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A Monocysteine Approach for Probing the Structure and Interactions of the UmuD Protein

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UmuD participates in a variety of protein-protein interactions that appear to be essential for its role in UV mutagenesis. To learn about these interactions, we have initiated an approach based on the construction of a series of monocysteine derivatives of UmuD and have carried out experiments exploring the chemistry of the unique thiol group in each derivative. In vivo and in vitro characterizations indicate that these proteins have an essentially native structure. In proposing a model for the interactions of UmuD in the homodimer, we have made the following assumptions: (i) the conformations of the mutant proteins are similar to that of the wild type, and (ii) the differences in reactivity of the mutant proteins are predominantly due to the positional effects of the single cysteine substitutions. The model proposes the following. The region including the Cys-24–Gly-25 cleavage site, Val-34, and Leu-44 are closer to the interface than the other positions tested as suggested by the relative ease of dimer cross-linking of the monocysteine derivatives at these positions by oxidation with iodine (I_2) and by reaction with *bis*-maleimidohexane. The mutant with a Ser-to-Cys change at position 60 (SC60) is similar in iodoacetate reactivity to the preceding derivatives but cross-links less efficiently by I_2 oxidation. This suggests that Ser-60, the site of the putative nucleophile in the cleavage reaction, is located further from the dimer interface or in a cleft region. Both Ser-19, located in the N-terminal fragment of UmuD that is removed by RecA-mediated cleavage, and Ser-67 are probably not as close to the dimer interface, since they are cross-linked more easily with *bis*-maleimidohexane than with I_2. The SC67 mutant phenotype also suggests that this position is less important in RecA-mediated cleavage but more important in a subsequent role for UmuD in mutagenesis. Ala-89, Gln-100, and Asp-126 are probably not particularly solvent accessible and may play important roles in protein architecture.

The process of UV and chemical mutagenesis requires the participation of the products of three genes, *umuD*, *umuC*, and *recA* (15, 27, 46, 53, 57, 63, 64). The *umuDC* operon is repressed by the LexA repressor (4, 15, 53) and is regulated as part of the *recA*$^+$ *lexA*$^+$-dependent SOS response (36, 46, 63, 64, 66). The SOS response is induced when RecA, activated by single-stranded DNA generated by the cell's attempts to replicate damaged DNA, mediates the proteolytic cleavage of the bond between Ala-84 and Gly-85 of LexA (35), apparently by facilitating the otherwise latent capacity of LexA to autodigest (34). Activated RecA (designated RecA*) also activates UmuD for its role in mutagenesis by mediating the posttranslational cleavage of UmuD at its Cys-24–Gly-25 bond by a similar mechanism (9, 52). The C-terminal fragment, UmuD′, has been shown genetically to be necessary and sufficient for its role in mutagenesis (41).

Evidence has been presented suggesting that intact UmuD functions as an inhibitor of mutagenesis (6, 47) and may be important as part of a posttranslational mechanism to regulate the cell's capacity to carry out SOS mutagenesis (6). UmuD shares homology with the C-terminal regions of LexA, the repressors of bacteriophages λ, φ80, 434, and P22, and with the analogous mutagenesis proteins MucA and ImpA (6, 14, 45, 51). This homology has functional significance in that all these proteins undergo RecA-mediated cleavage and autodigestion at alkaline pH. The cleavage reaction for this family of proteins is proposed to occur by a mechanism similar to that of serine proteases in which the nucleophile, a conserved serine residue, is activated by a lysine residue (54). Various genetic experiments indicate that RecA has a third role in SOS mutagenesis beyond mediating the cleavage of LexA and UmuD (13, 20, 41, 60).

Progress has been made recently in understanding the roles of UmuD′, UmuC, and RecA in SOS mutagenesis. Cohen-Fix and Livneh (11) have reported the development of an extract in which UV-irradiated plasmid DNA is processed to yield mutated DNA. The extract is made from SOS-induced cells and requires the *umuD*, *umuC*, and *recA* gene products. Rajagopalan et al. (47) have demonstrated that the addition of UmuD′, renatured UmuC, and RecA will permit DNA polymerase III holoenzyme to carry out limited bypass synthesis on a primed DNA substrate with a single abasic site in the template strand. Experiments indicating interactions between a RecA–single-stranded DNA complex and UmuD′ (20) or UmuC (21) have been used to suggest that UmuD′ and UmuC might play roles in targeting the polymerases to the lesions. Sommer et al. (56) have suggested that binding of UmuD′ and UmuC to the RecA-coated single-stranded DNA at the site of the lesion might cause it to switch from being a substrate for recombination to being a substrate for bypass mutagenesis. Other observations (5, 61) have led to the suggestion that UmuD′ and UmuC might alter the behavior of DNA polymerase III on damaged DNA by altering the molecular mechanism responsible for its processivity.

