

Ubiquitin Function Studied by Disulfide Engineering*

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Disulfide engineering was used to probe the role of conformational mobility in ubiquitin-mediated proteolysis. Six genes that encode cysteine-containing mutants of ubiquitin were constructed, expressed in *Escherichia coli* and the proteins purified. Single cysteine-containing mutants and a 4/14 disulfide were active in degradation of a substrate protein *in vitro*, while the 4/66 disulfide, which cross-links the NH₂- and COOH-terminal strands of the protein, was only 20–30% active. The solution structure of the 4/66 mutant was solved: the disulfide is left-handed with no perturbations in the backbone from that of wild type ubiquitin. The results suggest that conformational mobility is required for the activity of ubiquitin in signaling proteolysis.

Conformational changes in proteins are essential for the action of many enzymes and signal transducing receptors (1, 2). Dynamic changes in proteins, however, are often difficult to study under conditions of biological activity. A technique that is now available to study conformational changes is insertion of cysteine at strategic locations in the primary sequence by site-directed mutagenesis. If pairs of cysteines are inserted at positions that are spatially close but widely spaced in the primary sequence, the cysteines may oxidize to form a covalent disulfide bond, which severely constrains the flexibility of the protein in solution. Engineered disulfides have recently been introduced into several proteins in attempts to increase their stability (reviewed in Ref. 3), but not to study the effects of conformational restriction in enzymatic reactions. Cysteines, however, have been introduced into a membrane-bound receptor protein to probe conformational changes upon ligand binding by trapping the conformational intermediates as disulfides (2).

Here we use disulfide engineering to study the possible role of conformational mobility in ubiquitin, a small protein involved in cytoplasmic protein degradation (4–6). In a series of enzymatic steps, ubiquitin is covalently attached at its carboxyl terminus to the amino terminus and amino groups of lysine side chains on target proteins. Ubiquitinated proteins may be stably modified, de-ubiquitinated, or degraded by endoproteases that recognize ubiquitinated proteins. It has been proposed that conformational changes in ubiquitin, while conjugated to the target protein, determine whether a target protein is stable, de-ubiquitinated, or degraded (6, 7, 16, 17). To test this hypothesis, we inserted disulfide bonds

in ubiquitin via modular mutagenesis (10, 18). Ubiquitin is an ideal protein for disulfide engineering studies because its structure is known at high resolution from crystallographic (8) and NMR studies (see below) and because it does not naturally contain any cysteine residues. We constructed and expressed genes encoding six cysteine-containing mutants of ubiquitin, three of which formed disulfide bonds. All of the mutants were activated by E1, but substantial differences between the two disulfide mutants were seen in the *in vitro* degradation assay. The three-dimensional structure of the disulfide mutant (4/66) was determined by NMR methods. These results are discussed in terms of the current models for the mechanism of ubiquitin-targeted proteolysis.

MATERIALS AND METHODS

Gene Construction, Mutagenesis, and Expression—Construction of a cassette adapted human ubiquitin gene and the strategy for modular mutagenesis was described previously (10, 18). To generate the six new mutants described in this study, modules M1, M2, and M7 of the synthetic human ubiquitin gene were replaced with synthetic DNA that encodes the desired amino acid substitutions (Table I). Double mutations were constructed by module switching as previously described (Fig. 2 in Ref. 18). The DNA sequence of each of the mutagenized genes was confirmed by sequencing directly the double-stranded plasmids as previously described (18). The mutant genes were inserted into vectors for expression in *Escherichia coli* under the heat inducible P_L promoter (pNMHUB, Ref. 18). Expression and purification of the mutant genes in *E. coli* was done as previously described (18), except that 100 mM DTT¹ was added to the crude *E. coli* lysate to prevent cross-linking of the expressed ubiquitin mutants to *E. coli* proteins.

