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Structural and Functional Changes Associated with Modification of the Ubiquitin Methionine

Shakti Bamezai, Mary Ann T. Banez, and Esther Breslow*

Department of Biochemistry, Cornell University Medical College, New York, New York 10021
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ABSTRACT: The effects of oxidation and cleavage of Met-1 of ubiquitin on conformation and biological activity were individually investigated. Proton NMR studies demonstrated that oxidation to the sulfone led to restricted structural perturbations at neutral pH, particularly in the vicinity of Ile-61. Below pH 3, in the presence of acetic acid, oxidation to the sulfone facilitated a conformational expansion demonstrable by retardation on gel electrophoresis and CD changes below 210 nm. The predominant phase of the low-pH transition did not involve significant changes in α-helix content, indicating the capacity of ubiquitin for limited structural transitions. Cleavage of Met-1 by CNBr, on the other hand, was associated with a global unfolding transition below pH 4 that involved a major loss of α-helix. Differences in the behavior of the native and des-Met proteins at low pH indicate that Met-1 contributes a minimum of 3.4 kcal/mol to the stability of the native conformation. Two Met-1 sulfoxide isomers, of markedly different conformational stability, were formed by treatment of ubiquitin with H_2O_2. One isomer was similar in stability to the sulfone, while the other was intermediate in stability between the sulfone and des-Met proteins, the differences potentially interpretable in terms of the geometry of the Met-1–Lys-63 hydrogen bond. The overall activities of the oxidized and des-Met derivatives in ATP-dependent proteolysis differed subtly from that of native ubiquitin. The unresolved sulfoxides exhibited an approximately 50% increase in activity, while the sulfone and des-Met proteins exhibited a 50% decrease in activity at low concentrations and normal activity at higher concentration. The results demonstrate that Met-1 is not essential to ubiquitin activity, but allow the possibility that it influences activity via its effects on local conformation or conformational stability.

The 76 residues of ubiquitin represent a sequence uniquely conserved in evolution (Ozkaynak et al., 1984). This conservation presumably reflects the multiple components to which ubiquitin must be recognizable during its activation and conjugation to protein in the process of ATP-dependent proteolysis and in other ubiquitin-dependent events (Bush & Goldknopf, 1981; Hershko, 1986; Hershko & Ciechanover, 1986; St. John et al., 1986; Rechsteiner, 1987). The role of the carboxyl terminus of ubiquitin in its activation and subsequent conjugation of proteins has been characterized (Wilkinson & Audhya, 1981; Sobhanadity et al., 1988) and the importance of several other residues investigated by chemical modification and site-specific mutagenesis (Cox & Wilkinson, 1986; Ecker et al., 1987; Chau et al., 1989). Apart from Lys-48 and residues at or near the carboxyl terminus, however, no single residue strictly essential to ubiquitin activity has been localized.

Ubiquitin (Ub)[1] contains a single conserved methionine at position-1 (Schlesinger et al., 1975). Crystallographic analysis indicates that the methionine sulfur is hydrogen bonded to the backbone NH of Lys-63 (Vijay-Kumar et al., 1987). In a preliminary study, this laboratory reported that oxidation to the sulfone was without significant effect on ubiquitin biological activity, but was associated with an unusual low-pH conformational transition (Breslow et al., 1986a). Because this conformational change appeared to represent a molecular expansion unaccompanied by a significant change in α-helix or β-sheet content, it was suggested that ubiquitin contained two domains, the separation of which was facilitated by

[1] Abbreviations: Ub, ubiquitin; HPLC, high-performance liquid chromatography; CD, circular dichroism; NMR, nuclear magnetic resonance; TPCK, *N*-tosylphenylalanine chloromethyl ketone; TSP, sodium 3-(trimethylsilyl)propionate-d_4.

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weakening of the hydrogen bond between Met-1 and Lys-63. It was also suggested that a similar conformational transition might occur upon ubiquitin conjugation. This suggestion was based on immunological evidence that the conformation of conjugated ubiquitin differed from that of native ubiquitin, the conjugated protein reacting with antibodies directed against denatured ubiquitin and not with antibodies directed against the native protein (Haas & Bright, 1985; St. John et al., 1986). A rationale for such a conformational change would invoke the need for ubiquitin-directed proteases to discriminate between free and conjugated ubiquitin.

We have now further investigated the role of Met-1 in modulating ubiquitin conformational stability by preparing and characterizing other derivatives altered at this position. The conformational and functional properties of the sulfoxides and CNBr cleavage products have been investigated, and the nature of the low-pH conformational changes associated with modification of Met-1 has been further explored.

Materials and Methods

Preparation of Modified Ubiquitins. Native ubiquitin was purchased from Sigma. Amino acid analysis and electrophoresis indicated a homogeneous protein of the correct composition and it was used without further purification.

Sulfoxide Synthesis and Characterization. Native ubiquitin (6 mg) was dissolved in 3 mL of 0.2 M acetic acid and 90 μL of 30% H_2O_2 added. The reaction mixture was stirred for 1 h at room temperature, diluted with deionized water, and lyophilized. The lyophilized product was redissolved and relyophilized twice, with a resultant yield of 6 mg of ubiquitin sulfoxide. After hydrolysis in 3 N *p*-toluenesulfonic acid, which prevents conversion of methionine sulfoxide to methionine, amino acid analysis indicated the presence of methionine sulfoxide and the absence of either methionine or the sulfone; other amino acids were normal. The sulfoxide could be reduced back to methionine, as demonstrated by amino acid analysis. For reduction, 1 mg of sulfoxide was dissolved in 2 mL of 1 M NH_4HCO_3 (pH 7.7) in the presence of 2.2 M β-mercaptoethanol in a sealed tube flushed with nitrogen. The reaction mixture was kept at 60 °C for 24 h, diluted with 7 mL of water, and lyophilized. The lyophilized product was redissolved and relyophilized three times before characterization. Treatment of the sulfoxide with mercaptoethanol in the absence of heat failed to give a significant yield of the reduced product.

