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Interactions of *Escherichia coli* UmuD with Activated RecA Analyzed by Cross-Linking UmuD Monocysteine Derivatives

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SOS mutagenesis in *Escherichia coli* requires the participation of a specialized system involving the activated form of UmuD (UmuD′), UmuC, RecA, and DNA polymerase III proteins. We have used a set of monocysteine derivatives of UmuD (M. H. Lee, T. Ohta, and G. C. Walker, J. Bacteriol. 176:4825–4837, 1994) and the cysteine-specific photoactive cross-linker *p*-azidoiodoacetanilide (AIA) to study not only the interactions of intact UmuD in the homodimer but also the interactions of UmuD with activated RecA. The reactivities of the individual UmuD monocysteine derivatives with AIA were similar to their reactivities with iodoacetate. The relative efficiencies of cross-linking of the AIA-modified monocysteine UmuD derivatives in the homodimer form are also consistent with our previous conclusions concerning the relative closeness of various UmuD residues to the dimer interface. With respect to the UmuD-RecA interface, the AIA-modified VC34 and SC81 monocysteine derivatives cross-linked most efficiently with RecA, indicating that positions 34 and 81 of UmuD are closer to the RecA interface than the other positions we tested. The AIA-modified SC57, SC67, and SC112 monocysteine derivatives cross-linked moderately efficiently with RecA. Neither C24, the wild-type UmuD that has a cysteine located at the Cys-24–Gly-25 cleavage site, nor SC60, the UmuD monocysteine derivative with a cysteine substitution at the position of the putative active-site residue, was able to cross-link with RecA, suggesting that RecA need not directly interact with residues involved in the cleavage reaction. SC19, located in the N-terminal fragment of UmuD that is cleaved, and LC44 also did not cross-link efficiently with RecA.

Mutagenesis in *Escherichia coli* resulting from exposure to UV radiation and various chemicals is not a passive process but rather requires the participation of a specialized system involving the activated form of UmuD (designated UmuD′), UmuC, RecA, and DNA polymerase III (8). The production of the UmuD, UmuC, and RecA proteins is regulated as part of the *recA*$^+$-*lexA*$^+$-dependent SOS response (8). This is induced when RecA, activated by single-stranded DNA (ssDNA) generated by the cell's attempt to replicate damaged DNA (23), mediates the proteolytic cleavage of LexA at the Ala-84–Gly-85 cleavage site (16), apparently by facilitating the otherwise latent capacity of LexA to autodigest (15). RecA*, the activated form of RecA, also mediates the posttranslational cleavage of UmuD at its Cys-24–Gly-25 bond by a similar mechanism (3, 25), removing the first 24 amino acids to generate UmuD′ for its role in mutagenesis (18). UmuD shares homology with the C-terminal regions of LexA, with the repressors of bacteriophages λ, φ80, 434, and P22, and with UmuD analogs that play roles in mutagenesis, such as MucA and ImpA (1, 6, 20, 24). This homology has functional significance in that all these proteins undergo RecA-mediated cleavage and autodigestion at an alkaline pH. The cleavage reaction for this family of proteins has been proposed to occur by a manner similar to that of β-lactamases, in which a nucleophile, apparently a serine residue conserved in all members of the family, is activated by a lysine residue (19, 26). Various genetic experiments indicate that RecA plays a third direct role in mutagenesis beyond mediating the proteolytic cleavage of LexA and UmuD (5, 7, 18, 30).

The role of RecA* in mediating the cleavage of repressor and mutagenesis proteins implies that a direct interaction between these proteins and RecA must occur which leads to the cleavage of the protein. This class of interaction has been visualized for the complex of LexA and the RecA filament by electron microscopy (32). In the study, the LexA repressor was found to bind within the deep helical groove of the activated RecA filament. The strikingly different effects of certain RecA mutations on the ability of this protein to mediate the cleavage of different repressor and mutagenesis proteins suggest that some contacts between the repressor or mutagenesis proteins and RecA might be specific for a particular protein. This view is supported by the observation that the RecA430 mutant (which has a glycine-to-serine substitution at position 204) is deficient at mediating the cleavage of LexA and very deficient at mediating the cleavage of UmuD (25) and λ repressor (22) but is proficient at mediating the cleavage of φ80 repressor (6).

Other evidence suggests that direct physical interactions also occur between RecA and the cleavage product of a mutagenesis protein (i.e., UmuD′ or MucA′). In DNA mobility shift assays, UmuD′ or MucA′ as well as UmuD could be cross-linked by glutaraldehyde to a RecA–ssDNA complex (7). In addition, it has been observed that the overproduction of UmuD′ and UmuC proteins in the recipient in an Hfr × F$^-$ conjugal cross inhibits recombination but that this inhibition can be substantially suppressed by overproduction of RecA. These experiments have led to the suggestion that the interaction of UmuD′ and UmuC with the growing end of a RecA nucleofilament inhibits recombination and switches the RecA-coated DNA from being a substrate for recombination to being a substrate for bypass mutagenesis (28). It is not yet understood whether the natures of the UmuD-RecA interactions and UmuD′-RecA interactions are similar or different. One observation which suggests that the interactions might be different is the finding that RecA430 fails to mediate the cleavage of UmuD but is functional for mutagenesis when UmuD′ is directly produced (18).

SOS mutagenesis appears to be due to a process of transle-

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sion synthesis in which the replicative machinery, involving UmuD′, UmuC, RecA, and DNA polymerase III, encounters a noncoding or miscoding lesion, inserts an incorrect nucleotide across from the lesion, and then continues elongation (8). Biochemical approaches for the study of the mechanistic process of SOS mutagenesis have recently been developed. Rajagopalan et al. (21) have reconstituted limited replicative bypass in an in vitro system with purified UmuD′, renatured UmuC, RecA, and DNA polymerase III proteins and a DNA substrate with a single abasic lesion. In another approach, Cohen-Fix and Livneh (4) have reported the development of a crude cell-free system made from SOS-induced cells that is capable of processing UV-irradiated plasmid DNA to yield mutated DNA in a fashion that requires the *umuD*, *umuC*, and *recA* gene products.

In an effort to gain insights into the interactions of UmuD with other proteins and its structure-function relationships, we initiated a monocysteine approach for studying the UmuD protein (12). UmuD has one cysteine in its amino acid sequence, located at the Cys-24–Gly-25 cleavage site. The substitution of an alanine residue for this cysteine results in a derivative whose function is indistinguishable from that of the wild type (12). This observation has allowed us to construct a family of UmuD proteins differing only in the position of the unique cysteine residue. In designing this set of monocysteine derivatives, we attempted to maximize the probability of obtaining biologically active molecules by making cysteine substitutions at sites which (i) represented conservative substitutions or (ii) were located in regions of the amino acid sequence which were not conserved in related proteins (UmuD analogs and repressors subject to RecA-mediated cleavage). The locations of the cysteine substitutions were also chosen to sample regions along the entire length of the UmuD polypeptide chain. From our initial characterizations of the UmuD monocysteine derivatives, we had made several inferences concerning the relative topological arrangement of certain residues of UmuD in relation to the homodimer interface (12). The assignments were primarily made on the basis of the solvent accessibility of the cysteines at these positions, as determined by iodoacetate reactivities, and the relative ease of homodimer cross-linking of the monocysteine derivatives by formation of disulfide bonds upon mild oxidation with iodine or by reaction with the cysteine-specific cross-linker *bis*-maleimidohexane (BMH).