Disulfide Modeling and NMR Structure Determination—Two disulfide mutations were modeled using the native ubiquitin crystal structure (8) coordinates on an Evans and Sutherland PS330 graphics system running the MOGLI software package. Cysteines were placed at positions Phe⁴ and Thr¹⁴ or Phe⁴ and Thr⁶⁶ in conformations that allowed for the disulfide to be formed. No attempts were made to put them in “preferred” conformations. The two modeled disulfide mutants were then energy minimized using AMBER 3.0 (developed by U. C. Singh, P. K. Weiner, J. Caldwell, and P. A. Kollman, University of California, San Francisco) for 1000 steps (*in vacuo*) using the all atom potentials, a 6-Å residue-based non-bonded cutoff, a distance-dependent (1/r⁴) dielectric with no additional constraints on the disulfide geometry. Next 2 ps of molecular dynamics at 300 K using the same parameterization was performed for each structure, followed by minimization. The dynamics runs were sufficiently long for the disulfide side chains to rearrange into relatively low-energy conformations.

The structure of the 4/66 disulfide was calculated independently from NMR data (12). NMR resonance assignments of the 4/66 mutant were obtained by comparison to those of wild type ubiquitin (13). The mutant structures were determined using about 650 nuclear Overhauser effect distance constraints, 35 hydrogen bond constraints, and 20 × 1 torsion angle constraints. The constraints were used for

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¹ The abbreviations used are: DTT, dithiothreitol; SDS, sodium dodecyl sulfate; DTNB, 5,5'-dithio-(2-nitrobenzoic acid); BSA, bovine serum albumin.

distance geometry calculations using the program DSPACE (9) (licensed from Hare Research, Inc., Woodinville, WA). Four distance geometry structures were further optimized against the experimental constraints using AMBER (see Ref. 9 for further information on the NMR, distance geometry, and AMBER methods).

Biochemical Characterization of Mutant Proteins—Amino composition analysis of purified ubiquitin mutants was performed on a Beckman amino acid analyzer. The ubiquitin-activating enzyme E1 was purified from bovine reticulocytes and the PP_i-ATP exchange assay was performed as described previously (18). Preparation of enzymes which support the *in vitro* ubiquitination and degradation of exogenous proteins were made and degradation assays were performed as previously described (18). Polyclonal antibodies to ubiquitin (isolated from bovine blood) were raised in rabbits and affinity purified on an Affi-Gel column (Bio-Rad) that contained immobilized ubiquitin. Immunochemical detection of ubiquitin was done by electrophoretically transferring proteins from SDS gels to nitrocellulose and then binding by anti-ubiquitin antibodies followed by [¹²⁵I]iodinated protein A (Amersham) and autoradiography.

Protein determinations for the ubiquitin mutants were done by measuring the tyrosine absorbance at 280 nm of the purified proteins ($A_{1\%} = 1.6$) and by gel electrophoresis followed by Coomassie staining and densitometric scanning.

Reduced sulfhydryls on the purified proteins were measured using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent) as follows: DTNB (10 mg) was dissolved in 9 ml of water and titrated into solution with 1 ml of 1 M sodium acetate. Immediately before addition to the protein, an aliquot of this stock DTNB solution was diluted 100-fold with a buffer solution containing TRIS (100 mM) and EDTA (1 mM), pH 8.0. Protein samples in 100 μ l of water were reacted with 400 μ l of the pH 8.0 DTNB solution and vortexed. The solution was transferred to a cuvette and the A_{412} was followed with time until it stabilized. Titratable sulfhydryls in the proteins were determined by using cysteine as a primary standard ($\epsilon_M = 13,600$). Alternatively, sulfhydryls were titrated with [¹⁴C]ethylmaleimide (Du Pont-New England Nuclear) in sodium phosphate buffer, pH 7.0, followed by removal of unincorporated sulfhydryl reagent by gel filtration with Sephadex G-25 (Pharmacia LKB Biotechnology Inc.) and liquid scintillation counting.

RESULTS

Investigation of the ubiquitin structure (8) revealed two promising sites for disulfide insertion. Residues Phe⁴, Thr¹⁴, and Thr⁶⁶ could form disulfide bridges across anti-parallel β -strands (4/14) or parallel β -strands (4/66). These sites were selected on the basis that they were on the surface, did not involve residues previously shown to be important for proteolytic activity (18), and that a nonperturbing disulfide could be formed between residues distant in the primary sequence. It is likely that if any conformational change occurs that it would originate from the NH₂- or COOH-terminal strands, since the rest of the molecule is stabilized by numerous hydrogen bonds and hydrophobic contacts (8). Furthermore, when molecular dynamics calculations (AMBER) are performed on ubiquitin, it starts to unfold at the contact formed by residues 1–2 and 63–64.²

Energy minimization and molecular dynamics calculations were performed on the modelled disulfides using the program AMBER (version 3.0). The minimized structures (Fig. 1) predict that the 4/66 disulfide could be formed without disrupting the backbone structure and the 4/14 disulfide could be accommodated by small local changes in protein conformation. Mutagenesis of a synthetic human ubiquitin gene was accomplished as described previously (18). Six mutants were constructed (Table I). The mutant genes were expressed in *E. coli* and the proteins were purified as previously described (18).