UmuD (15 kDa) and UmuD′ (12 kDa) participate in a variety of protein-protein interactions that appear to be important for their biological roles. Both proteins form ho-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Reference or source
Strains		
AB1157	*argE3*	15
GW3200	As AB1157, but *umuD44*	41
SG1611	JM101 derivative; Δ(*lac-pro*) Δ*gal* Δ*lon-510 supE thi*/(F′ *traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*)	24
Plasmids		
pGW2101	*umuDC* containing *Hpa*1-*Hpa*1 of pSE117 (15) cloned into *Eco*RV-*Pvu*II fragment of pZ150 (69). *rop* gene of pBR322 has been deleted. Vector contains M13 ori	41
pGW2020	pGW2101 with *umuC* deleted	41
pGW2021	pGW2101 derivative with *Fsp*I site generated 2 nucleotides 5′ to the initiation codon of *umuD*	This work
pVSR	pBR322 derivative carrying the T7 promoter and Shine-Dalgarno (SD) sequence with a *Hin*dIII restriction site 5 bp 3′ from the SD sequence	28
pAC-T7	Encodes IPTG-inducible T7 RNA polymerase, Km^r; pACYC184 derivative	28
pGW6050	pVSR derivative with *umuDC* under control of T7 promoter. *umuDC* subcloned from pGW2021; Ap^r	This work
pGW6060	pGW6050 derivative with *umuC* deleted	This work
pGW6070	pGW6060 derivative with M13 ori. M13 ori from pZ152	This work
pGW6100	70TGT to GCC; Cys-24 to Ala; pGW6070 derivative; *umuD131*	This work
pGW6111	178TCT to TGT; Ser-60 to Cys; pGW6100 derivative; *umuD132*	This work
pGW6121	100GTT to TGT; Val-34 to Cys; pGW6100 derivative; *umuD133*	This work
pGW6131	130TTG to TGT; Leu-44 to Cys; pGW6100 derivative; *umuD134*	This work
pGW6141	376GAT to TGT; Asp-126 to Cys; pGW6100 derivative; *umuD137*	This work
pGW6151	241AGC to TGC; Ser-81 to Cys; pGW6100 derivative; *umuD136*	This work
pGW6161	55AGC to TGC; Ser-19 to Cys; pGW6100 derivative; *umuD138*	This work
pGW6171	169AGT to TGT; Ser-57 to Cys; pGW6100 derivative; *umuD139*	This work
pGW6181	199AGT to TGT; Ser-67 to Cys; pGW6100 derivative; *umuD135*	This work
pGW6191	265GCT to TGT; Ala-89 to Cys; pGW6100 derivative; *umuD140*	This work
pGW6211	298CAA to TGT; Gln-100 to Cys; pGW6100 derivative; *umuD141*	This work
pGW6221	334AGC to TGC; Ser-112 to Cys; pGW6100 derivative; *umuD142*	This work

modimers and heterodimers (68), and the interactions of the UmuD · D′ heterodimers are more stable than that of either of the homodimers (6). It seems likely that all three forms of the dimers interact with UmuC (40, 68). UmuD′ also appears to undergo a special interaction with the RecA filament (20) and may interact with one or more components of DNA polymerase III holoenzyme (7, 25, 26, 37, 47). In addition, intact UmuD interacts with RecA* in a fashion that results in cleavage of its Cys-24–Gly-25 bond, and it is capable of autodigestion of the same bond if incubated at alkaline pH (9).

Structural information for UmuD certainly would be valuable in elucidating its roles and interactions in this complex process. In the current absence of any direct physical information concerning the structure of UmuD, we have initiated an approach for investigating the structure and interactions of *Escherichia coli* UmuD that is based on the construction of a set of monocysteine derivatives. This type of approach has previously been used successfully in investigations of topography and subunit interactions of such systems as chemoreceptor proteins (16, 17, 38, 43), bacteriorhodopsin (3, 19), troponin C (44, 62, 65), and subunits of the *E. coli* F_1 ATPase (1, 2). For example, single cysteines were introduced into locations representative of different structural domains of bacteriorhodopsin, and the topography as well as the orientation of the α-helices in the transmembrane regions was investigated by using various cysteine-specific reagents (19). In another example, disulfide cross-linking of monocysteine derivatives of the transmembrane portion of the *E. coli* Tar receptor led investigators to suggest a helical-bundle structure for the transmembrane region in which the four helices of this region are not structurally equivalent, i.e., two helices interact closely, while the other two are more peripherally located (43). With any missense mutant, one can carry out standard genetic characterizations of the mutant phenotypes and biochemical characterizations of the mutant proteins. However, the power of the monocysteine approach comes from the fact that one can also carry out an additional set of chemical investigations that take advantage of the presence of a single thiol group in each of the mutant proteins (1–3, 16, 17, 19, 38, 44, 62, 65). These have the potential to yield insights into such issues as the accessibility of particular amino acids to solvent, conformational changes undergone by the protein, and the nature of subunit interactions in multiprotein complexes. This type of experimentation is not intended to replace direct physical examinations of structure; moreover, if the three-dimensional structure of UmuD is eventually solved by crystallographic or nuclear magnetic resonance techniques, it will be possible to use results obtained in these studies to evaluate the proposed structure and to develop additional models concerning the nature of UmuD's interactions with various proteins. In the meantime, since such a structural model for UmuD is not presently available, the results obtained from studies of monocysteine derivatives can be used to make significant inferences about the nature of UmuD's three-dimensional structure in solution and about the nature of its intermolecular interactions. In this paper, we describe the construction and characterization of these monocysteine derivatives and discuss the qualitative structural inferences made from this type of experimentation.

MATERIALS AND METHODS

Construction of monocysteine *umuD* mutant plasmids and characterization of in vivo UmuD mutant phenotypes. Table 1 lists the bacterial strains and plasmids described in the text. To facilitate the overproduction and purification of the UmuD mutant proteins, all the *umuD* mutants we constructed were

under the control of the T7 promoter. pGW6050 was constructed by cloning the *umuD*-containing *Fsp*I-*Dra*I fragment of pGW2021 into the *Hin*dIII site of pVSR (28) by filling in the 5′ overhangs at the *Hin*dIII restriction site and ligating the blunt ends. pGW6060 was derived by deleting *umuC* from pGW6050 by *Bam*HI digestion, partial *Bgl*II digestion, and religation. pGW6070 was constructed by cloning the *umuD*-containing *Apa*LI-*Apa*LI fragment of pGW6060 into the *Apa*LI-*Apa*LI fragment of pZ152 (69) containing the M13 origin of replication. Mutant derivatives of *umuD* were constructed by using an oligonucleotide-directed mutagenesis system (Bio-Rad) with uracil-containing single-stranded DNA and oligonucleotides 21 bases in length, and each construct was confirmed by sequencing the entire *umuD* gene.