We wanted to extend these investigations to study interactions of not only UmuD in the homodimer but also UmuD with RecA and eventually with other proteins involved in the complex process of UV-induced translesion synthesis. Use of cysteine-specific homobifunctional cross-linking reagents, however, would not be adequate in these studies since it would require the interacting protein to contain a cysteine residue at the site of interaction. We therefore adopted a strategy used in many investigations of protein-protein interactions which requires neither prior knowledge of the interacting sites of adjacent proteins nor mutagenesis of the interacting protein. This approach involves chemically modifying a unique cysteine residue of one protein with a cysteine-specific photoactivatable cross-linker and then using the derivatized residue as a probe of the local environment when the protein interacts with other molecules (2). In such experiments, the modified protein is first incubated with another protein (or proteins) with which it can interact. Exposure of the resulting complex to UV light results in covalent cross-linking of the complex. In this paper, we have extended our study of UmuD interactions by probing interactions of the $UmuD_2$ homodimer as well as interactions of UmuD with RecA. We have used the cysteine-specific photoactive cross-linker *p*-azidoiodoacetanilide (AIA) (33) in these investigations of UmuD interactions.

MATERIALS AND METHODS

UmuD mutant derivatives were produced and purified as described previously (12). Unlabeled AIA and [2-^{14}C]AIA were synthesized as described previously (33).

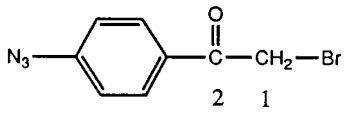
Incorporation of [2-^{14}C]AIA into the UmuD protein. UmuD mutant derivatives at a monomer concentration of 20 μM in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 8.0)–500 mM NaCl were incubated with a 10-fold molar excess of [2-^{14}C]AIA in the dark at 37°C for 1 h. To quench the reaction, an equal volume of sodium dodecyl sulfate (SDS) sample buffer containing 10% β-mercaptoethanol was added to the reaction mixture. Reagents were separated from the samples by electrophoresis on an SDS–13% polyacrylamide gel. The extent of labeling was determined by staining the gel with Coomassie blue, cutting out the band, and extracting the protein from the band by incubating it at 55°C for >18 h in 0.5 ml of Solvable (DuPont-New England Nuclear) and 0.5 ml of H_2O. Subsequently, 10 ml of Formula 989 (Dupont-New England Nuclear) was added, samples were vigorously mixed, and ^{14}C disintegrations were counted with a Beckman LS 6000SC liquid scintillation counter.

Cross-linking of UmuD with AIA. UmuD derivatives at a monomer concentration of 80 μM in 50 mM HEPES (pH 8.0)–500 mM NaCl were incubated with a 10-fold molar excess of AIA in the dark at 37°C for 1 h. The reaction mixture was dialyzed in a microdialyzer for 40 min against 40 mM Tris buffer (pH 8.0)–100 mM NaCl–0.1 mM EDTA to remove excess reagent. UmuD derivatives at a final concentration of 60 μM were then placed on ice and exposed to UV light at 320 nm and a power output of 90 μW/cm^2 for 15 min to initiate the photolysis reaction. The reaction was quenched by removal of the reaction mixtures from light and the addition of an equal volume of SDS sample buffer containing 10% β-mercaptoethanol. Cross-linked species were resolved from non-cross-linked species by electrophoresis on a 13% polyacrylamide gel. Densities of Coomassie blue-stained bands corresponding to the monomeric and dimeric forms were quantitated with an LKB Bromma 2202 Ultroscan laser densitometer.

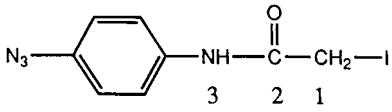
Normalization of UmuD homodimer cross-linking data. UmuD cross-linking data were normalized to account for the differences in UmuD modification by AIA. The normalized data are expressed as the maximum percentage of UmuD cross-linked for 100% UmuD modification, calculated as follows: let X = the fraction of UmuD monomers modified with AIA (observed), let c = the fraction of total UmuD cross-linked (observed), and let r = the intrinsic reactivity of AIA on the modified UmuD derivative.

Given that the fraction of modified UmuD (designated UmuD*) of the total population is X, the fraction of unmodified UmuD is then $1 - X$. Most of the UmuD proteins exist as dimers in solution (1, 31). A small percentage (<1%) of the UmuD derivatives C24 (wild-type UmuD) and VC34 was found in dimers of dimers ($UmuD_4$). To simplify calculations, this small population of $UmuD_4$ for these derivatives was not included in the equation. The population of modified and unmodified dimers would therefore consist of the following: $UmuD^*_2$, $UmuD_2$, and UmuD*-UmuD. This population can be described by the equation $X^2 + (1 - X)^2 + 2X(1 - X) = 1$, where X^2 is the fraction of $UmuD^*_2$, $(1 - X)^2$ is the fraction of $UmuD_2$, and $2X(1 - X)$ is the fraction of UmuD*-UmuD in the population. The cross-linked population consists of doubly modified dimers having one or two cross-links and singly modified dimers having one cross-link and can be described by the equation $c = X^2[1 - (1 - r)^2] + 2X(1 - X)(r)$, where $(1 - r)^2$ is the probability that a doubly modified dimer will not be cross-linked and $[1 - (1 - r)^2]$ is the probability that a doubly modified dimer will have any cross-linkages. This simplifies to a quadratic equation in which r has only one root between 0 and 1: $r = 1 - \sqrt{(1 - c)}/X$. The maximum percentage of UmuD cross-linked for each given UmuD derivative that is 100% modified by AIA can then be calculated from the equation % UmuD cross-linked$_{max} = 1 - (1 - r)^2$.

Cross-linking of AIA-modified UmuD derivatives to RecA. UmuD derivatives were modified with AIA as described above for UmuD homodimer cross-linking. After dialysis against 40 mM Tris buffer (pH 8.0)–100 mM NaCl–0.1 mM EDTA, modified UmuD derivatives at a final concentration of 45 μM were incubated for 5 min at 37°C in the dark in a reaction volume of 30 μl with 8 μM RecA, activated in the presence of 180 μM ATPγS, 8.8 ng of poly(dT)$_{27}$ (Pharmacia) per μl, and 18 mM $MgCl_2$. Reaction mixtures were then quickly transferred to a 96-well tissue culture serocluster with U-bottom wells (Costar) which was placed on ice and exposed to 320-nm UV light at a power output of 90 μW/cm^2 for 15 min. The photolysis reaction was quenched by removal of the plate from the light and addition of 15 μl of SDS sample buffer containing 10% β-mercaptoethanol to each well. Species with different molecular weights were resolved by electrophoresis on an SDS–13% polyacrylamide gel and visualized by Coomassie blue staining or by chemiluminescence after Western blotting (immunoblotting). For the latter, samples of cross-linked mixtures from the above-mentioned reactions were resolved by electrophoresis as described above, transferred to a polyvinylidene difluoride transfer membrane (Immobilon-P), and blotted with affinity-purified antibodies raised against UmuD′ or RecA; cross-reacting material was visualized by chemiluminescence (Tropix).



p-Azidophenacyl bromide (APB)



p-Azidoiodoacetanilide (AIA)

FIG. 1. Chemical structures of APB and AIA.