On 18% polyacrylamide gel electrophoresis in the presence of SDS, in the absence of 5% mercaptoethanol (Fig. 2, panel B), the three mutants containing only one cysteine (Phe⁴ →

Cys, Thr¹⁴ → Cys, Thr⁶⁶ → Cys) and the mutant containing all three of these changes showed two bands corresponding in molecular weight to ubiquitin monomer and dimer (lanes 1, 2, 3, and 6). Both bands were reactive with anti-ubiquitin antibody. In the presence of 5% mercaptoethanol (Fig. 2, panel A), only a single band corresponding to mono-ubiquitin remained on the gel, suggesting that the "dimer" band was actually a dimer of ubiquitin via an intermolecular disulfide bond.

No dimerization of the two double cysteine mutants was detected either by Coomassie staining or by Western analysis (lane 4, Phe⁴ → Cys + Thr¹⁴ → Cys; lane 5, Phe⁴ → Cys + Thr⁶⁶ → Cys). This suggests that intramolecular disulfide bonds are formed preferentially to intermolecular disulfide bonds, as expected from thermodynamic considerations.

Neither of the mutants containing two cysteines had any titratable sulfhydryl groups by Ellman's (11) reagent (Table I) or [¹⁴C]ethylmaleimide (not shown), confirming the presence of intramolecular disulfide bonds in both mutants. In contrast, approximately one titratable sulfhydryl was found on each of the single cysteine-containing mutants/mol of protein monomer. The mutant with three cysteines also had approximately one titratable sulfhydryl group, consistent with the formation of a disulfide bond and one free sulfhydryl. We treated 0.5 mg of each disulfide mutant with 1 mM DTT (an 8.5-fold stoichiometric excess) for 30 min, and then, the DTT was removed by gel filtration under an argon atmosphere. The protein fraction was collected directly into tubes containing [¹⁴C]ethylmaleimide under argon and reacted for 30 min. Disulfides 4/66 and 4/14 had 1.2 and 1.5 titratable cysteines/mol of protein immediately after DTT treatment. Hence, the disulfide containing mutants remained partially oxidized in the presence of DTT.

We studied the activities of the ubiquitin mutants in the PP_i-ATP exchange assay with purified E1. E1 catalyzes the activation of ubiquitin in the presence of ATP in the first step of the ubiquitination pathway. All six of the cysteine mutants were active in stimulating pyrophosphate exchange in the presence of purified E1. The maximum velocities of PP_i-ATP exchange for the mutants were identical to wild type ubiquitin (5 pmol/min) within experimental error (data not shown, standard deviation 1.2 pmol/min). All six mutants were also conjugated to endogenous substrates in a partially purified preparation of ubiquitin conjugating enzymes from reticulocytes (18) as determined by Western blotting (not shown).

The ability of the ubiquitin mutants to support the degradation of ¹²⁵I-bovine serum albumin (BSA) *in vitro* was measured (18). All of the mutants with single cysteine substitutions and the 4/14 disulfide (Table I) were as active as wild type ubiquitin. However, disulfide 4/66 and the mutant containing three cysteine substitutions were consistently 20–30% as active as wild type ubiquitin. Because substantial activity is lost in the 4/66 disulfide, while single cysteine substitutions at these sites do not result in decreased activity, these results suggest that a disulfide cross-link in this area of the molecule interferes with the function of ubiquitin in proteolysis. Fig. 1 shows that the 4/14 disulfide cross-links ubiquitin only at the first and second strands of the β -pleated sheet near the amino terminus. In contrast, the 4/66 disulfide cross-links much more widely separated regions of the molecule, linking regions near the carboxyl and amino terminus. It is likely that this disulfide more severely constrains the number of conformations available to ubiquitin, which may be important in signaling the proteolysis of a target protein.

Although the 4/66 disulfide mutant was fully active in the

² F. Brown, unpublished data.