Gel electrophoretic analysis of the sulfoxide gave a single band at pH 4 with the same mobility as that of native ubiquitin, but gave two well-separated bands of equal intensity under conditions of partial unfolding such as 0.9 M acetic acid (Results) or on acid–urea gels. These are assigned to the two configurational sulfoxide isomers. Reduction completely restored electrophoretic properties to those of unmodified ubiquitin. Failure to reverse the altered mobility of oxidized ubiquitin in studies elsewhere (Loir et al., 1984) may reflect the presence of species other than sulfoxide (Breslow et al., 1986a) and/or the use of milder reduction conditions than used here.

Sulfone Synthesis and Characterization. Native ubiquitin (15 mg) was dissolved in 0.2 mL of a 1:1 mixture of methanol and formic acid and cooled to −5 °C, and 1 mL of performic acid (prepared fresh by stirring a mixture of 950 μL of formic acid and 50 μL of 30% H_2O_2 for 2 h at room temperature) was added. The reaction mixture was stirred for 2.5 h at −5 °C, after which the reaction was stopped by the addition of 0.15 mg of catalase in 15 mL of deionized water, and the resultant mixture was lyophilized. The lyophilized product was chromatographed on Sephadex G-50 in 0.2 M acetic acid and the protein peak relyophilized to give 14 mg of the sulfone. Amino acid analysis of the sulfone, which behaved as a single component when analyzed electrophoretically (Results), indicated the absence of methionine and the presence of 1 residue methionine sulfone; other amino acids were identical with those of the unmodified protein. Control studies in which native ubiquitin was carried through the same procedure, but with the omission of H_2O_2, indicated that the altered physical properties of the sulfone (Results) did not result from nonspecific effects of acid.

Cleavage of Met-1 by CNBr and Purification and Characterization of Des-Met-ubiquitin. The procedure used to prepare des-Met-ubiquitin, unless otherwise specified, was as follows: Ubiquitin (11 mg) was dissolved in 2 mL of 20% formic acid under N_2 and 22 mg of CNBr added. The reaction mixture was stirred for 24 h in the dark at room temperature, diluted with water, and lyophilized. The crude lyophilized product was chromatographed on Sephadex G-50 (fine) in 0.2 M acetic acid. Two protein peaks were obtained, one with the retardation of native ubiquitin chromatographed at the same concentration[2] and a less retarded peak. The peak with the mobility of the native protein was demonstrated to be the sulfoxide by amino acid analysis, by its electrophoretic behavior, which was identical with the sulfoxide in different solvent systems, and by reduction back to native ubiquitin. The less retarded peak was demonstrated to be des-Met-ubiquitin by the absence of methionine or methionine derivatives on amino acid analysis, by mass spectrometry (see below), and by peptide mapping of tryptic digests using the HPLC system of Panneerselvam et al. (1987). Tryptic fragment I of native ubiquitin, representing the sequence Met-1–Lys-6 eluted at 81.5 min and was absent from the des-Met protein. A fragment of retention time 73 min, unique to the des-Met protein, was demonstrated by amino acid analysis, sequencing, and mass spectrometry to represent the sequence Gln-2–Lys-6. Other conditions used to prepare des-Met-ubiquitin included cleavage in 0.1 N HCl with a 10-fold higher CNBr concentration and cleavage in formic acid in the presence of 6.5 M urea.

Electrophoresis of the des-Met protein at pH 4 indicated two components, one with the mobility of native ubiquitin and the second retarded in migration to the cathode by 20%. Cleavage of Met-1 leaves an amino-terminal Gln residue, suggesting that the fast and slow components respectively represent the uncyclized and cyclized (pyroglutamate) forms of the amino-terminal Gln. Sequencing of the slow component, prepared under conditions yielding this component almost exclusively (e.g., CNBr cleavage in 0.1 N HCl at high CNBr concentrations) and further purified by cation-exchange HPLC (using a linear gradient of 0.02% ammonium acetate, pH 3.5, to 0.5 M ammonium acetate, pH 7.6), confirmed that it had a blocked amino terminus. By contrast, sequencing of des-Met-ubiquitin enriched in the faster electrophoretic component gave a high yield of amino-terminal glutamine. Mass spectral analysis of the latter product gave a mass of 8430.8 amu as the principal species, which corresponds to the expected mass

[2] Unmodified ubiquitin shows concentration-dependent behavior on Sephadex-G-50 in 0.2 M acetic acid, the degree of retardation increasing with increasing concentration. This behavior suggests aggregation associated with limited unfolding and exposure of hydrophobic sites under these conditions. Although CD data provide no evidence of ubiquitin unfolding in acetic acid (Results), it is possible that the low pH change in the resolvability of Met-1 protons by NMR (Results) reflects a change sufficient to allow adsorption to Sephadex.

of 8434 amu for the noncyclized des-Met product. A minor component with a mass of 8416 amu, corresponding to the des-Met cyclized product, was also seen. For most of the studies here, the two des-Met products were not resolved, but their relative proportions were analyzed electrophoretically.

Proteolysis Assays. Rabbit reticulocytes were obtained from Pel-Freez. Lysates were prepared as previously described (Breslow et al., 1986b). Fraction II, which contains all factors known to be necessary for ATP-dependent proteolysis except ubiquitin, also was prepared as previously described (Breslow et al., 1986b) with the exception that concentration was achieved by precipitation with $(NH_4)_2SO_4$ according to the method of Ciechanover et al. (1978).

Studies of Alcohol-Induced Helix Formation. Stock solutions of native ubiquitin or the sulfone (9 mg/mL) were prepared in H_2O. Aliquots at pH 2.5 in HCl were diluted into water–2-propanol solutions to give a final protein concentration of 1 mg/mL and a range of 2-propanol concentrations from 25 to 90%. CD spectra were monitored in the far-UV at each 2-propanol concentration. Alcohol-induced CD changes were reversed on lowering the 2-propanol concentration.