RESULTS

Modification of UmuD derivatives with [^{14}C]AIA. We initially chose the commercially available photoactive cross-linker *p*-azidophenacyl bromide (APB) for these investigations because of its length (only 9 Å [0.9 nm]) and the high reactivity of its photoactive end. However, cross-linking and incorporation studies of CA24, the UmuD derivative lacking cysteine, using APB suggested that APB did not react exclusively with cysteines (33). We therefore synthesized and used a new reagent, AIA for the following investigation (Fig. 1). AIA is comparable to APB in length and reactivity but has greater cysteine specificity (33).

We continued our investigations of UmuD interactions by using the subset of UmuD derivatives which reacted well with [^{3}H]iodoacetate (12). Efficient reactivity with iodoacetate implies that the sulfhydryl group is exposed and can be readily modified by our cross-linking reagent. We used [^{14}C]AIA to check the extent of incorporation into each derivative. The length of incubation and conditions were chosen to maximize specific incorporation and minimize nonspecific incorporation (33). The results are shown in Fig. 2. Most of the derivatives reacted to an extent of 60 to 80%. The reactivities of SC19 and wild-type UmuD were only slightly lower (approximately 50% modification after 1 h). The values for incorporation of AIA into SC19 and UmuD are slightly lower than the values for the extent of incorporation of iodoacetate; however, for the most part, AIA reactivities are comparable to the previously reported [^{3}H]iodoacetate reactivities of the UmuD derivatives (12). This result is to be expected assuming that the cysteine-specific functional group of the photo-cross-linker reacts in a manner, and with a reactivity, similar to iodoacetate. The observed small differences in reactivity between the two reagents might be due to the negative charge on iodoacetate or to the presence of the hydrophobic phenyl ring in AIA.

Use of AIA for cross-linking of UmuD derivatives in the homodimer. When a UmuD derivative that has been modified with AIA is allowed to form a complex with another protein (or proteins) and is UV irradiated, the photoactive end of the cross-linker will react with any nucleophilic group in the vicinity. The photoactivatable end of AIA is an azido group which, when activated on exposure to UV light, is reactive for only about 0.1 to 5 μs (2). Therefore, cross-linking of two interacting proteins by this reagent suggests a close spatial relationship of the regions of the proteins which are cross-linked (within the 9 Å [0.9 nm] length of the cross-linker). The results of UmuD cross-linking in the $UmuD_2$ homodimer when this reagent was used are shown in Fig. 3A. A small percentage (<1%) of the UmuD derivatives C24 (wild-type UmuD) and VC34 migrated to a position on the gel consistent with a dimer of dimers ($UmuD_4$) (data not shown).

These results were normalized to account for the differences in degree of modification by [^{14}C]AIA (Fig. 3B), since a population of UmuD with a greater degree of modification will have a higher probability of being cross-linked in the UmuD homodimer. The normalized data are presented as the maxi-

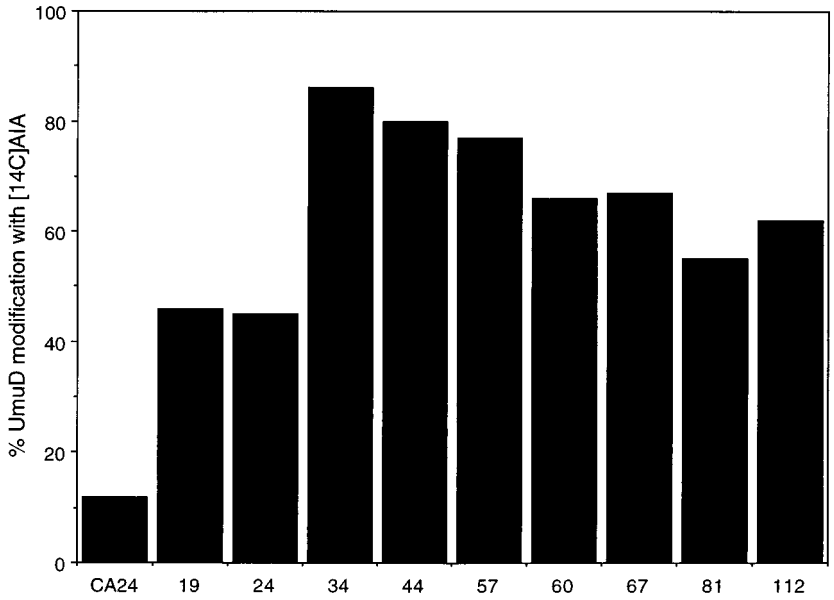


FIG. 2. Reactivity of UmuD monocysteine mutant proteins with [^{14}C]AIA. The percentage of total protein modified by AIA in 60 min was measured. UmuD mutant derivatives, at a monomer concentration of 20 μM in 50 mM HEPES (pH 8.0)–500 mM NaCl, were incubated with a 10-fold molar excess of [2-^{14}C]AIA in the dark at 37°C for 1 h. The numbers along the *x* axis correspond to the affected amino acids in the UmuD monocysteine derivatives.