UV mutagenesis was carried out as described previously (15). We found the mutability of a *umuD44* strain producing UmuD under T7 control in the absence of isopropyl-β-D-thiogalactopyranoside (IPTG) to be only slightly greater than that of the *umuD44* strain producing UmuD under the control of its own promoter (see Fig. 1).

In vivo RecA-mediated cleavage was assessed by the following method. *E. coli* SG1611 cells (24) harboring helper plasmid pAC-T7 encoding the IPTG-inducible T7 RNA polymerase and a plasmid containing *umuD* under T7 control were grown at 37°C in 2× YT broth (50) to an optical density at 600 nm of 1.0, after which production of UmuD was induced with a 0.5 mM final concentration of IPTG. After a 1-h incubation, cells were centrifuged, resuspended in fresh 2× YT broth and incubated for another hour. After UV irradiation of cells in 0.85% saline at 50 J/m^2, cells were centrifuged, resuspended in 2× YT broth, and incubated for 45 min. This procedure produces roughly 10 to 20 times the number of UmuD molecules in an induced cell. UmuD cleavage was assessed by centrifuging the cells, resolving the protein from 5×10^9 cells by electrophoresis on a 13% polyacrylamide gel containing sodium dodecyl sulfate (SDS), transferring the protein to polyvinylidene difluoride transfer membrane (Immobilon-P), and blotting with affinity-purified antibodies raised against UmuD′. The antibody reacted equally well with UmuD and UmuD′ at the 1:5,000 dilution used in these studies. Cross-reacting material was visualized by chemiluminescence (Tropix). Visualized UmuD and UmuD′ bands were quantitated by using the LKB Bromma 2202 Ultroscan Laser densitometer.

Overproduction and purification of UmuD proteins. Overnight cultures of SG1611 containing pAC-T7 and a *umuD*-containing plasmid in M9-glucose medium (50) supplemented with 0.1 mM $CaCl_2$, 0.1 mM $FeCl_3$, 0.1 mM $ZnSO_4$, 4 g of glucose per liter, 5 μg of thiamine per ml, 25 μg of kanamycin per ml, and 100 μg of ampicillin per ml (for selection of cells harboring pAC-T7 and the *umuD*-containing plasmid) were diluted 1:20 into 2× YT broth supplemented with 100 μg of ampicillin per ml and 25 μg of kanamycin per ml and incubated at 37°C. At an optical density at 600 nm of 0.7 to 0.8, IPTG was added to a final concentration of 0.5 mM to induce the production of T7 RNA polymerase. After 1 h of incubation at 37°C, rifampin was added to a final concentration of 200 μg/ml. Cells were harvested after an additional 4-h incubation, centrifuged at 4,000 rpm in a Beckman J-6B centrifuge with a JS-4.2 rotor at 4°C, and resuspended in lysis buffer (50 mM Tris, pH 8.0; 2.0 mM dithiothreitol [DTT]; 5 mM EDTA; 1 mM phenylmethylsulfonyl fluoride; and 10 μg of pepstatin A per ml). Cells were lysed by addition of 0.5 mg of lysozyme per ml and 100 mM NaCl, incubation for 30 min at 0°C with agitation, followed by addition of 10 μg of DNase I per ml and 10 mM $MgCl_2$, incubation for 60 min at 0°C, and centrifugation at 14,000 rpm.

UmuD in the supernatant was precipitated by the addition of $(NH_4)_2SO_4$ to 35% saturation and incubation with stirring for 20 min. The pellet was resuspended in buffer H_A (10 mM Na phosphate, pH 6.8; 0.1 mM EDTA; 1 mM DTT; 100 mM NaCl) and applied to a hydroxylapatite column. The column was washed with buffer H_A, and the proteins were eluted with 30 mM Na phosphate (pH 6.8)–0.1 mM EDTA–1 mM DTT–100 mM NaCl. The UmuD-containing fractions were applied to a Mono Q ion-exchange column, and the proteins were eluted with a linear gradient of 100 to 460 mM NaCl in buffer H_A. The UmuD-containing fractions eluted at about 300 mM NaCl. Buffer of UmuD-containing fractions was exchanged by applying fractions to a 10-ml Bio-Rad Econopac 10 DG gel filtration column and eluting with 10 mM Na phosphate, pH 6.8, containing 0.1 mM EDTA, 100 mM NaCl, and 0.1 mM DTT.

In vitro RecA-mediated cleavage reaction. RecA protein was purified as described elsewhere (12). Reactions were carried out in buffer D (40 mM Tris-HCl, pH 8.0; 10 mM $MgCl_2$; 30 mM NaCl; 2 mM DTT) with 50 ng of a 20-mer oligonucleotide per 20-μl sample volume and 1 mM adenosine-5′-*O*-[γ-thio] triphosphate (ATPγS) as described previously (9). UmuD (10 μM) was incubated with 3.5 μM RecA at 37°C for 30 min. The cleavage reaction was quenched by the addition of SDS sample buffer with 10% β-mercaptoethanol, the mixture was heated to 100°C for 5 min, and the proteins were resolved by electrophoresis on an SDS–13% polyacrylamide gel. The amounts of UmuD and UmuD′ were quantified from the Coomassie blue-stained gels by using the LKB Bromma 2202 Ultroscan Laser densitometer. In these studies, UmuD (CA24) (UmuD with a Cys-to-Ala mutation at position 24) was found to behave identically to the UmuD$^+$ protein. This single-time-point assay does not necessarily reflect initial rates; therefore, differences in cleavage rates may be underestimated in this assay.