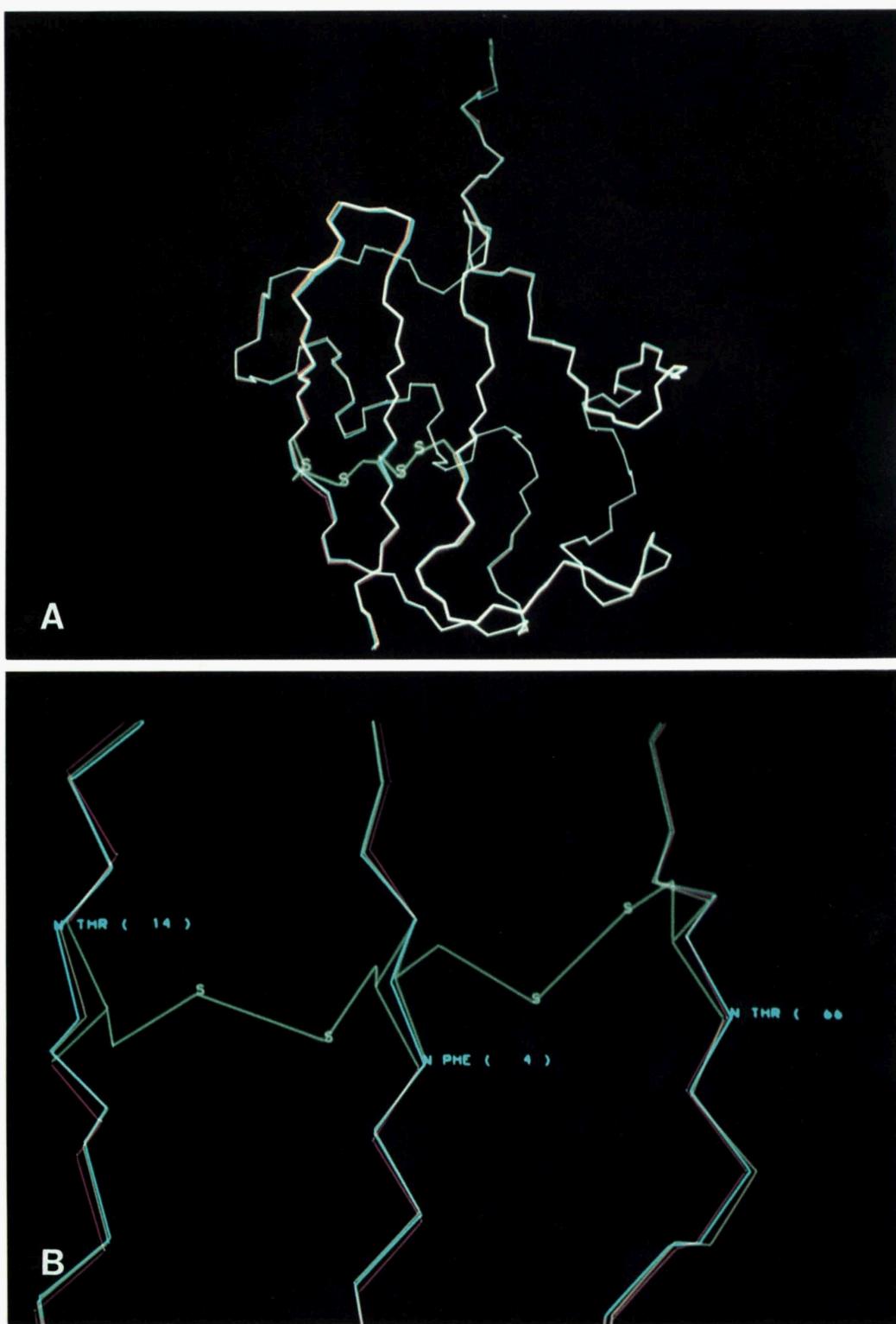


FIG. 1. Placement of disulfide bonds in ubiquitin by molecular modeling. Panel A, modeling Phe⁴ → Cys + Thr¹⁴ → Cys (on the left) and Phe⁴ → Cys + Thr⁶⁶ → Cys (on the right) the crystal structure backbone trace is shown in blue superimposed upon the energy minimized disulfide mutant structure shown in red. Where the two structures directly superimpose the combination of colors appears as white. The disulfide region is shown in green. Panel B, a closer view of the crystal structure backbone trace and the two mutant structures in the same color scheme as panel A.