Other Methods. Amino acid analyses were routinely performed following 24-h hydrolysis in 6 N HCl at 110 °C. For quantitative analysis of methionine sulfoxide, hydrolysis was performed by substituting 3 N *p*-toluenesulfonic acid for 6 N HCl. Gas-phase sequencing of ubiquitin derivatives was performed with a Model 470A Applied Biosystems sequencer equipped with a Model 120A phenylthiohydantoin analyzer.

Electrophoresis on 15% polyacrylamide gels in acetic acid or lactic acid, in the absence or presence of urea, according to the method of Panyim and Chalkley (1986) was performed using either tubes as described earlier (Breslow et al., 1986a) or slabs. Pyronin Y as was used as the tracking dye. Electrophoresis under nondenaturing conditions at pH 4 was performed in 15% polyacrylamide gels using a continuous system with a running buffer of 0.05 M Tris-HCl adjusted to pH 4.

Circular dichroism studies were performed on a Jobin-Yvon Mark 5 CD spectrometer as previously described (Breslow et al., 1986a). During the course of these studies, improvement of the optical system by replacement of the existing mirrors alllowed improvement in UV penetration so that the sulfone could be studied in absorbing solvents at lower wavelengths than reported earlier. All samples were run at approximately 1 mg/mL in a 0.02-cm path with exact concentrations determined by UV absorbance. In each figure, the spectra shown are normalized to the identical concentration on the basis of the UV data.

Proton NMR studies were performed at 500 MHz. Samples were repeatedly lyophilized from D_2O, perdeuterated acetic acid was added at the concentration indicated, and the pH was adjusted with NaOD or DCl. This procedure leaves a large number of the NH protons unexchanged (Cary et al., 1980), but does not otherwise alter the interpretability of the data. The des-Met protein was relatively insoluble in D_2O at neutral pH and was lyophilized from 0.9 M perdeuterated acetic acid, a procedure that led to complete exchange of NH protons. All spectra are reported in ppm downfield from the internal TSP standard.

Results

Gel Electrophoresis at Low pH Indicates Diminished Conformational Stabilities of Methionine-Modified Ubiquitins. Native and modified ubiquitins show large differences in gel electrophoretic behavior in acetic acid. Figure 1 compares their electrophoretic behavior in 0.9 M acetic acid (pH 2.4) on slab gels. The sulfone and des-Met proteins are retarded by approximately 25%[3] and 55%, respectively, relative to native ubiquitin, and the two sulfoxide isomers (see Materials and Methods) exhibit remarkable differences, one behaving similarly to the sulfone and the second just slightly less retarded than the des-Met protein. The mobility differences between the native and modified proteins are dependent on

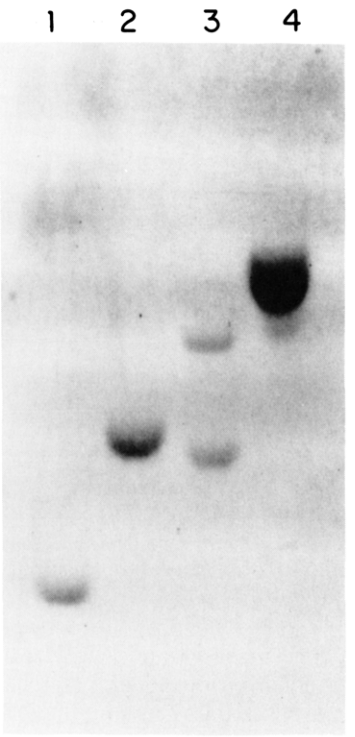

FIGURE 1: Gel electrophoretic behavior of native and modified ubiquitin derivatives in 0.9 M acetic acid in the absence of urea. Direction of migration is from top to bottom. Lane 1, native Ub; lane 2, ubiquitin sulfone; lane 3, ubiquitin sulfoxide; lane 4, des-Met-Ub. Bottom of gel is not shown.

Table I: Electrophoretic Mobilities of Native Ubiquitin and Ubiquitin Sulfone in Different Buffer Systems[a]

pH	running buffer	relative mobilities: native/dye	sulfone/dye	native/sulfone
3.3	0.9 M acetic acid, 0.025 M Tris	0.80	0.81	0.99
3.0	0.9 M acetic acid, 0.012 M Tris	0.88	0.83	1.06
2.6	0.9 M acetic acid, 0.006 M Tris	0.86	0.86	1.00
2.4	0.9 M acetic acid	0.84	0.74	1.14
2.2	1.8 M acetic acid	0.87	0.60	1.45
2.0	0.9 M lactic acid, 0.025 M Tris	0.98	0.85	1.16
1.8	0.45 M lactic acid	1.05	0.76	1.37
1.5	0.9 M lactic acid	1.18	0.79	1.50

[a] Data were obtained by using 15% polyacrylamide tube gels as described further in Materials and Methods.

[3] Retardation of the sulfone relative to the native protein under these conditions has been reported previously (Breslow et al., 1986a), but the differences now found are significantly less than those seen originally. We have since learned that quantitative mobilities of the different ubiquitin preparations are sensitive to gel preparation, varying with such factors as the length of preelectrophoresis and thickness of the gel (tube vs slab gels, etc.). However, the qualitative electrophoretic relationships among the different derivatives is independent of these variables. In studies reported here, where mobilities under different buffer conditions are compared, other variables were held constant.