Reactivity of mutant UmuD proteins to [^{3}H]iodoacetate. UmuD proteins at a 20 μM concentration in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 8.1) containing 500 mM NaCl were incubated with a 40× molar excess of [^{3}H]iodoacetate (150 mCi/mmol; Amersham) at 37°C for 60 min in the dark (19). Reactions were quenched by adding an equal volume of SDS sample buffer with 10% β-mercaptoethanol to destroy the unreacted iodoacetate and 4% SDS to denature the protein. Reagents were separated from samples by electrophoresis on a 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)–0.5 ml of H_2O. Subsequently, 10 ml of Formula 989 (DuPont-New England Nuclear) was added, samples were vigorously mixed, and ^{3}H disintegrations were counted with the Beckman LS 6000SC Liquid Scintillation counter.

Cross-linking of UmuD mutant derivatives with glutaraldehyde, I_2, Cu^{2+}-phenanthroline (CuP), and *bis*-maleimidohexane (BMH). Glutaraldehyde cross-linking studies with UmuD derivatives were carried out essentially as described previously (6). Solutions of UmuD (10 μM) in 10 mM sodium phosphate buffer, pH 6.8–100 mM NaCl were incubated with a 0.05% final concentration of glutaraldehyde (Sigma) for 5 min. The reactions were quenched by the addition of 0.13 M Tris-HCl to the SDS sample buffer. For the cross-linking of mutant UmuD proteins with UmuD′, equimolar amounts of the two proteins were preincubated for 30 min at 37°C and then treated with glutaraldehyde.

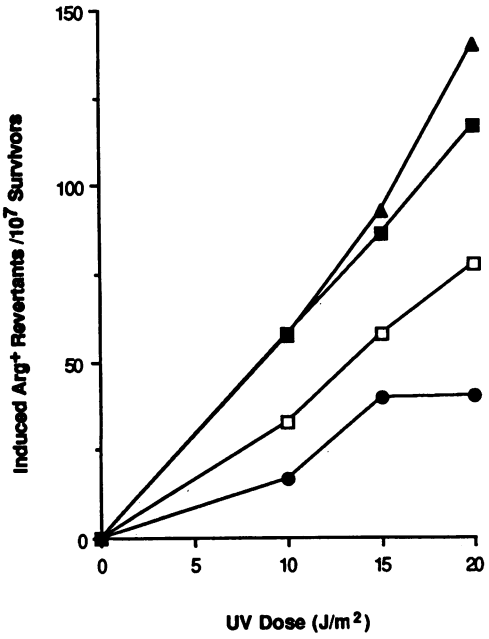

FIG. 1. Effect of plasmids encoding UmuD mutant proteins on UV mutagenesis in an AB1157 *umuD44* strain (GW3200). Assays were conducted in the absence of IPTG. Open squares, pGW2020 ($UmuD^+$, under LexA control); solid squares, pGW6070, pAC-T7 ($UmuD^+$, under T7 control); solid triangles, pGW6100, pAC-T7 (UmuD [CA24], under T7 control); solid circles, pGW6111 (CA24, SC60, under T7 control).

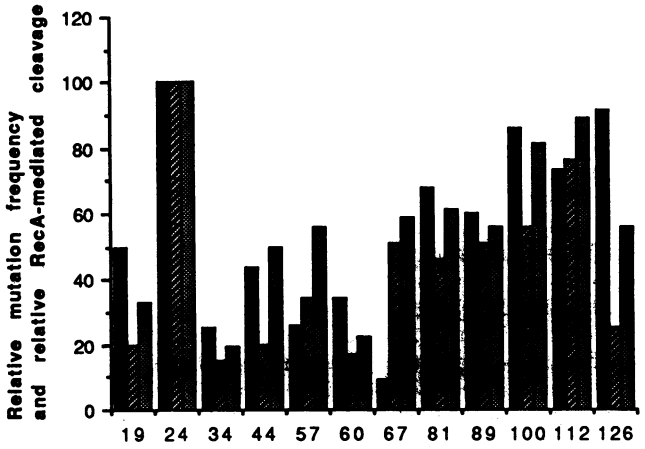

FIG. 2. Relative mutation frequency and in vivo and in vitro RecA-mediated cleavage. Mutagenesis was determined for cells irradiated with a UV dose of 20 J/m^2. In vivo and in vitro RecA-mediated cleavage assays were conducted as described in Materials and Methods. Solid bars, relative mutation frequency (percentage of wild-type level); hatched bars, relative in vivo RecA-mediated cleavage (percentage of wild-type level); dotted bars, relative in vitro RecA-mediated cleavage (percentage of wild-type level). Extent of in vivo RecA-mediated cleavage for CA24 and $UmuD^+$ is 60%. Extent of in vitro RecA-mediated cleavage for CA24 and $UmuD^+$ is 80%. Numbers along the *x* axis represent amino acid positions.

Disulfide formation reactions were carried out by treatment of UmuD with iodine or CuP. Reactions with iodine were initiated by the addition of 0.5 mM aqueous I_2 to 10 μM UmuD (in 50 mM HEPES [pH 8.1]–100 mM NaCl), mixtures were incubated at 22°C for 20 min, and reactions were quenched by the addition of 50 mM *N*-ethylmaleimide (NEM; Sigma) to block the remaining free sulfhydryl groups and SDS sample buffer (43). Oxidations with O_2 catalyzed by CuP were conducted by reacting UmuD (at 0.1, 1, and 10 μM) with 0.48 mM Cu^{2+} and 0.65 mM phenanthroline for 5 or 10 min at 0°C and quenched by adding 10 mM EDTA to chelate the Cu^{2+}, 50 mM NEM to block unreacted sulfhydryl groups, and sample buffer (18). Reactions with CuP were conducted in 50 mM HEPES–100 mM NaCl, pH 8.1, or 10 mM Na phosphate–100 mM NaCl, pH 7.3.

