partial identity of the two postulated domains is also suggested to us by the X-ray structure. Examination of this structure suggests that the Met-1 hydrogen bond helps to stabilize a limited series of interactions between the carboxyl-terminal region (residues 63-76) and the amino-terminal β-sheet region (residues 1-17). Accordingly, we propose that these regions separate from each other at low pH when the hydrogen bond is broken. Additionally, a potential functional significance of such an inter-domain separation is suggested by recent immunological studies. These indicate that Ub conjugated to protein amino groups does not react with antibodies directed against unconjugated native Ub, but does react with antibodies directed against SDS-denatured Ub (18). The latter include a monoclonal antibody specifically

[3] Chloramine T is used for Ub iodination. If it indeed oxidizes Met-1, the similar conjugation patterns noted here for ^{125}I-labelled native and performic acid-oxidized Ub are to be expected.

directed against the region 64-76 (4), indicating that this region is more available in the conjugated state. Therefore, we suggest that separation of the amino-terminal and carboxyl-terminal domains may accompany Ub conjugation. The alternative possibility, that Ub completely unfolds upon conjugation, seems less likely given the demonstrated stability (6,7) of its 2° structure to denaturants.

In sum, studies of the side-chain of Met-1 have demonstrated a new conformation of ubiquitin, characterized by significant molecular expansion with limited change in secondary structure. The results suggest an expandable two-domain structure which may explain changes in ubiquitin properties associated with its conjugation to proteins.

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