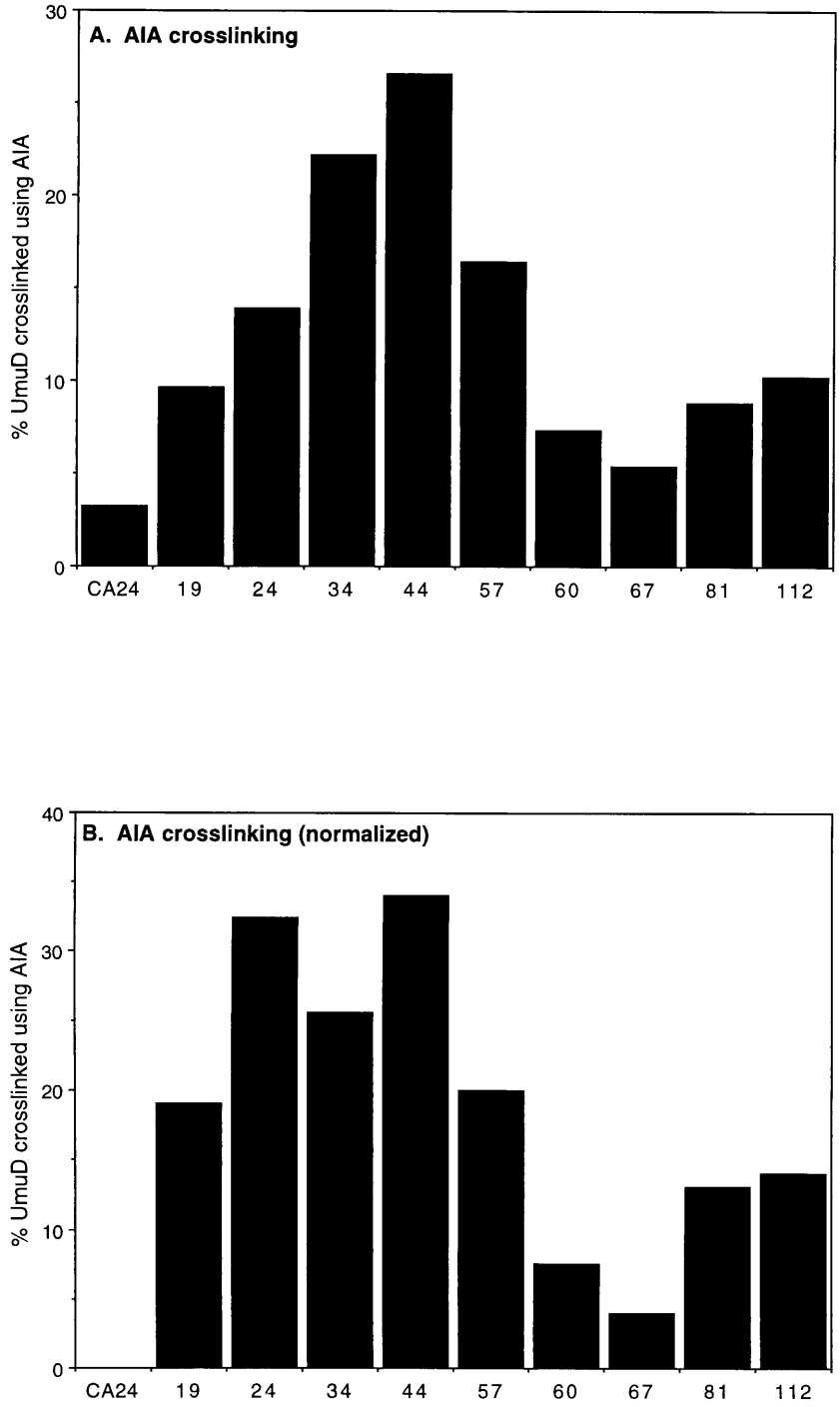


FIG. 3. Percentage of UmuD cross-linked by AIA. (A) Quantitation of UmuD cross-linking from densitometric scans of Coomassie blue-stained gels. UmuD derivatives, at an 80 μM monomer concentration in 50 mM HEPES (pH 8.0)–500 mM NaCl, were incubated with a 10-fold molar excess of AIA in the dark at 37°C for 1 h. Each reaction mixture was dialyzed in a microdialyzer to remove excess reagent. UmuD derivatives at a final concentration of 60 μM were then exposed to UV light at 320 nm on ice for 15 min to initiate the photolysis reaction. (B) Normalization of cross-linking data. UmuD cross-linking data were normalized to account for the differences in UmuD modification by AIA as described in Materials and Methods. Data are presented as the maximum percentage of cross-linking for a given UmuD derivative that is fully modified by AIA. The numbers along the *x* axis correspond to the affected amino acids in the UmuD monocysteine derivatives.

mum percentage of cross-linking in the homodimer for a fully modified population of a given derivative.

Of the derivatives tested, the UmuD mutants that had the greatest ability to be cross-linked were C24 (wild type), VC34, and LC44, which cross-linked 32, 26, and 34%, respectively. The efficient cross-linking of these derivatives is consistent with our previously reported cross-linking results (12). Other UmuD monocysteine derivatives that also cross-linked to a moderate extent were SC19 and SC57, which cross-linked 19 and 20%, respectively. In the experiments examining cross-

linking by disulfide bridges after oxidation with iodine, SC57 resulted in very little cross-linking (12). It is possible that the position of this substitution is in contact with the adjacent UmuD in the $UmuD_2$ homodimer, though the point of contact may be too far from position 57 of the adjacent protein to permit disulfide bond formation. The other mutant derivatives (SC60, SC67, SC81, and SC112) cross-linked between 4 and 14%, just slightly higher than background levels (as determined by cross-linking of the CA24 derivative which lacks cysteines). These results are consistent with the previously reported results from disulfide cross-linking after iodine oxidation. This demonstrates the usefulness of this reagent in identifying the points of protein interactions.

Use of AIA for cross-linking of UmuD to RecA*. For these experiments, we wanted to optimize conditions for interactions of UmuD with RecA and, as a result, maximize the probability of cross-linking. Preliminary experiments examining UmuD-RecA interactions under the conditions routinely used for RecA-mediated cleavage (3) suggested that under these conditions, at equilibrium, the UmuD-RecA complex was not present in abundance. In studies of UmuD reactivities to [^{3}H]iodoacetate, addition of RecA* did not protect any of the UmuD monocysteine derivatives from reacting with [^{3}H]iodoacetate (data not shown). In addition, we were not able to obtain significant cross-linking with either of two commercially available homobifunctional cross-linkers, BMH, which cross-links at cysteine residues, or glutaraldehyde, which cross-links at lysine residues. We therefore tried to optimize conditions by taking into account the following factors: (i) the molar ratio of UmuD to RecA in solution, (ii) the concentration of cofactor used for RecA activation, (iii) length of time for incubation of UmuD with activated RecA, and (iv) the temperature for the photolysis reaction.

On the basis of their electron microscopic studies of RecA-LexA complexes, Yu and Egelman (32) suggested that LexA does not bind RecA with a stoichiometry of 1:1 but rather binds with some cooperativity at random locations along the RecA filament, saturating at about 40% occupancy. In their image analysis of negatively stained filaments, they observed (i) no binding with 3.4 μM LexA fragment (with 6 μM RecA) and (ii) nearly saturating binding at 6 μM intact LexA (with 1.5 μM RecA), assuming that the binding parameters are the same for the intact LexA and the fragment. Their model of cooperative binding predicted that in the first case they would have 18% occupancy of LexA binding sites and that there would be 36% occupancy in the second case. Since a variety of lines of evidence indicate that RecA mediates the cleavage of UmuD in a manner similar to that by which it mediates LexA cleavage, we thought it possible that this cooperative model for binding might also apply to the interaction of UmuD with RecA. After unsuccessful attempts to cross-link UmuD to RecA using UmuD/RecA molar ratios of less than 4:1, we found that we were able to obtain the most successful cross-linking of these complexes with UmuD/RecA molar ratios of greater than or equal to 4:1.