TABLE I
Position of mutations, titratable sulphydryls/protein monomer by Ellman's reagent
and activity in *in vitro* degradation of ^{125}I -BSA

Measurement of titratable sulphydryls using Ellman's reagent is described under "Materials and Methods." The relative concentration of ubiquitin monomers and dimers was determined by running the protein sample on nonreducing polyacrylamide gel electrophoresis as shown in Fig. 2, followed by scanning a photographic negative of the gel. The activities of the mutants in degrading ^{125}I -BSA are reported relative to that of wild type human ubiquitin, defined at 100%. The assay was performed as described previously (18). The standard shown in parentheses is from five experiments.

	Codon			SH monomer	% control degradation
	4	14	66		
Wild type ubiquitin	-TTC-PHE	-ACT-THR	-ACC-THR	0.0	100 (20)
Phe ⁴ → Cys	-TGT-CYS	-ACT-THR	-ACC-THR	1.4	90 (20)
Thr ¹⁴ → Cys	-TTC-PHE	-TGT-CYS	-ACC-THR	0.8	68 (60)
Thr ⁶⁶ → Cys	-TTC-PHE	-ACT-THR	-TGT-CYS	1.1	110 (18)
Phe ⁴ → Cys, Thr ¹⁴ → Cys	-TGT-CYS	-TGT-CYS	-ACC-THR	0.0	100 (18)
Phe ⁴ → Cys, Thr ⁶⁶ → Cys	-TGT-CYS	-ACT-THR	-TGT-CYS	0.0	30 (10)
Phe ⁴ → Cys, Thr ¹⁴ → Cys, Thr ⁶⁶ → Cys	-TGT-CYS	-TGT-CYS	-TGT-CYS	0.8	20 (15)

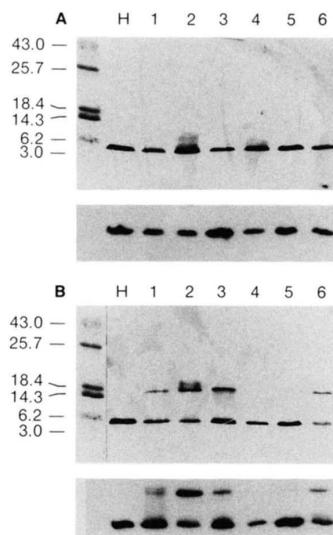


FIG. 2. Panel A (top), Coomassie-stained 18% acrylamide gel, containing SDS and 2-mercaptoethanol of purified human ubiquitin and the cysteine mutants. The lanes are identified as follows (also shown in Table I): H, human ubiquitin; 1, Phe⁴ → Cys; 2, Thr¹⁴ → Cys; 3, Thr⁶⁶ → Cys; 4, Phe⁴ → Cys, Thr¹⁴ → Cys; 5, Phe⁴ → Cys, Thr⁶⁶ → Cys; 6, Phe⁴ → Cys, Thr¹⁴ → Cys, Thr⁶⁶ → Cys. Panel A (bottom), an identically prepared gel transferred to nitrocellulose and probed with anti-ubiquitin antibody and [^{125}I]iodinated protein A followed by autoradiography. Panel B, identical to the gels shown above, except that 2-mercaptoethanol was deleted from the gel buffer.

E1 assay, the lower activity in the proteolysis assay (above) might be due to some structural perturbation introduced by the disulfide. To test this possibility, the structure of the 4/66 disulfide was determined in a series of distance geometry calculations using NMR-derived information (12). These cal-

culations have also been performed on wild type ubiquitin, and are essentially in agreement with the x-ray results (Fig. 3).³ The average root mean square deviation between the three lowest energy structures was 1.6 Å for backbone atoms and 2.7 Å for all atoms (protons included). These values are somewhat inflated due to global variance between structures (14); when comparing the parallel β -strands alone, the average root mean square deviation values are 0.7 and 2.0 Å for backbone and all atoms, respectively, while the average deviation of the backbone atoms in the parallel strands from the x-ray structure (8) is 0.8 Å.