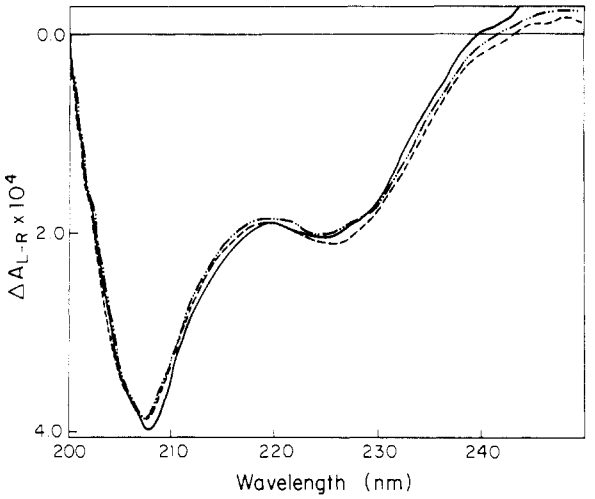

FIGURE 2: Circular dichroism comparison of native and oxidized ubiquitin at pH 7 in 0.05 M ammonium acetate. Data were obtained at 1.0 mg/mL with a 0.02-cm light path. —, native Ub; - - -, ubiquitin sulfoxide; – ·· –, ubiquitin sulfone.

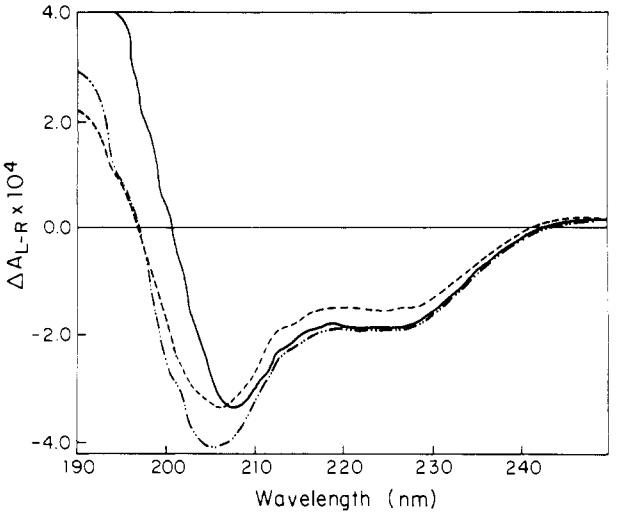

FIGURE 3: Circular dichroism comparison of native and oxidized ubiquitin in 0.9 M acetic acid, pH 2.4. Data were obtained with a 0.02-cm path and are normalized to 0.86 mg/mL (0.1 mM). —, native Ub; - - -, ubiquitin sulfoxide; – ·· –, ubiquitin sulfone.

pH, total acid concentration, and acid identity. This was monitored electrophoretically for the sulfone in tube gels, keeping other known variables constant (Table I). Increasing the acetic acid concentration from 0.9 (pH 2.4) to 1.8 M (pH 2.1) increased the relative electrophoretic retardation of the sulfone from 14% to 50%. At constant (0.9 M) acetic acid concentration, increasing the pH from 2.4 to 3 obliterated mobility differences between the native protein and the sulfone. In lactic acid, lower pH values were required to induce electrophoretic differences between the native protein and sulfone than in acetic acid.

The different mobilities of the native and modified proteins at low pH in acetic acid reflect conformational changes in the modified protein at low pH, assignable to an increase in molecular size, that are absent in the native protein. This can be seen from protein mobilities relative to the pyronin Y dye front (Table I). Since pyronin Y does not titrate below pH 7 (we have independently determined its pK_a as 11.1), changes in protein mobility relative to dye reflect changes in the protein. The data in Table I indicate that, between pH 2.6 and 3, the native protein and sulfone have similar mobilities. Between pH 2.6 and 2.2, the mobility of the native protein is constant, while that of the modified protein diminishes. In lactic acid, the effects appear more complex. Effects of pH and acid identity are explored further below.

Native and modified proteins were also studied on acid–urea gels as described under Materials and Methods (data not shown). The same relative stabilities to unfolding among the different derivatives were seen under these conditions as in acetic acid alone.

Circular Dichroism Comparison of Native and Modified Ubiquitin. Electrophoretic differences between the native and modified ubiquitins at low pH in acetic acid are paralleled by circular dichroism differences. At neutral pH, the far-ultraviolet CD spectra of native ubiquitin, the sulfone, and the sulfoxides are almost indistinguishable (Figure 2), while significant differences are present at pH 2.4 in 0.9 M acetic acid (Figure 3). At the lower pH, the sulfone spectrum remains the same as that of native ubiquitin above 210 nm, consistent with a lack of difference in α-helix content (Breslow et al., 1986a), but the 208-nm negative peak seen at neutral pH, or in native ubiquitin, reproducibly shifts to 206 nm and marked differences from native ubiquitin are seen at shorter wavelengths. The lack of difference between native ubiquitin and the sulfone under these conditions above 210 nm has been noted previously (Breslow et al., 1986a), but spectra were not previously compared at shorter wavelengths in acetic acid because of earlier light transmission problems (see also Materials and Methods). Differences between the sulfone and native protein are eliminated by increasing the pH of 0.9 M acetic acid to 3 (data not shown), in accord with the normalization of electrophoretic mobility under similar conditions (vide supra). The change at pH 3 is a pH effect and not a simple salt effect since addition of NaCl at pH 2.4 has no effect on the spectrum. CD differences between the native protein and sulfone in acetic acid are not paralleled in HCl. When the native protein and sulfone are compared at low pH in HCl, minor differences are seen below 210 nm that are nonidentical with those in acetic acid and that appear to reflect events that occur above pH 3.

Sulfoxide spectra in 0.9 M acetic acid (Figure 3) show 206-nm minima similar to that of the sulfone, but exhibit a reduction in negative ellipticity at 225 nm indicative of a decrease in α-helix content. Differences between the sulfoxide(s) and sulfone are consistent with the greater electrophoretic retardation, representing a greater degree of unfolding, of one of the sulfoxide isomers under these conditions.