RecA requires the presence of ssDNA and a nucleotide cofactor to be active for cleavage. We encountered two problems when utilizing the commonly used cofactor ATPγS to activate RecA. First, the photoactive azido group of AIA is very sensitive to and can be quenched by reducing agents such as β-mercaptoethanol or dithiothreitol (2). We found that an excess amount of ATPγS introduced a trace amount of reducing agent, which seemed to quench the azido group of the cross-linker during UV irradiation. Also, in comparison to other cofactors, ATPγS seemed to be most efficient at activating RecA for mediation of proteolytic cleavage. We wanted to avoid excessive conversion of UmuD to UmuD′. In an attempt to circumvent both of these difficulties, we tried various other cofactors, including dATP. In addition, we tried forming a RecA-ssDNA-ADP-AlF_4^- complex (17) with the hope of achieving a more stable association of UmuD with the RecA nucleoprotein filament. However, these methods did not seem to be as effective at promoting UmuD-RecA interactions, as evidenced by a substantial decrease in the rate of RecA-mediated cleavage and a low yield of UmuD-RecA cross-linked complexes. We therefore decided to use ATPγS, but at significantly reduced concentrations compared with the concentrations routinely employed in RecA-mediated cleavage reactions (10-fold less), and to incubate the UmuD with activated RecA at 37°C for a brief time period (5 min) to initiate formation of UmuD-RecA complexes yet minimize cleavage.

We carried out a preliminary screen of the ability of the various AIA-modified UmuD monocysteine derivatives to cross-link with activated RecA and found that VC34 cross-linked most efficiently. We therefore focused first on the VC34 derivative to test cross-linking conditions and to compare its cross-linking ability with that of the UmuD derivative lacking cysteines, CA24. Figure 4A shows representative cross-linking data for the mutants VC34 and CA24. Photolysis of derivatized VC34 with activated RecA (shown in lane 1) resulted in the appearance on the gel of several new higher-molecular-mass species. The most prominent species had an apparent molecular mass of approximately 72 kDa and constituted about 1% of the total protein in the reaction. A cross-linked complex containing RecA (38 kDa) and two UmuD monomers (30 kDa total) might migrate to a position corresponding to this approximate molecular mass. Other species which appear very faint on the Coomassie blue-stained gel migrate to positions corresponding to molecular masses of 64, 97, and >100 kDa. Western analysis of the cross-linking of the UmuD derivative VC34 to RecA is shown in Fig. 4B, lanes 1 and 3. The higher-molecular-mass species migrating to the positions described above cross-reacted with both αUmuD (lane 1) and αRecA (lane 3) antibodies, supporting the suggestion that these complexes contain both UmuD and RecA. It has not yet been established whether the different species represent complexes differing in the number of UmuD monomers per UmuD-RecA complex, different complexes containing UmuD and UmuD′ cross-linked to RecA, or different conformational isomers of the same UmuD-RecA complex. It is possible that such complexes would not have the mobility corresponding to their calculated molecular mass, since such cross-linked species, when denatured, will not assume a totally linear conformation. For example, $UmuD_2$, which has a calculated molecular mass of 30 kDa, has a mobility on an SDS-acrylamide gel corresponding to approximately 40 kDa.

In Fig. 4A, lane 2, the AIA-modified UmuD derivative VC34 is photoactivated for cross-linking in the absence of RecA. The absence of the appearance of the same pattern of higher-molecular-mass species in this reaction indicates that the formation of these complexes is dependent on the presence of activated RecA. This result suggests that the presence of RecA* causes a complex change in the ability of AIA-modified VC34 to react, possibly by allowing UmuD to react with another UmuD in a new way and/or allowing the attachment of multiple UmuD monomers onto a single RecA monomer. It seems unlikely that the UmuD-RecA complexes also contain DNA, since the single cross-linker present on VC34 must cross-link to another VC34 UmuD or RecA molecule in order to become attached to the complex. In addition, cross-linking of a UmuD derivative in the presence of DNA and the absence of RecA did not result in new species which might be consis-

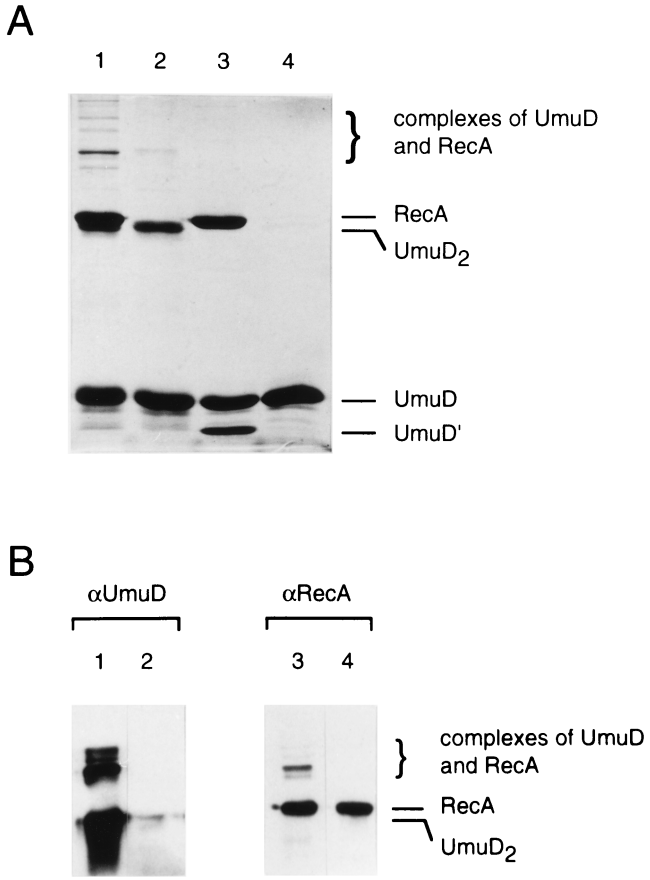


FIG. 4. Cross-linking of the UmuD derivatives VC34 and CA24 to RecA* by AIA. UmuD derivatives, at a monomer concentration of 80 μM, were modified with AIA and then dialyzed to remove excess reagent. Modified UmuD derivatives at a final concentration of 45 μM were incubated with 8 μM RecA, activated in the presence of ATPγS and ssDNA, for 5 min at 37°C in the dark and then exposed to UV light for 15 min to initiate the photolysis reaction. (A) Coomassie blue staining of cross-linked species resolved by electrophoresis on an SDS-polyacrylamide gel. Lanes: 1, VC34 in the presence of activated RecA; 2, VC34 only; 3, CA24 in the presence of activated RecA; 4, CA24 only. (B) Western analysis of cross-linked complexes by blotting with αUmuD or αRecA antibodies and visualizing by chemiluminescence. For lanes 1 and 2, samples were visualized with αUmuD antibodies; for lanes 3 and 4, samples were visualized with αRecA antibodies. Lanes 1 and 3: VC34 in the presence of activated RecA; lanes 2 and 4: CA24 in the presence of RecA.

tent with UmuD-DNA complexes (data not shown). As suggested earlier, the appearance of the faint band in the cross-linking reaction in the absence of RecA* which migrates to a position corresponding to about 72 kDa is consistent with the formation of cross-linked dimers of dimers ($UmuD_4$ complexes).