UmuD was cross-linked with BMH (Pierce) by the addition of 1 mM BMH to 10 μM UmuD (in 10 mM Na phosphate–100 mM NaCl, pH 7.3) and incubation for 5 min at 22°C, and the reaction was quenched by the addition of 50 mM DTT and sample buffer. Cross-linked dimers of UmuD were resolved from monomers by electrophoresis on a 13% polyacrylamide gel. For I_2, CuP, and BMH experiments, densities of Coomassie blue-stained bands corresponding to the monomeric and dimeric forms were quantitated with the LKB Bromma 2202 Ultroscan Laser densitometer. For the experiments measuring CuP cross-linking of UmuD at 1 and 0.1 μM concentrations, bands corresponding to the monomeric and dimeric forms were visualized on a Western blot (immunoblot) by chemiluminescence (Tropix). Visualized bands were then quantitated with the densitometer.

RESULTS

Construction of a *umuD* mutant encoding a UmuD derivative without a cysteine. We constructed a series of *umuD* derivatives that encode mutant UmuD proteins, each of which has a single cysteine at a unique site. In order to do this, we took advantage of the fact that the only cysteine in UmuD is Cys-24 at the Cys-24–Gly-25 cleavage site. In the family of phage repressors and mutagenesis proteins, most of the members have an Ala-Gly cleavage site, but a subset, UmuD and the bacteriophage φ80 repressor, has a Cys-Gly bond as the site for RecA-mediated cleavage. This suggested that changing the Cys-24 to alanine would result in a fully functional protein that contained no cysteine in its amino acid sequence. Site-directed oligonucleotide mutagenesis was used to construct a *umuD* derivative, *umuD131*, that encodes a mutant UmuD protein that has an Ala-24–Gly-25 cleavage site.

We found the ability of the UmuD (CA24) derivative to participate in UV mutagenesis in vivo to be essentially indistinguishable from that of wild-type UmuD (Fig. 1). Furthermore, UmuD (CA24) behaved identically to the $UmuD^+$ protein during purification and undergoes RecA-mediated cleavage in an apparently identical fashion.

Activity of UmuD monocysteine mutant proteins in UV mutagenesis and RecA-mediated cleavage. We then used site-directed oligonucleotide mutagenesis to make 11 derivatives of *umuD131* in which a codon for some particular amino acid was replaced by a cysteine codon. In order to maximize the probability of obtaining biologically active UmuD proteins, we chose (i) sites that were not strongly conserved within the UmuD-MucA-LexA-phage repressor family of proteins (6), (ii) sites of serine residues in UmuD, and (iii) sites of cysteine residues in the homologous proteins (6). All the mutations were confirmed by sequencing of the entire gene.

We then characterized the different in vivo properties of these monocysteine UmuD derivatives and compared them

with that of wild-type UmuD (Fig. 2). The ability of the mutant UmuD proteins to participate in UV mutagenesis was determined by expressing them in the *umuD44* strain and measuring the reversion of an *argE3* mutation to Arg^+. Most of the monocysteine UmuD derivatives retained substantial activity for mutagenesis. The most severely impaired was the SC67 derivative, which was only 9% as active as the parental protein UmuD (CA24) in UV mutagenesis. The next most impaired derivatives (VC34, SC57, and SC60) still had about one-quarter to one-third of the activity of the wild type in UV mutagenesis.

Since RecA-mediated cleavage of UmuD is needed to activate it for its role in UV mutagenesis, we also determined the ability of the derivatives to undergo RecA-mediated cleavage in vivo. Cells carrying the UmuD mutant plasmids were induced for UmuD production and irradiated with UV light at a dose of 50 J/m^2. After a 45-min incubation at 37°C, the extent of cleavage was detected by Western blotting with affinity-purified UmuD antisera (6) and was found to be ~60% for the wild-type UmuD under these conditions. Although we recognize that this approach is not sensitive to small differences in extent of cleavage, we did find, nevertheless, that the monocysteine derivatives of UmuD were stable in vivo and, in general, that the activity of the UmuD mutant proteins in UV mutagenesis correlated well with their ability to undergo RecA-mediated cleavage in vivo. The only exception was the SC67 derivative, which was cleaved 60% as well as the parental UmuD protein, UmuD (C-24), but was only 9% as active in mutagenesis. This suggests that this position is important for the subsequent role of UmuD′ in mutagenesis. The reduction in cleavage noted with the SC60 derivative was expected since, by analogy to LexA, Ser-60 has been implicated as the possible nucleophile in the RecA-mediated cleavage reaction activating UmuD for its role in mutagenesis (54). Consistent with this hypothesis, Nohmi et al. (41) had shown that SA60 and SC60 derivatives of wild-type UmuD showed an impaired ability to participate in UV mutagenesis, while the SA60 mutation introduced into the truncated protein, UmuD′, was much less deficient in mutagenesis. The strain expressing DC126 was almost as mutable as a strain expressing the parental UmuD protein, yet the DC126 derivative was cleaved only 25% as much as the wild-type proteins were. A possible explanation is discussed below (see Discussion).

RecA-mediated cleavage of the UmuD monocysteine mutant proteins in vitro. All of the UmuD monocysteine derivatives were purified to homogeneity by a set of procedures identical to those used to purify both the wild-type UmuD and the UmuD (CA24) proteins. We obtained the same level of production for these derivatives in vivo as for the wild type, indicating that they are similar in stability to the wild-type protein. In addition, that they could be purified by the same procedure as that used for $UmuD^+$ suggests that their conformation is very similar to that of the wild-type protein. These purified UmuD derivatives were assayed for their ability to undergo RecA-mediated cleavage in vitro. As shown in Fig. 2, the ability of the various UmuD derivatives to undergo RecA-mediated cleavage in vitro correlated well with that determined in vivo.