Analysis of the CD spectrum of the des-Met protein at neutral pH is complicated by low solubility. The best data (not shown) indicate slightly reduced ellipticity at 225 nm and a slight shift in the 208-nm negative extremum to shorter wavelengths relative to native ubiquitin, representing either a difference in average conformation or the presence of a small amount of irreversibly denatured protein. Data in 0.9 M acetic acid, in which the des-Met protein is soluble, accord with the electrophoresis data in indicating major unfolding relative to either the native or oxidized proteins (Figure 4). In 0.9 M acetic acid, the des-Met protein exhibits a minimum at 201–202 nm and 225-nm ellipticity is reduced by approximately 40% relative to native ubiquitin. Titration of the des-Met protein in 0.9 M acetic acid with base indicates that the low-pH changes are reversed by pH 5 (Figure 4, inset). In contrast to the sulfone and sulfoxides, the same low-pH differences between the des-Met and native protein are also manifest in HCl. The 225-nm ellipticity of the des-Met protein at pH 2 in HCl is the same as that in 0.9 M acetic acid, while the native protein exhibits no change in this region between neutral pH and pH values as low as 0.5 (data not shown).

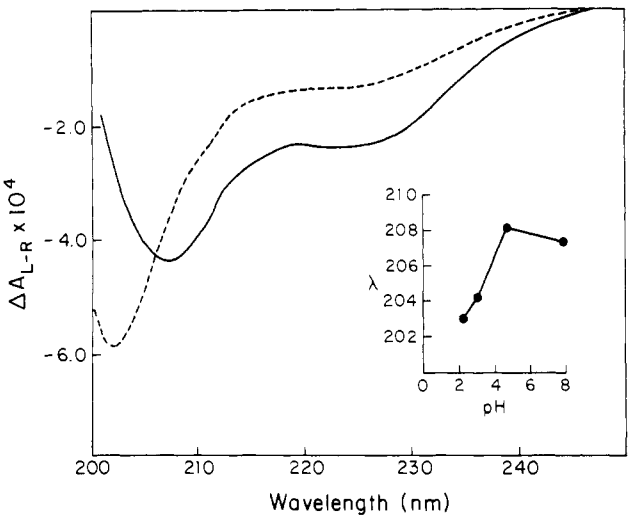

FIGURE 4: Circular dichroism comparison of native and des-Met-ubiquitin in 0.9 M acetic acid, pH 2.4. Data are normalized to 1.1 mg/mL (0.125 mM) in a 0.02-cm light path. —, native Ub; ---, des-Met-Ub. Inset shows the change in wavelength of the low-wavelength trough of des-Met-Ub as a function of pH in 0.9 M acetic acid.

The spectra in Figures 3 and 4 indicate that the conformations of the sulfone and des-Met proteins induced by 0.9 M acetic acid are fundamentally different. This is most clearly evidenced by the 207-nm crossover point between the spectra of the native and des-Met proteins that is consistently absent from the corresponding spectra of the native protein and sulfone. Such a crossover point is also seen with the sulfoxides, again consistent with a closer similarity of one of the sulfoxide forms than of the sulfone to the des-Met protein.

Effect of Methionine Modification on Alcohol-Induced Helix Formation. The α-helix content of native ubiquitin increases in a biphasic manner in the presence of organic alcohols, reflecting the influence of the decreased dieletric constant (Wilkinson & Mayer, 1986). Comparison of the effects of dielectric constant change on the native protein and the sulfone indicates that the sulfone transition occurs at higher dielectric constant than that of the native protein. The first 2-propanol-induced folding transition for native ubiquitin occurred with a dielectric constant midpoint of 63 under our conditions (Materials and Methods), while that for the sulfone had a midpoint of 68. This result differs from the lack of effect of methionine oxidation on this transition reported elsewhere (Wilkinson & Mayer, 1986), but is significantly principally because it indicates the decreased conformational constraints in the oxidized protein.

Proton NMR Comparisons of Native and Methionine-Modified Ubiquitin. Proton NMR studies demonstrate local structural differences between the native protein and sulfone at all pH values not seen by CD and support the thesis (Breslow et al., 1986a) that the altered low-pH CD spectrum of the sulfone can involve only a minor conformational rearrangement. These studies incidentally demonstrate a new aspect of the behavior of native ubiquitin.

Figure 5 compares segments of the aliphatic proton spectra of native ubiquitin and the sulfone in 0.9 M acetic acid at pH 3, where their conformations are the same as monitored by CD and electrophoresis, and at pH 2.3, where their average conformations differ. Since NMR spectra were obtained in D_2O, it is relevant that CD spectra indicate the same low-pH differences between the sulfone and native protein in D_2O as in H_2O. At pH 3, the two proteins show localized differences in the aliphatic region (selectively indicated by lines). Of these,

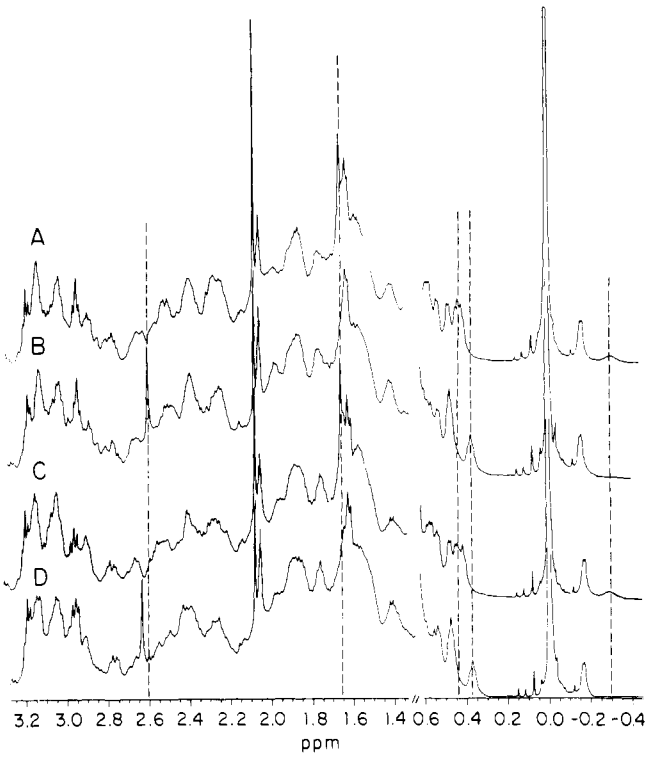

FIGURE 5: Comparison of selected regions of the aliphatic proton NMR spectra of native ubiquitin and ubiquitin sulfone in 0.9 M acetic acid at pH 2.3 and 3.0. Abscissa is in ppm downfield from TSP. From top to bottom: A, native Ub, pH 3.0; B, ubiquitin sulfone, pH 3.0; C, native Ub, pH 2.3; D, ubiquitin sulfone, pH 2.3. Lines define constant chemical shift for peaks discussed in the text.