Control experiments with CA24, the UmuD derivative that lacks cysteines, are shown in Fig. 4A, lanes 3 and 4. The CA24 UmuD protein was treated in a manner identical to that by which the VC34 monocysteine derivative was treated, in that it was incubated with AIA, dialyzed, and mixed with RecA*. Photolysis of this mixture did not result in the appearance of any new cross-linked species. This is also evident from the Western analysis of this reaction (Fig. 4B, lanes 2 and 4). This indicates that protein-protein or protein-DNA cross-linking did not occur upon exposure of the reaction mixture to UV light in the absence of a photo-cross-linker and that the formation of the new, higher-molecular-mass complexes was not due to RecA-RecA cross-linking. Furthermore, in the experiments involving the CA24 UmuD derivative, photolysis resulted in negligible homodimer cross-linking, as expected (see Fig. 4A, lane 4, and Fig. 4B, lane 2), thereby demonstrating the specificity of the reagent. It is also interesting to note that in the case of the CA24 UmuD derivative, incubation with RecA* resulted in some cleavage of UmuD to UmuD′, indicating that these reaction conditions for cross-linking were favorable for RecA*-UmuD interactions (Fig. 4A, lane 3).

We next examined the rest of the set of UmuD monocysteine derivatives under the same conditions. The results are shown in Fig. 5. The UmuD monocysteine derivatives in this set, modified with AIA, displayed different abilities to cross-link with RecA. Photolysis of the UmuD derivatives which were able to cross-link with RecA resulted in the appearance of higher-molecular-mass complexes. The most prominent species had apparent molecular masses of approximately 64 and 72 kDa. Apart from the differences in the overall efficiency of cross-linking to RecA, the number of such species and their exact mobilities varied quite strikingly between these derivatives. This observation suggests that the position of the cross-linker on the particular UmuD derivative affects its ability to react in particular ways with RecA and/or UmuD (i.e., it affects the position of the specific attachment of the cross-linker to a neighboring protein), which in turn influences the exact mobility of the resulting complexes.

With respect to the overall efficiency of cross-linking, the modified monocysteine derivatives VC34 and SC81 appear to cross-link most efficiently with RecA. To a lesser extent, others (SC57, SC67, and SC112) also formed cross-linked species of approximately the same molecular mass. SC19, C24, LC44, and SC60 did not result in significant cross-linking. With the exception of SC19 and the wild-type protein, C24, all of the monocysteine UmuD derivatives were modified by [^{14}C]AIA to approximately the same degree; therefore, the amounts of cross-linked UmuD-RecA can be qualitatively compared. SC19 and the wild-type UmuD incorporated roughly half the amount of [^{14}C]AIA as the other mutants. However, this lower degree of AIA incorporation cannot fully account for the absence of the higher-molecular-mass species corresponding to UmuD-RecA, since even with this level of modification a significant amount of cross-linked $UmuD_2$ is present in the same reaction mixture.

With respect to the formation of complexes with different molecular masses, the cross-linking of the UmuD derivatives SC81, SC67, VC34, SC57, and SC112 (in order of decreasing intensity) all resulted in the appearance of a species with an apparent molecular mass of about 64 kDa. The mobilities of these complexes varied within the range corresponding to 55 to 65 kDa. A prominent band corresponding to a molecular mass of around 67 to 73 kDa was also present for the UmuD derivatives VC34, SC57, SC67, SC81, and SC112. In the case of VC34, this band was of a greater intensity than the lower-molecular-mass band, of approximately equal intensity for SC67, SC81, and SC112, and of considerably lower intensity for SC57. Cross-linking of the derivatives VC34 and SC81 to RecA resulted in the appearance of a faint band corresponding to about 97 kDa, and only cross-linking of VC34 resulted in the appearance of a band corresponding to a mass of greater than 100 kDa.

DISCUSSION

We have extended our investigations of the interactions of UmuD by using the monocysteine approach to study not only interactions of UmuD in the homodimer but also interactions of UmuD with RecA. We have used the cysteine-specific pho-

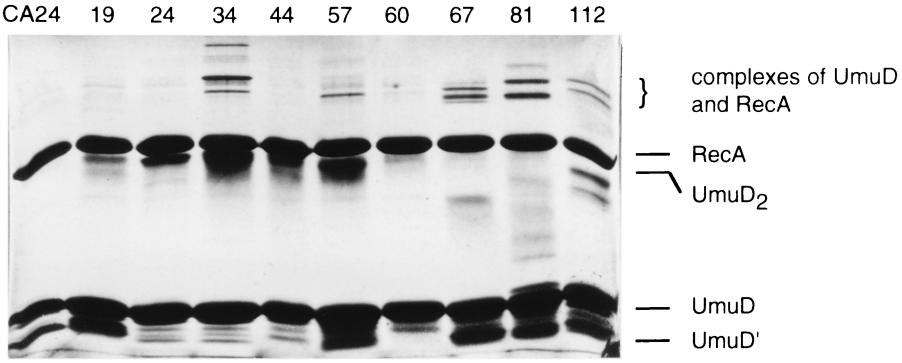


FIG. 5. AIA cross-linking of UmuD monocysteine derivatives to activated RecA. UmuD derivatives modified with AIA were dialyzed to remove excess reagent and then incubated with activated RecA for 5 min at 37°C in the dark. The photo-cross-linking reaction was then initiated by exposing the reaction mixtures to UV light for 15 min on ice as described in Materials and Methods. All lanes contain both a UmuD derivative and activated RecA. Lanes are identified by the position of the cysteine substitution of each particular monocysteine mutant in the cross-linking reaction. CA24 is the UmuD derivative lacking cysteines.

toactivatable cross-linker AIA (33) for these investigations. All the UmuD monocysteine derivatives in this set had previously been tested for their reactivity with [^{3}H]iodoacetate and had been found to be quite accessible to solvent (12). We found the reactivities of these derivatives to [^{14}C]AIA to be similar to their reactivities to iodoacetate, with most of the derivatives reacting to an extent of 60 to 80% in 1 h. The reactivities of SC19 and C24 (wild type) were only slightly lower (approximately 50% modification after 1 h). These results are consistent with our previous inference that the unique cysteines at these positions were exposed to solvent (12).

UmuD interactions in the $UmuD_2$ homodimer. In contrast to the rather similar reactivities of the monocysteine derivatives to iodoacetate and AIA, the various monocysteine derivatives modified with AIA displayed striking differences in their abilities to cross-link to another UmuD in the $UmuD_2$ homodimer and in turn differed in their abilities to cross-link into a RecA-containing complex if activated RecA was present. In interpreting these results, we have taken into account the following factors: (i) cross-linking is highly dependent on the distance from the reactive radical of the activated cross-linker to the adjacent residue because the half-life of the activated AIA-derived cross-linker is rather short (only 0.1 to 5 μs) (2) and (ii) cross-linking is dependent on the chemical nature of the residue with which it is to react (i.e., this residue must be nucleophilic). AIA is only 9 Å (0.9 nm) long; therefore, those UmuD monocysteine derivatives which were able to be cross-linked in the homodimer by this cross-linker were probably within about 9 Å (0.9 nm) of the adjacent UmuD monomer. The results of the studies on the abilities of this set of monocysteine derivatives to be cross-linked by AIA support our previous inferences concerning the relative closeness of these positions to the $UmuD_2$ homodimer interface (12). We found the UmuD monocysteine derivatives C24 (wild type UmuD), VC34, and LC44 to cross-link most efficiently with this reagent. On the basis of the results of cross-linking studies with iodine and BMH, we suggested that residues at positions 24, 34, and 44 are closer to the dimer interface than the other residues tested. Our results of homodimer cross-linking with AIA are consistent with this inference. The results of $UmuD_2$ homodimer cross-linking with AIA also suggest that the residue at position 57 is relatively closer to the dimer interface. Because cross-linking with AIA does not require that there be a nearby cysteine residue in the other interacting protein, this strategy of probing protein interactions should be a better predictor of the relative closeness of particular residues to an intermolecular interface than the use of cysteine-specific homobifunctional reagents. Thus, position 57 in one UmuD monomer might be fairly close to the dimer interface but not necessarily as close to position 57 of the adjacent UmuD monomer. The monocysteine derivative with a cysteine substitution at position 19 also cross-linked with moderate efficiency. We found previously that SC19 was cross-linked rather efficiently with BMH but was cross-linked less efficiently upon oxidation with iodine (12). These observations led to the suggestion that the residues at positions 19 in the homodimer are not as close to the dimer interface as residues 24, 34 and 44 but are within the 13.9-Å (1.39-nm) span of BMH. The observation that SC19 can be cross-linked in the homodimer by AIA (which is 9 Å [0.9 nm] in length) is consistent with this previous finding. Our present results also suggest that residues at positions 60, 67, 81, and 112 are relatively farther from the dimer interface than the others tested, and again this supports our previous inferences made on the basis of iodine and BMH cross-linking (12).