Formation of homodimers and heterodimers between UmuD derivatives and UmuD′. To survey the abilities of the UmuD mutant proteins to dimerize, we examined the abilities of the UmuD derivatives to be cross-linked by glutaraldehyde (6, 30). Glutaraldehyde cross-links the amino groups of proteins, and this reaction is rapid and specific. We expected that if the monocysteine derivatives retained structures that are similar to that of the wild type, they would exhibit the same extent of cross-linking as the wild type did. We found that most of the UmuD derivatives cross-link to the same extent as the wild type, indicating that most monocysteine mutants retain an essentially native structure that is able to dimerize effectively. However, it is possible that small differences in dimerization constants might have escaped detection by this approach, because most of the UmuD protein under these conditions is probably in dimeric form. Differences in dimerization constants were detected in the LC44 and DC126 mutants, which were observed to show a partial reduction in homodimer formation (Fig. 3A).

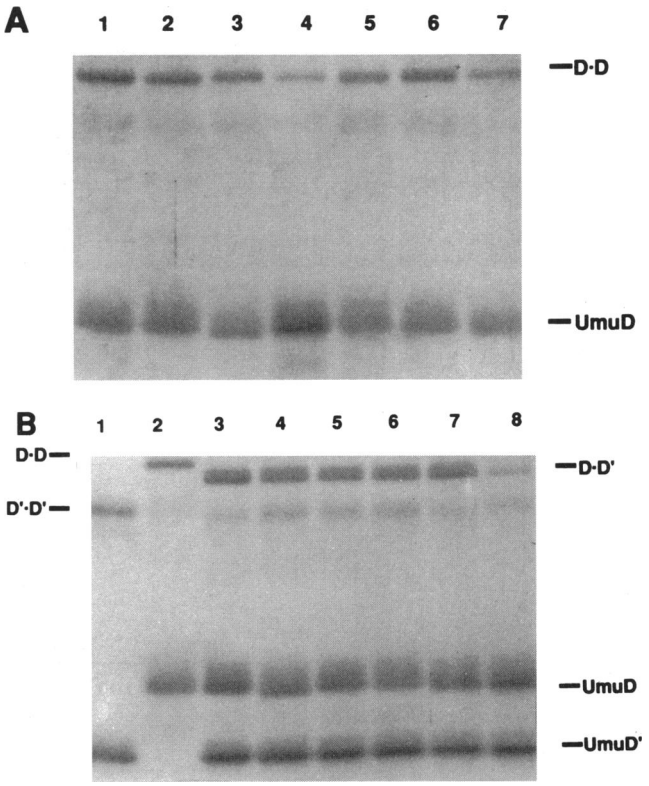

FIG. 3. Glutaraldehyde cross-linking of UmuD monocysteine mutant homodimers and UmuD · UmuD′ heterodimers. (A) Glutaraldehyde cross-linking of $UmuD_2$ was carried out by adding a final concentration of 0.05% glutaraldehyde to 10 μM UmuD and incubating for 5 min. Lanes: 1, CA24; 2, $UmuD^+$; 3, VC34; 4, LC44; 5, SC60; 6, SC81; 7, DC126. (B) For cross-linking of mutant UmuD to wild-type UmuD′, 10 μM UmuD derivative was incubated with 10 μM UmuD′ for 30 min at 37°C and then treated with glutaraldehyde. Lanes: 1, UmuD′ only; 2, $UmuD^+$ only; 3 to 8, UmuD′ and UmuD derivatives $UmuD^+$ (3), VC34 (4), LC44 (5), SC60 (6), SC81 (7), and DC126 (8). Data shown are representative of duplicate experiments.

We also surveyed the abilities of the monocysteine UmuD derivatives to form heterodimers with UmuD′. Previously, Battista et al. (6) had shown that glutaraldehyde cross-linking experiments performed 15 min after mixing equimolar amounts of $UmuD_2$ and $UmuD'_2$ homodimers resulted in the detection of only UmuD · UmuD′ heterodimers, a result which indicated that the UmuD · UmuD′ heterodimer is more stable than either of the homodimers. Equimolar amounts of the intact monocysteine UmuD proteins and wild-type UmuD′ protein were mixed and incubated at 37°C for 30 min in order to allow them to reach equilibrium. All the derivatives formed heterodimers (Fig. 3B). However, the AC89 (data not shown)