two are interpretable, reflecting differences in methionine CH_3 protons and those of Ile-61. With respect to Met-1, a singlet peak at 2.60 ppm in the sulfone, corresponding in intensity to approximately three protons, is absent in the native protein (and can also be shown to be absent in the des-Met protein and sulfoxides) and is assigned to the methionine sulfone CH_3 group. These protons are located at 3.16 ppm in the sulfone of the free amino acid (data not shown). The position of the methionine CH_3 protons in the native protein is also relevant. This peak is unresolvable at higher pH [Di Stefano and Wand (1987) and Weber et al. (1987); unpublished observations]. However, a sharp peak at 1.66 ppm in the pH 3 spectrum of the native protein is absent in the sulfone (and also absent in the des-Met protein and sulfoxides) and is accordingly assigned to the methionine CH_3. The assignment is consistent with the fact that this chemical shift is 0.5 ppm upfield from methionine CH_3 protons in unstructured peptides (Bundi & Wüthrich, 1979), analogous to the displacement of the corresponding sulfone proteins from those in the free amino acid (vide supra). Additionally, in the sulfoxide, the 2.6 and 1.66 ppm peaks of the sulfone and native proteins, respectively, are replaced by two new peaks, at 2.14 and 2.28 ppm, each of approximately half the intensity of the 2.6 ppm sulfone peak, in keeping with these assignments (data not shown). The appearance of a peak at pH 3 in the native protein assignable to the methionine CH_3, which is absent at pH 6, indicates a conformational change in the native protein amino terminus between pH 6 and 3 not previously observed.

Several resonances assigned to Ile-61 (Di Stefano & Wand, 1987; Weber et al., 1987), particularly those located at −0.32, +0.4, and +0.42 ppm in the native protein, are shifted in the sulfone at pH 3 (Figure 5); comparable differences between the sulfone and native protein can also be seen at neutral pH (data not shown). The large upfield shift of these resonances

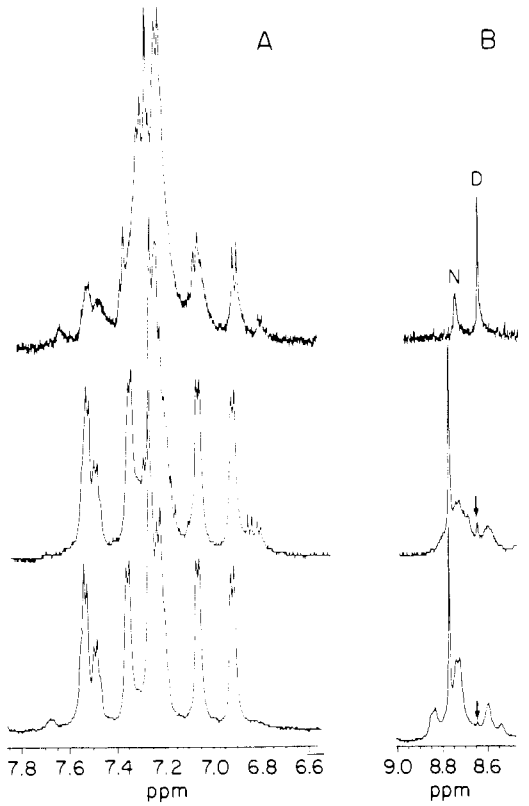

FIGURE 6: Comparison of aromatic proton NMR spectra of native ubiquitin, ubiquitin sulfone and des-Met-Ub in 0.9 M acetic acid at pH 2.3. Abscissa is in ppm downfield from TSP. (A) 6.6–7.8 ppm region; B) 8.5–9.0 ppm region. Arrows indicate chemical shift of His C-2 proton in the completely unfolded (D) state. From top to bottom: des-Met-Ub; ubiquitin sulfone; native Ub.

in the native protein reflects their shielding by the ring of Phe-45 (Vijay-Kumar et al., 1987).[4] Accordingly, the change in these resonances in the sulfone indicates a change in the orientation of Ile-61 relative to Phe-45 associated with alteration of the Met-1–Lys-63 hydrogen bond. Note that the Met-1 sulfur appears too distant from the side chain of Ile-61 as judged by the crystal structure (Vijay-Kumar et al., 1987) for the sulfone to directly perturb its protons. It is of interest that Ile-61 protons are also shifted relative to the native protein in the folded conformations of the des-Met protein and sulfoxide, but differently from that in the sulfone. For example, the −0.32 ppm band of the native protein, which is shifted to an unassigned position in the sulfone, can be located still further upfield at −0.48 ppm in the folded des-Met protein at pH 4 (data not shown).

At pH 2.3, aliphatic proton spectra of both the native protein and sulfone (Figure 5) change slightly relative to pH 3, reflecting some protonation over this pH interval. However, differences between the two proteins are altered so slightly that most changes are unassignable. The clearest change is a downfield shift of the methionine sulfone CH_3 protein from 2.60 ppm at pH 3 to 2.63 ppm at pH 2.3. The peak assigned to the methionine CH_3 of the native protein (1.66 ppm) does not shift over this pH interval.