All of the experiments described in this paper were carried out in the absence of structural information from X-ray crystallographic or nuclear magnetic resonance studies, and the inferences we have discussed to this point have been drawn solely from our solution studies of UmuD and its family of monocysteine derivatives. However, Peat et al. (19) have now solved the crystal structure of the cleaved form of UmuD, UmuD′, to 2.5 Å (0.25 nm). The structure of the UmuD′ monomer consists of a globular head (residues 50 to 135) and an extended amino-terminal tail. In the $UmuD'_2$ homodimer present in the crystal, the two UmuD′ monomers are oriented so that their extended N-terminal tails point in opposite directions. Residues Tyr-52, Val-54, Ile-87, Phe-94, and Phe-128 are involved in hydrophobic interactions at the $UmuD'_2$ dimer interface, and Glu-93 and Lys-55 form salt bridges with their dimer partners on both sides of the interface of $UmuD'_2$ (19). In contrast, our AIA cross-linking studies of the intact $UmuD_2$ homodimer, whose crystal structure has not been solved, reinforce our previous conclusion that Cys-24, Val-34, and Leu-44 seem to be closer to the $UmuD_2$ homodimer interface than the other positions we tested. As discussed more fully in the accompanying paper by Guzzo et al. (10), the residues we have concluded are near the $UmuD_2$ homodimer interface on the basis of our UmuD monocysteine studies (references 10 and 12 and this paper) are clearly not near the interface of the $UmuD'_2$ homodimer. This suggests that the structure of the $UmuD_2$ homodimer (the inactive form in SOS mutagenesis) in solution is radically different from that of the cleaved $UmuD'_2$

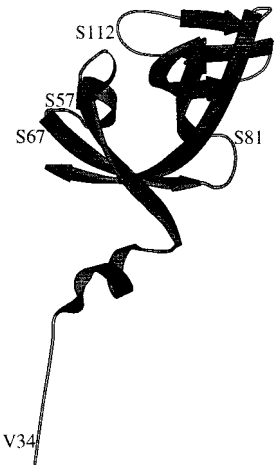


FIG. 6. Ribbon diagram of the UmuD′ monomer solved by Peat et al. (19). The structure of UmuD′ consists of a globular domain with an extended amino-terminal tail. The positions indicated (34, 57, 67, 81, and 112) are those that we have found to cross-link efficiently to activated RecA. Our cross-linking results suggest that residues on opposite sides of the UmuD monomer are close to the RecA interface.

homodimer (the active form in SOS mutagenesis) seen in the crystal.

UmuD interactions with RecA. When AIA-modified UmuD derivatives were incubated with activated RecA and then irradiated with UV light to initiate the photolysis reaction, only a subset of the modified derivatives cross-linked to activated RecA. Of these derivatives, VC34 and SC81 cross-linked most efficiently, while SC57, SC67, and SC112 cross-linked moderately efficiently to RecA. Figure 6 shows a ribbon diagram of the UmuD′ monomer solved by Peat et al. (19) as well as the positions (34, 57, 67, 81, and 112) that our results suggest lie near the UmuD-RecA interface during the interaction of intact UmuD with the RecA nucleoprotein filament. Although the structure of the intact UmuD monomer is not yet known, it seems reasonable to assume that the globular C-terminal domain of UmuD′ will be similar to the corresponding region of intact UmuD. If this assumption is correct, our results indicate that residues on opposite sides of the UmuD monomer (57, 67, and 112 on one side and 81 on the other) are close to the RecA interface.

It is interesting to consider our above-stated inferences concerning the nature of UmuD-RecA interactions in the light of the UmuD′ structure reported by Peat et al. (19) together with our conclusion from one of the accompanying papers (11) that $UmuD_2$ homodimers are preferentially cleaved as monomers in the RecA*-mediated reaction. Our findings could be explained by a model in which the UmuD monomer lies in a groove in the RecA nucleoprotein filament and thus interacts with different regions of the RecA protein. This suggestion is consistent with Yu and Egelman's determination (32) that LexA binds within the deep groove of the activated RecA filament, with two strong contacts with the RecA filament surface spanning adjacent RecA protomers. If UmuD also binds within the deep groove of the activated RecA filament, our observation that residues from different sides of the UmuD molecule cross-linked to RecA would not be surprising. In the case of LexA, the first site, site A, is a discrete contact on the inner surface of a pendulous lobe on each RecA subunit. The identification of this site is in agreement with the repressor binding site proposed in the RecA crystal structure (29), including residues 229 and 243, positions at which mutations affect repressor cleavage. The second contact, site B, maps in the region of the RecA crystal structure containing residues 156 and 165 and an intervening disordered loop region, L1, which has been suggested to be the secondary DNA binding site within the RecA filament (29).

One caveat to note when interpreting the UmuD-RecA cross-linking data is that cross-linking is performed under conditions that result in some cleavage of UmuD to UmuD′. Thus, it is possible that some of the cross-linked species actually contain UmuD′. Future analysis of the compositions of the complexes may help to resolve such issues.

It is intriguing that all of the UmuD derivatives that cross-linked to RecA yielded higher-molecular-weight complexes that could be resolved by SDS-polyacrylamide gel electrophoresis. Because there is only one photoactivatable group per UmuD molecule, it seems most likely that further work will show that the more slowly migrating species contain multiple UmuD molecules in addition to a RecA molecule. This suggests the interesting possibility that the RecA-mediated cleavage of UmuD is bimolecular with respect to UmuD rather than unimolecular, as has been demonstrated for the cleavage of this class of molecule under alkaline conditions (15, 27). This issue is more fully addressed in the accompanying paper by Lee et al. (11).