The disulfide geometry is left-handed, although some deviation from a canonical left-handed spiral is present. The average cysteine torsion angles (15) are $X_1 = +5.0 \pm 10^\circ$, $X_2 = 144 \pm 3^\circ$, $X_3 = -60 \pm 8^\circ$, $X'_2 = -39 \pm 16^\circ$, $X'_1 = -46 \pm 20^\circ$, with an average $\text{C}^\alpha\text{-C}^\alpha$ distance of 4.46 ± 0.26 Å (4.63 Å in the native x-ray structure). The unusual X_1 angle appears necessary to accommodate the relatively close $\text{C}^\alpha\text{-C}^\alpha$ distance; the average left-handed $\text{C}^\alpha\text{-C}^\alpha$ distance is 5.88 ± 0.49 Å (15). The unusual geometry was seen in both the modeling and from the independent NMR calculations. The overall folding of the molecule is identical to native ubiquitin. The only deviation is a slight translocation of the β -carbon position of Cys⁴.

DISCUSSION

Ubiquitin, when conjugated to target proteins, is generally believed to serve as a signal for proteolysis, although the mechanism of signaling is unknown. At least three possibilities can be envisaged. First, ubiquitin may simply act as a signal that is recognized by a specific protease that has a binding site for ubiquitin and degrades the attached protein. Second, ubiquitin may participate in the unfolding of attached proteins by perturbing the molecular center mass beyond that

³ P. L. Weber, manuscript in preparation.