Figure 6 compares the aromatic proton spectra of the sulfone and native protein with that of des-Met-ubiquitin at pH 2.3 in 0.9 M acetic acid. The principal difference between the sulfone and native protein is a large change in peak shape near 7.25 ppm, reflecting environmental changes in either Phe-4 or Tyr-59 ring protons [protons in this region have been assigned by Di Stefano and Wand (1987) and Weber et al. (1987)], but these differences can also be seen at pH 3 (not shown). The sole pH-induced differences are small sharp peaks in the sulfone, particularly evident near 7.2 and 7.3 ppm, representing one or more minor new species in slow chemical exchange with the folded form. One of these species is assigned to the completely unfolded form, as evidenced by comparison with the largely unfolded des-Met protein in 0.9 M acetic acid (Figure 6). The existence and fraction of completely unfolded form present can be calculated most clearly from examination of the C-2 proton resonance of His-68. This resonance shifts from 8.77 to 8.66 ppm upon thermal unfolding at pH 3.9 (Cary et al., 1980). In the des-Met protein (Figure 6B), 35% of the C-2 proton intensity is located at 8.73 ppm (folded form) and 65% at 8.63 ppm (unfolded form), indicating 65% unfolding. In both the native protein and sulfone, the C-2 proton of the folded form is located at 8.74 ppm at pH 3 (not shown) and at 8.76 ppm at pH 2.3 (Figure 6B), atop a background of nonexchanged peptide bond NH protons. However, at pH 2.3, a very small peak is evident at 8.65 ppm in the sulfone that is absent at pH 3 and barely perceptible in the native protein at pH 2.3; this is assigned to the unfolded form. Examination of the relative intensities of the 8.65 and 8.76 ppm protons indicates the presence of 7% unfolded protein in the sulfone and 1% in the native protein under these conditions. The relative intensities of the other sharp bands are generally consistent with this conclusion. NMR spectra of the sulfone in 1.8 M acetic acid (not shown) are similar to those obtained in 0.9 M acetic acid with the exception that the content of completely unfolded protein is increased to 11%.

It is significant that the unfolded form is only a minor contributor to the properties of the sulfone in 0.9 M acetic acid. This accords with the CD results (vide supra), which indicate a conformation for the sulfone under these conditions that is intrinsically different from the mixture of native and totally unfolded species represented by the des-Met protein.

Biological Activity of Methionine-Modified Ubiquitins. The ability of native and modified ubiquitins to restore ATP-dependent proteolysis to lysates depleted of endogenous ubiquitin (i.e., to fraction II of reticulocyte lysates) was measured (Table II). Within a rather large experimental variation, the sulfoxide appeared more active than the native protein at both 5 (subsaturating concentration) and 10 μg/mL. Because the sulfoxide is an equimolar mixture of two isomers, the results are consistent with the possibility that the activity of one isomer

Table II. Relative Activity of Methionine-Modified Ubiquitins in ATP-Dependent Proteolysis[a]

derivative	concn (μg/mL)	rel activity (%)
sulfone	5	46 ± 16
sulfone	10	104 ± 45
des-Met-ubiquitin[b]	0.5	67 ± 18
des-Met-ubiquitin[b]	5	43 ± 6
des-Met-ubiquitin[b]	20	113 ± 15
sulfoxide	5	152 ± 40
sulfoxide	10	177 ± 51

[a] The ability of native and modified ubiquitins to restore ATP-dependent protelysis in fraction II of reticulocyte lysates was compared with ^{125}I-labeled casein as substrate (Materials and Methods). The activity of each derivative relative to that of the same concentration of native ubiquitin in the same experiment is given. Results represent the average of data obtained in two separate studies. Within each study, data were averaged over readings obtained at 60 and 90 min. Results for des-Met-ubiquitin at 0.5 μg/mL respresent data from a single study. [b] The sample of des-Met-ubiquitin used for biological assay contained approximately 50% of the pyroglutamate (N-terminal cyclized) form.

[4] The γ-carbon of Ile-61 is 3.8 Å above the center of the ring of Phe-45 [Vijay-Kumar et al. (1987) and personal communication].

is more than twice that of unmodified ubiquitin. Both the des-Met protein and sulfone exhibited decreased activity relative to the native protein at low concentrations, but were indistinguishable from the native protein at high concentration. Studies of the ability of ^{125}I-labeled native and modified ubiquitins to form protein conjugates (at high ubiquitin concentrations) suggest that the steady-state level of conjugates formed with the sulfone is lower than that of the other derivatives, raising the possibility that the sulfone might be conjugated less efficiently than the other derivatives, but that its conjugates are degraded more rapidly (data not shown).

Discussion

The results demonstrate that Met-1 is not essential to the role of ubiquitin in ATP-dependent proteolysis, but suggest that the methionine influences recognition, perhaps in part by its effects on ubiquitin conformation or conformational stability. At neutral pH, the global conformations of native and modified ubiquitin are alike, but the modified proteins show local differences in the vicinity of Ile-61, as demonstrated by NMR and diminished conformational stability as evidenced by their low-pH behavior.

At least two types of conformational change at low pH are facilitated by alterations of Met-1. In the des-Met product, the predominant change is an unfolding below pH 4 characterized by a significant loss of α-helix and major change in the proton NMR spectrum. The unfolding is pronounced in the CD spectrum at pH values at least as high as 3 and is not evident in the native protein at pH values as low as 0.5, indicating that Met-1 contributes a minimum of 3.4 kcal/mol to ubiquitin stability. On the other hand, oxidation of the Met-1 sulfur to the sulfone predominantly facilitates an initial low-pH conformational expansion that does not involve a significant loss of α-helix content and that is also distinguishable from the unfolding seen in the des-Met protein by its apparent dependence on the presence of an organic acid and modest effects on the proton NMR spectrum. The role of organic acid is unclear, the acid potentially either altering solvent structure or directly binding to the altered form of the protein. Oxidation of Met-1 to the sulfoxide also destabilizes the native conformation, with a pronounced difference in stability of the two sulfoxide isomers. As monitored by gel electrophoresis and supported by CD, one isomer is similar to the sulfone in stability, while the other is intermediate in stability between the sulfone and the markedly less stable des-Met derivative.