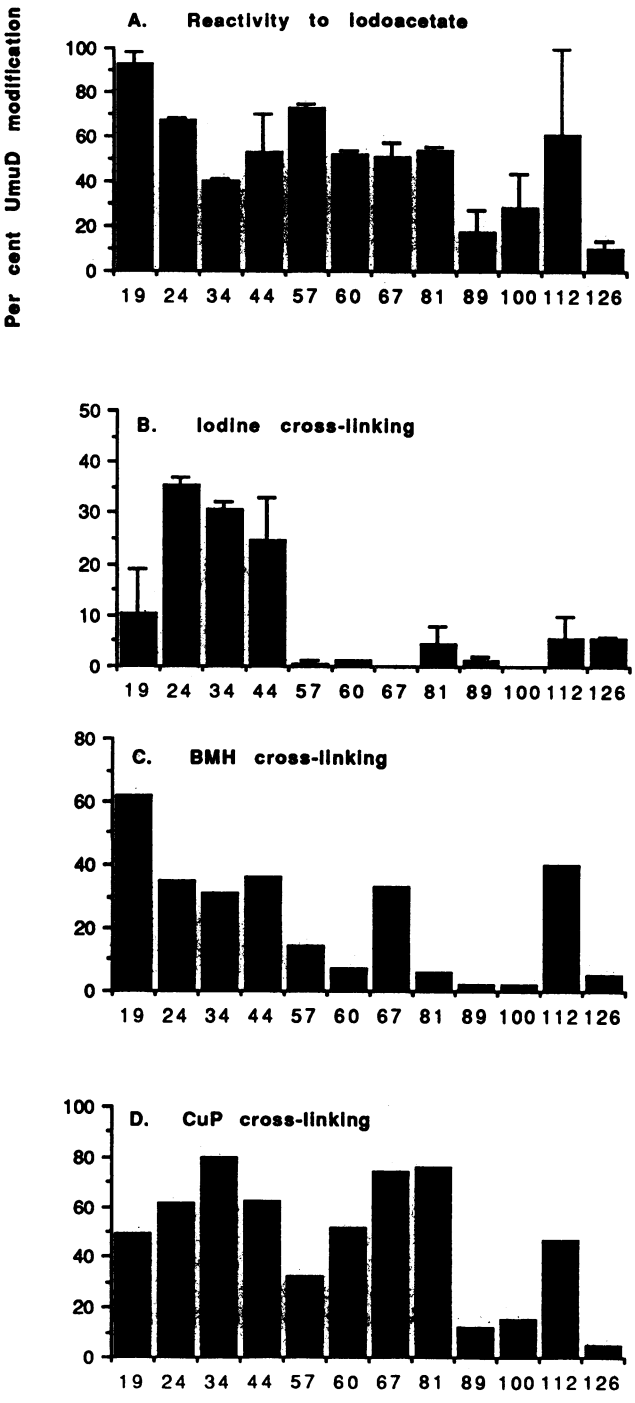

FIG. 4. (A) Reactivity of UmuD monocysteine mutant proteins with [^{3}H]iodoacetate. The percentage of total protein modified by iodoacetate in 60 min was measured. UmuD at a concentration of 20 μM was incubated with a 40-fold molar excess of [^{3}H]iodoacetate in 50 mM HEPES (pH 8.1)–500 mM NaCl for 60 min in the dark. The counts determined for UmuD (CA24) were only slightly above background level (250 cpm in comparison with 16,500 cpm for fully reacted UmuD) and were subtracted as background. (B) Percent UmuD cross-linked by using iodine (I_2). UmuD (10 μM) was incubated with 0.5 mM iodine for 20 min at 22°C as described in Materials and Methods. (C) Percent UmuD cross-linked by using BMH. BMH (1 mM) was added to 10 μM UmuD, and the mixture was incubated for 5 min at 22°C as described in Materials and Methods. (D) Percent UmuD cross-linked by using CuP. Oxidations with O_2 catalyzed by CuP were conducted by reacting 10 μM UmuD with 0.48 mM Cu^{2+} and 0.65 mM phenanthroline for 10 min at 0°C in 50 mM HEPES–100 mM NaCl, pH 8.1, as described in Materials and Methods.

and DC126 derivatives displayed a substantially decreased ability to form heterodimers with UmuD′. The effects of both of these mutations on heterodimer formation were greater than their effects on homodimer formation. In contrast, the LC44 mutation, which impaired homodimer formation, did not appear to impair heterodimer formation. The observation that both the $UmuD_2$ homodimer and the UmuD · UmuD′ heterodimer can be cross-linked with glutaraldehyde indicates that most likely, the same general region is involved in dimerization on UmuD and UmuD′. The different effects of the different cysteine substitutions on homodimer and heterodimer formation, however, suggest the possibility that the specific surface contacts in the homodimer and the heterodimer are different. Thus, while a slight conformational change resulting from the introduction of a cysteine substitution might lead to either (i) a shift in the positioning of the lysine residues involved in the cross-linking or (ii) a subtle change in the protein's surface structure involved in homodimer or heterodimer formation, these effects need not affect the $UmuD_2$ homodimer or UmuD · UmuD′ heterodimer interface in the same way. A cysteine substitution at position 89 or 126 appears to affect the surface areas of UmuD involved in interactions with UmuD′ more dramatically than those involved in interactions with UmuD, while a cysteine substitution at position 44 seems to affect only the region involved with homodimer formation. While further investigations are required to elucidate these subtle interactions, these data do support the conclusion that these UmuD monocysteine derivatives retain structures that are very similar to the structure of the $UmuD^+$ protein.

Cysteine-specific reactivities of UmuD monocysteine mutant derivatives. In order to test for the accessibility and reactivity of the unique cysteines in UmuD, the purified UmuD derivatives were reacted with [^{3}H]iodoacetate. Generally, the extent of reactivity for each thiol group depends primarily on its exposure to solvent and also on its particular local electrostatic environment (16). In the case of UmuD, which favors dimer formation, consideration must also be given to the possibility that although a particular cysteine may be on the surface of the protein in the monomer, its accessibility may be reduced if it is located on the dimer interface where it may be partially protected from reaction with iodoacetate.

The results of these studies are summarized in Fig. 4A. In these studies, the control protein with no cysteine, UmuD (CA24), was also treated with [^{3}H]iodoacetate and the resulting counts were found to be only slightly above background level (250 cpm incorporated in comparison with 16,500 cpm incorporated for fully reacted wild-type UmuD). These counts were subtracted as background to control for any nonspecific reactions that might have occurred with this reagent. In most cases, we have made the assumption that the differences in reactivities primarily reflect differences in accessibility of the sulfhydryl group for the reagent (16). The exception might be the thiol group at position 60, which might have a higher degree of inherent reactivity in its electrostatic environment than the others because of the role of the serine in this position as the putative nucleophile (see Discussion). In this study, we found that SC19 is the most reactive, being almost completely modified by iodoacetate during the 60-min incubation at 37°C. SC19 is located in the 24-residue amino-terminal region of the

protein that is cleaved. The high-level reactivity of SC19 in this fragment suggests that it is in a well-exposed region. Those derivatives that have low reactivities, AC89, QC100, and DC126, are most likely buried within the interior of the protein or at least minimally exposed to the exterior environment. Sulfhydryls located at any of the other locations on the protein, i.e., positions 24, 34, 44, 57, 60, 67, 81, and 112, had reactivities ranging from 40 to 80% modification in 60 min. We interpret this as meaning that these sulfhydryls are quite exposed to the solvent. The reductions in the reactivities of these sulfhydryls compared with that of SC19 may be explained by one or more of the following: (i) the sulfhydryl may not be fully exposed because of the folding of the protein, (ii) the reactivity of the sulfhydryl with iodoacetate could be slightly influenced by the local electrostatic environment, or (iii) the sulfhydryl could be partially protected from reaction with iodoacetate by the dimerization of UmuD.