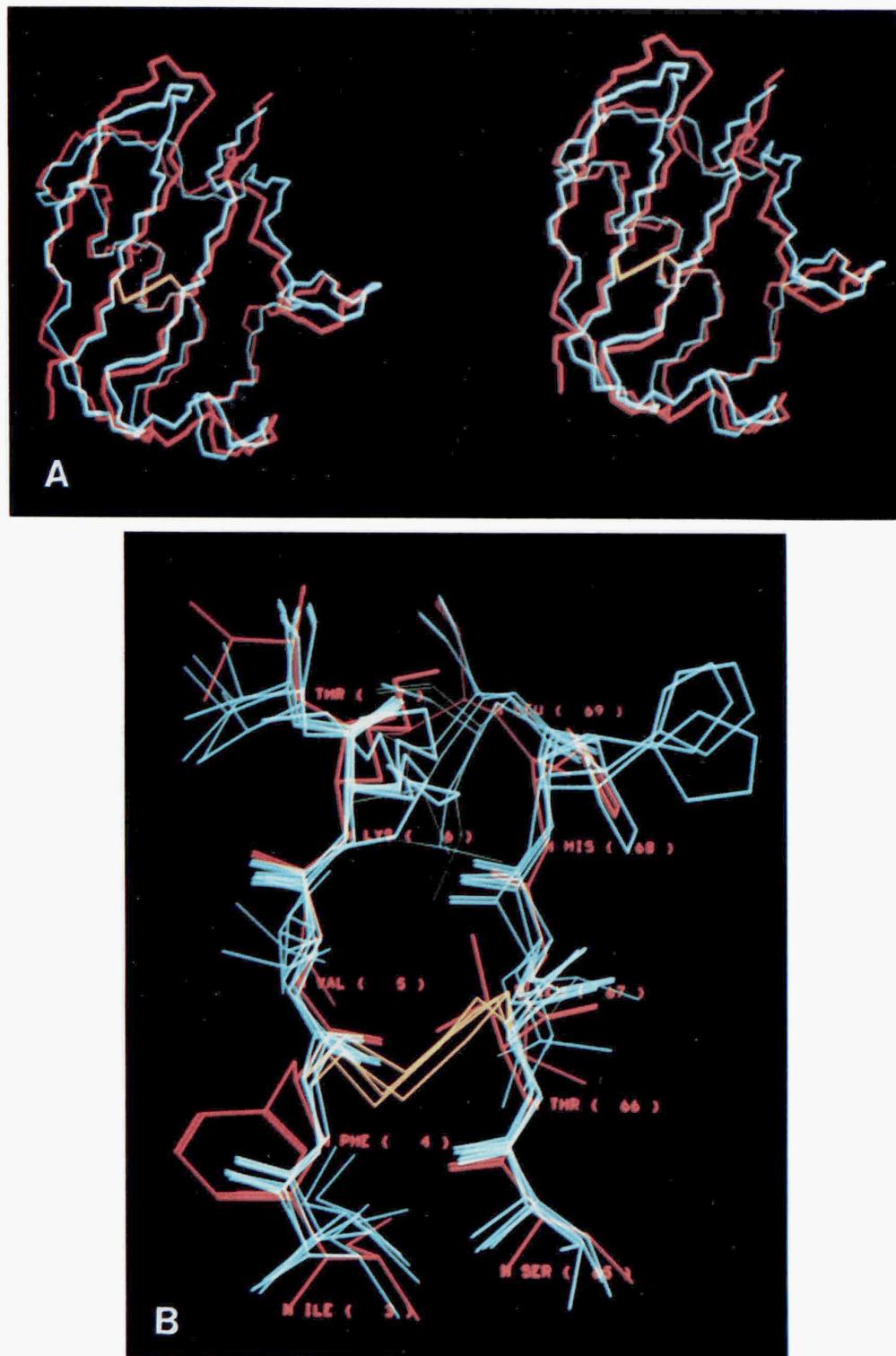


FIG. 3. *Panel A*, the backbone atoms of the average of four AMBER refined structures (blue) are shown with the x-ray structure (red) superimposed. The NH₂ terminus is at the bottom left, the COOH terminus at the top right (residues 72-76 are not included, as they are freely mobile in solution). *Panel B*, non-hydrogen atoms of residues 2-7 and 64-69, the parallel sheet interface, are shown. The four NMR structures (blue) and wild type x-ray structure (red) are superimposed. The slight displacement of the β -carbon of residue 4 in the disulfide is visible.

tolerated by normal thermal motion (7). The unfolded target protein is then more accessible to proteases. In each of these models the decision to degrade a specific target protein is made at the level of the enzymes that conjugate ubiquitin to the target proteins and no conformational change in ubiquitin is required for activity.

A third model has been proposed in which ubiquitin plays an active "decision making" role in determining the fate of proteins to which it is attached (6, 16, 17). When conjugated to partially denatured proteins, the exposed hydrophobic region from the denatured protein would trigger a conformational change in ubiquitin. This altered conformation of ubiquitin is then recognized by the protease and signals the degradation of the attached protein. According to this model, ubiquitin attached to undenatured proteins would not undergo the conformational change and, therefore, not signal proteolysis. The "normally" folded ubiquitin would be a good substrate for deconjugation enzymes that remove ubiquitin from proteins.

To investigate the mechanisms of ubiquitin-mediated proteolysis we created a mutagenesis and expression system for ubiquitin and used the system to produce a series of site-specific changes in ubiquitin. We have previously focused on the effects of changes on the protein surface, hydrophobic core, and tail region which becomes covalently attached to the target protein (18). Changes in these regions probe the effects on the folded structure of ubiquitin and the *in vivo* interactions between ubiquitin and the enzymes that activate and conjugate ubiquitin to other proteins.

In this paper we inserted disulfide bonds in ubiquitin in an attempt to influence the ability of ubiquitin to undergo conformational changes which may be involved in its activity in signaling the proteolysis of attached target proteins. Disulfide bonds occur naturally in both intracellular and extracellular proteins and may have evolved to stabilize particular conformational states of proteins. More recently, disulfide bonds have been engineered into several proteins by site-directed mutagenesis in an attempt to increase their stability (reviewed in Ref. 3). Engineered disulfide bonds can increase the stability of proteins by decreasing in the degrees of freedom of the *unfolded* state. The resulting decreased entropy of unfolding provides thermodynamic stabilization to the folded state. A second effect of disulfide bonds is that they place restrictions on the number of folded protein conformations in solution (3). This effect is relevant to proposed mechanisms of action in signaling proteolysis. Disulfide cross-linked ubiquitin might be restricted from achieving a conformation that is active in signaling proteolysis.

An important consideration in selecting the locations of the disulfide bonds was that they have not strained dihedral angles or force higher energy structural constraints in the protein backbone. This is important because the disulfide mutants of ubiquitin are tested for enzymatic function in partially reducing environments both *in vivo* and *in vitro*, where the reduced and oxidized forms of the protein would be in equilibrium. It was recently shown that the structural context of the disulfide is an important factor in stabilization of the disulfide bond in equilibrium with the reduced form. Studies on engineered disulfides in subtilisin (19), and the λ repressor protein (20) have indicated that a disulfide with unstrained dihedral angles favors the oxidized form of the protein in the presence of reducing agents. In the case of an engineered disulfide in dihydrofolate reductase, where crystal structures of both the oxidized and reduced forms of the proteins are available, an unfavored dihedral angle in the 39–85 disulfide bond results in an inability to air oxidize the

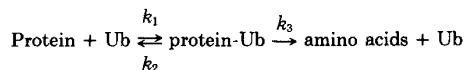
reduced form of the protein (21).