The stability relationships among the different ubiquitin derivatives in acetic acid reflect differences in bonding at Met-1. The greatest destabilization is seen with the des-Met protein, in which the hydrogen bond between the methionine sulfur and the backbone NH of Lys-63 is lost, together with β-sheet hydrogen bonds (Vijay-Kumar et al., 1987) involving the Met-1 amino and carbonyl. In the sulfone, only interactions at the sulfur are necessarily altered. We suggest that the sulfur–NH hydrogen bond of the native protein is replaced by a very weak oxygen–hydrogen bond in the sulfone (Figure 7), the weakness of the bond reflecting the weak hydrogen-bonding properties of sulfone (Durst, 1979). Additionally, insertion of the sulfone oxygen between the sulfur and the Lys-63 NH necessitates a local conformational change, manifest in the NMR spectrum by changes in the chemical shift of the protons of Ile-61, the overall effect destabilizing the native conformation. At the sulfoxide level of oxidation (Figure 7), one of the sulfoxide isomers should present an oxygen in the correct position for hydrogen bonding to the NH, as in the sulfone. We suggest that this is the more stable of

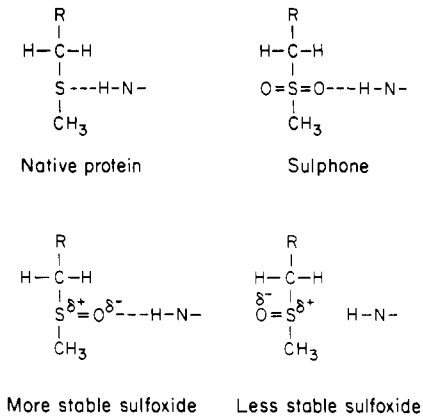

FIGURE 7: Schematic representation of possible orientations of the methionine sulfur in native ubiquitin, ubiquitin sulfone, and the two sulfoxide isomers. Note that bonds to sulfur are depicted as planar for simplicity, but are tetrahedral.

the isomers and that the effects of the normally stronger hydrogen bonds formed by sulfoxides relative to sulfones (Durst, 1979) are, in this case, offset by other differences between sulfones and sulfoxides; e.g., the partial positive charge on the sulfoxide sulfur should be destabilizing in a nonpolar environment. In the less stable sulfoxide (Figure 7), the oxygen is proposed to be on the wrong face of the sulfur to participate in hydrogen bonding to the NH, with a net dipole repulsion between the positively charged sulfur and the NH proton. The results additionally imply limited freedom of rotation around the ubiquitin C–S bonds.

The conformational changes that occur in the sulfone below pH 3, but which are absent in the native protein, argue that there is at least one low pK_a carboxyl group in the native protein, the protonation of which in the sulfone is associated with conformational change. This is not the α-carboxyl group, which is completely exposed in the native protein (Vijay-Kumar et al., 1987). Moreover, excision of the two carboxyl-terminal residues is without effect on the conformational properties of either the native protein or sulfone (unpublished results). The low pK_a carboxyl is therefore likely to be one (or more) of the four carboxyl groups (Asp-21, -52, and -58 and Glu-34) involved in intramolecular hydrogen bonds or salt bridges (Vijay-Kumar et al., 1987) in the native conformation. It was previously suggested that the sulfone conformational change involved separation of two ubiquitin domains (Breslow et al., 1986a). While the refined crystal structure of ubiquitin (Vijay-Kumar et al., 1987) does not indicate any clearly delineated domains, both the pattern of ubiquitin folding in the presence of organic alcohols (Wilkinson & Mayer, 1986) and the expanded, but still largely folded, low-pH conformation of the sulfone seen here indicate that limited conformational transitions can occur and suggest some degree of autonomy between different regions of the molecule. The CD and NMR data place constraints on the potential nature of the initial low-pH sulfone transition. In particular, the CD data, taken together with secondary structure reference spectra (Chang et al., 1978; Brahms & Brahms, 1980), allow only limited changes in β-sheet or β-turns if opposing spectral transitions do not cancel each other. An interesting candidate for the region involved is the series of reverse turns between Phe-45 and Ser-65 that includes both Asp-58 and Lys-63 (Vijay-Kumar et al., 1987). The integrity of one or more of these turns, compromised at neutral pH by the altered Met-1–Lys-63 bond in the sulfone, might be lost at low pH upon protonation of Asp-58. However, the NMR data suggest that the steric relationship between Ile-61 and Phe-45, while altered in the

sulfone, is not affected by the low-pH transition; i.e., no further changes in upfield Ile-61 resonances at 0.36 ppm are seen on lowering the pH from 3 to 2.3 (Figure 6) and the aromatic protons of Phe-45 and Tyr-59 (Di Stefano & Wand, 1987; Weber et al., 1987) also appear unaffected. Therefore, any change in this region is limited.

Conjugated ubiquitin reacts with antibodies directed against denatured ubiquitin approximately 10 times more strongly than does native ubiquitin (Haas & Bright, 1985; St. John et al., 1986), suggesting that ubiquitin might be partially unfolded by conjugation and that this unfolding is important to its activity. A genetically engineered disulfide, placed between residues 4 and 66 of ubiquitin, diminishes ubiquitin biological activity, and it has been suggested this disulfide prevents a needed conformational change (Ecker et al., 1989). Accordingly, it might be expected that increasing the ease of ubiquitin denaturation, particularly by weakening of the linkage between the segments containing residues 4 and 66 via alteration of the Met-1–Lys-63 hydrogen bond, would enhance biological activity. Some support for this thesis is found in the weakly enhanced activity of the sulfoxide, but is less evident in the overall activity of the sulfone and des-Met proteins. Therefore, either the ubiquitin unfolding associated with conjugation is different from that facilitated by methionine modification or the facilitation of conformational change associated with methionine modification is offset by diminished recognition of the ubiquitin amino terminus by one or more factors in the proteolytic pathway. Determination of the relative activities of the different modified ubiquitins in the individual steps of the proteolysis pathway should prove useful in further elucidating the potential role of ubiquitin unfolding in proteolysis.

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