Sites on UmuD that cross-link efficiently with RecA. Although the AIA-modified SC81 derivative cross-linked relatively well with RecA, indicating that position 81 appears to lie close to the UmuD-RecA interface, it does not lie in a region that is conserved within the UmuD-LexA-phage repressor family of proteins. It is possible that the small region around position 81 contributes to the specific interactions with RecA that distinguish the interactions of the various members of this family with RecA from one another (6, 22, 25). It should be noted, however, that the conservative SC81 mutation does not impair RecA-mediated cleavage, implying that this conservative mutation does not disrupt any critical contacts.

The SC57, SC67, and SC112 mutations all lie on the side of the UmuD′ monomer opposite from position 81. Since the incorporation of [^{14}C]AIA is roughly equivalent for each of these derivatives, the amount of UmuD-RecA cross-linking can be qualitatively compared. SC67 seemed to cross-link more efficiently than SC57 and SC112. Our observation that the SC67 mutation affects the UV mutagenesis phenotype more dramatically than it affects RecA-mediated cleavage led us to suggest that this position is more important for the subsequent role of UmuD′ in mutagenesis than it is for the cleavage reaction (12). Perhaps this role may involve interactions with RecA in its third role in mutagenesis. Alternatively, SC67 may interact with RecA during RecA-mediated cleavage; however, the substitution of serine for cysteine in this case does not greatly affect the interactions of RecA with UmuD which result in cleavage. It is interesting that amino acid changes that affect RecA-mediated cleavage in LexA (13), λ repressor (9), and UmuD (1, 18) have been found in the regions of these proteins that correspond to the Ser-57 and Ser-112 regions of UmuD.

Position 34 is located in the extended N-terminal tail seen in the $UmuD'_2$ crystal structure. Even in the case of UmuD′, the conformation of this region of the protein in solution is not yet known, although it seems likely that this region will be found to be able to adopt alternative conformational states; the unique extended conformation described by Peat et al. (19) is presumably due to crystal packing forces. With respect to the conformation of this region of the protein in intact UmuD, the situation is obviously even less clear, although, as discussed by Guzzo et al. (10), it appears that the region from position 30 to

44 forms part of the $UmuD_2$ homodimer interface. In experiments assessing the ability to perform in mutagenesis and the ability to undergo RecA-mediated cleavage, we found VC34 to be impaired in mutagenesis and the most severely deficient of all the monocysteine derivatives we tested in RecA-mediated cleavage (12). We interpreted these results to suggest that position 34 is important for the direct interactions with RecA which lead to cleavage (12). Our observation that the AIA-modified VC34 UmuD derivative cross-linked to RecA the most efficiently of all the derivatives we tested is consistent with this hypothesis. As we have discussed previously (12), it is also consistent with Sauer and Gimble's conclusions from their genetic studies (9) that the corresponding region of λ repressor is involved in RecA interactions.

Sites on UmuD that cross-link poorly with RecA. In the UmuD′ crystal structure (19), Ser-60, which apparently serves as the nucleophile for the cleavage of the position 24-25 peptide bond of UmuD, is largely buried in a cleft, with only 8% of its surface accessible to water. Although the AIA-modified SC60 UmuD derivative can cross-link in the $UmuD_2$ homodimer with low efficiency, an observation which indicates that the arylazide moiety is sufficiently exposed to undergo intermolecular reactions, it did not cross-link to activated RecA. This result suggests that the contacts between UmuD and RecA that result in UmuD cleavage do not involve a RecA residue being in the immediate vicinity of Ser-60 of UmuD. If UmuD is similar in conformation to UmuD′ in this region, the absence of cross-linking could be explained by the inaccessibility of the modified cysteine at position 60 to RecA. One possibility is that as the cleavage site is brought near the active site in the RecA-mediated cleavage reaction, the amino-terminal region covers the active-site residues and hinders direct interactions with RecA. Interestingly, wild-type UmuD, with a cysteine at position 24 (of the Cys-24–Gly-25 cleavage site), also did not cross-link to RecA but did cross-link very efficiently in the $UmuD_2$ homodimer. If, when UmuD interacts with RecA, the cleavage site becomes buried within the active-site cleft region, this cysteine would also be inaccessible for direct interactions with RecA. This is consistent with the role of RecA as a coprotease that acts by facilitating the otherwise latent capacity of UmuD to autodigest. Mutations have been found at the cleavage sites in LexA, λ repressor, and UmuD which severely affect the ability of these proteins to undergo RecA-mediated cleavage (1, 9, 13, 18). Since our results suggest that Cys-24, at the cleavage site, does not directly contact RecA, these mutations may affect cleavage by altering interactions between the cleavage site and the active site rather than by altering direct interactions with RecA (12, 14).

The relatively conservative substitution of cysteine for serine at position 19 resulted in a significant (70 to 80%) reduction in RecA-mediated cleavage (12). Mutations in the corresponding residue of LexA also caused severe impairment of the ability of the mutant proteins to undergo RecA-mediated cleavage and autodigestion (13, 14). However, the monocysteine derivative with the cysteine substitution at position 19, SC19, did not cross-link significantly with RecA. Assuming that the observed lack of cross-linking is predominantly due to the distance of the cross-linker from a nucleophilic residue on RecA, this result indicates that this position is farther from the UmuD-RecA interface than the other derivatives tested. This suggests that the substitution of cysteine for serine may not affect RecA-mediated cleavage simply by affecting the direct UmuD-RecA interactions. Ser-19 is located in the N-terminal domain of UmuD which is removed by RecA-mediated cleavage. Perhaps this N-terminal region is involved in maintaining the UmuD conformation, which is distinct from the UmuD′ conformation. The introduction of amino acid substitutions may alter the UmuD conformation which is favorable for interactions with RecA that lead to cleavage and not necessarily alter the specific contacts between UmuD and RecA.

From the results of the present and previous (12) cross-linking studies, we proposed that Leu-44, along with Cys-24 (of the Cys-24–Gly-25 cleavage site) and residue Val-34, is in a region which is closer to the $UmuD_2$ homodimer interface. LC44, however, did not cross-link with RecA in these experiments, whereas VC34, which also cross-linked very efficiently in the $UmuD_2$ homodimer, cross-linked with RecA the most efficiently of the UmuD monocysteine mutants we tested. In the crystal structure of the cleaved form, UmuD′ (19), Leu-44 appears to be located on the same face as Ser-57, Ser-67, and Ser-112, which we observed to cross-link with RecA. At least in the case of the unimolecular autodigestion and possibly in the case of the RecA-mediated cleavage reaction as well, the amino-terminal region is probably brought near the active-site cleft so that the cleavage site can be properly positioned inside the cleft (19). As pointed out by Peat et al. (19), this would necessarily entail UmuD adopting a very different conformation from that observed in the crystal of $UmuD'_2$ (19). Our results suggest that Leu-44 does not participate directly in RecA interactions as UmuD associates with RecA in the conformation necessary for cleavage of the position 24-25 peptide bond.

We have gained numerous insights into the interactions of intact UmuD by using the monocysteine approach. Future directions include determining the composition of the higher-molecular-weight RecA-UmuD complexes and identifying the sites of RecA which interact with UmuD. These investigations have the potential to advance our understanding of the interactions of UmuD in this complex process.

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