Both of the engineered ubiquitin dicysteine mutants could be modeled as disulfides with minimal perturbation of the structure (Fig. 1). The NMR solution structure of the 4/66 disulfide indicated that the ubiquitin backbone in this mutant was unchanged from the wild type ubiquitin structure (Fig. 3). Both the 4/66 and the 4/14 dicysteine mutants spontaneously air oxidized to disulfides. The reduced forms of these proteins could not be isolated, even after overnight dialysis against 10 mM DTT followed by degassed water. After treatment in an anaerobic environment with DTT, these disulfides could be partially reduced and titrated with [¹⁴C]N-ethylmaleimide, suggesting that there is an equilibrium between the disulfide and reduced form in the presence of reducing agent.

Another important objective in mutant selection was to insert disulfide cross-links into ubiquitin that did not interfere with the interactions between ubiquitin and the enzymes that activate and conjugate it to target proteins. Our previous mutagenesis experiments (18), and studies from chemical modifications of ubiquitin (6) have identified regions of ubiquitin important to the interactions with E1, particularly near the ubiquitin carboxyl terminus and these areas were avoided. All six of the mutants were successfully activated by the enzyme E1, suggesting that the minimal structural perturbations of these mutations permitted these proteins to get into the ubiquitin pathway. However, in the degradation assay the disulfide 4/66 and the triple cysteine mutant 4/14/66 were only from 20 to 30% as active as the control, suggesting that disulfide bond formation may have interfered with the function of ubiquitin in proteolysis. Because the location of the disulfide bond in the 4/14/66 triple cysteine mutant is not known, no further conclusions are drawn from this mutant. It is interesting that only the 4/66 disulfide mutant lost activity, while the 4/14 disulfide had the full biological activity of wild type ubiquitin.

Inspection of the location of these disulfides (Fig. 1) shows that the 4/14 disulfide cross-links ubiquitin only at the first and second strands of the β -pleated sheet near the amino terminus. In contrast, the 4/66 disulfide cross-links much more widely separated regions of the molecule, linking regions near the carboxyl and amino terminus. It is likely that this disulfide more severely constrains the number of conformations available to ubiquitin that may be important in signaling the proteolysis of a target protein. There are two caveats to this interpretation. First, the difference between the activities of the 4/14 and 4/66 disulfides in supporting proteolysis may be due to relative differences between the equilibrium between oxidized and reduced forms of the proteins in the assays. Second, substitution of the normal side chains with cysteines may result in decreased activity due to some important function of the lost side chains. However, the full activity of the mutants with the single cysteines at positions 4, 14, and 66 suggests that this is not the case.

Ubiquitinated proteins have two possible fates: deconjugation, where ubiquitin is removed and the previously conjugated protein is released intact, or degradation of the conjugated protein (7, 22–24). The overall equilibrium for this process is expressed as follows.



Where k_1 , k_2 , and k_3 are the grouped rate constants for the processes of ubiquitin activation and conjugation, deconjugation and degradation, respectively (22). We favor the interpretation that the 4/66 disulfide mutant of ubiquitin affects

the rates k_2 and k_3 in favor of deconjugation either by stabilizing ubiquitin in a conformational state that is a better substrate for deconjugation or preventing it from achieving a conformational state that is active in degradation.

In summary, we have constructed and expressed genes encoding six new mutants of ubiquitin containing cysteine(s), three of which formed disulfide bonds. All of the mutants were activated by the enzyme E1. Two of the disulfide mutants lost about 70–80% of the activity of wild type ubiquitin in supporting the degradation of BSA. The solution structure of one of these (disulfide 4/66) was solved by NMR. This mutant cross-linked across a large area of the protein and may have limited the ability of the molecule to undergo conformational changes. These studies, in conjunction with previous mutagenesis and chemical modification studies of ubiquitin provide insights to the complex process of ubiquitin-mediated protein degradation.

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