Disulfide cross-linking of UmuD monocysteine derivatives. In order to gain information concerning the positions of the various monocysteine substitutions relative to the dimer interface, we examined the susceptibilities of the homodimers of the UmuD monocysteine derivatives to becoming cross-linked by disulfide bonds. This cross-linking reaction can be carried out by the addition of iodine (I_2) (43) or CuP (8, 16–18, 38). The formation of disulfide-linked dimers of UmuD monocysteine derivatives occurs much more readily on the addition of CuP than on addition of I_2, and this difference is reflected in the results shown in Fig. 4B and D. For reactions catalyzed by CuP, mixtures were incubated at 0°C for 10 min before quenching with EDTA and NEM, while reactions catalyzed by iodine were carried out at 22°C for 20 min before quenching with NEM. Fig. 4B shows that disulfide formation upon iodine treatment of monocysteine mutant homodimers occurs efficiently for C-24, VC34, and LC44 (~30% cross-linked); moderately for SC19 (10%); and appreciably less for the other mutants (0 to 5%). The fact that the susceptibilities of the various monocysteine derivatives to cross-linking upon iodine treatment did not correlate at all with the susceptibilities of the same proteins to reaction with iodoacetate strongly suggests that the susceptibilities of the various derivatives to disulfide cross-linking are a result of the differences in the positions of the sulfhydryl pairs in the homodimers of the monocysteine UmuD derivatives rather than of their accessibility to reagents in solution. Thus, the observation that UmuD derivatives with cysteine at positions 24, 34, and 44 were most efficiently cross-linked suggests that the regions of these positions are closer to the dimer interface than the other positions tested. The less efficient cross-linking of SC19 compared with that of the derivatives, C-24, VC34, and LC44, suggests that the pair of sulfhydryls in the homodimer of this mutant might be further apart than those of C-24, VC34, or LC44.

This interpretation is also supported by the data from CuP cross-linking. Again, cross-linking occurs very readily for UmuD derivatives having sulfhydryls at positions 24, 34, and 44, and in fact can be effectively driven to completion on increase of the temperature from 0 to 22°C in the same reaction time (data not shown). This high level of disulfide cross-linking efficiency is consistent with the assignment of these positions to the dimer interface, and in light of these data, the modest reduction in iodoacetate reactivities of these residues can be reasonably explained by hypothesizing that dimerization causes the sulfhydryls in these positions to be partially protected from reaction with iodoacetate. The intermediate efficiency of cross-linking for SC19 with CuP is also consistent with data obtained from iodine cross-linking.

Sulfhydryls at positions that we deduced were buried or only partially exposed (positions 89, 100, and 126) cross-linked poorly with either reagent, as would have been expected. Sulfhydryls located at positions 57, 60, 67, and 81 have relatively high levels of reactivity to iodoacetate and yet cross-link poorly with I_2. A simple interpretation of these findings is that these positions are located on surfaces of the dimer that are exposed to solvent but are not sufficiently close to form disulfide cross-links efficiently. They could be located on the outer surface of the dimer away from the interface or else in clefts that would make the thiol group unavailable for cross-linking but still able to react well with iodoacetate. However, addition of CuP resulted in more efficient cross-linking of sulfhydryls at these positions. It is possible that the increased formation of disulfide-cross-linked dimers could have arisen either as a consequence of interdimer cross-linking or as a consequence of structural fluctuations within the UmuD dimer (17).

Dependence on UmuD concentration of disulfide cross-linking of UmuD monocysteine derivatives by CuP. To test whether the cross-linking by CuP resulted from inter- or intradimer interactions, we conducted experiments to study CuP cross-linking of the UmuD mutant derivatives at three different concentrations: 0.1, 1.0, and 10 μM UmuD. These cross-linking reactions were conducted at both pH 8.1 and pH 7.3. For most mutants, the results in Fig. 5 show no significant dependence of cross-linking ability of these mutant proteins on concentration under the conditions and at the concentration range tested. These results suggest that cross-linking of these UmuD derivatives occurs as a result of intradimer rather than interdimer disulfide bond formation.

It is interesting to note the difference in disulfide cross-linking ability of SC67 at pH 8.1 and pH 7.3. That this substantial difference in cross-linking ability is not evident in the results obtained from other UmuD derivatives indicates that this difference is not simply an artifact of the reagents or conditions tested but is in fact due to the properties of the specific monocysteine mutant protein. A possible explanation for this result is that the local environment around the cysteine substitution at position 67 is sensitive to changes in pH, such that decreasing the pH makes the thiol group in this position less susceptible to cross-linking with CuP by causing the sulfhydryls to become less accessible to each other in the dimer.

Cross-linking with BMH. Cross-linking UmuD with a cross-linker having a greater molecular span relaxes the requirement that the two sulfhydryls be within very close proximity. These cross-linkers can be used to identify those pairs of sulfhydryls that are within the maximum molecular span of the given cross-linker and can give an indication of possible interresidue distances. BMH used for these studies is a thiol-specific cross-linker with a 6-carbon spacer and has a maximum span of 13.9 Å (1.39 nm). However, because it is able to assume many different conformational states due to free rotation around the methylene carbons, it is very possible for this cross-linker to join sulfhydryls within its maximum molecular span but not beyond (67). The results of cross-linking with BMH are shown in Fig. 4C. Derivatives that were found to readily form disulfide bonds in the dimer were also found to cross-link relatively efficiently with this reagent, indicating that they are within the range of the cross-linking reagent. Positions of efficient cross-linking include positions 24, 34, and 44, which are probably close to the dimer interface, and also position 19 located in the N-terminal fragment. While the sulfhydryls of SC19 did not form disulfide bonds as efficiently as those of the derivatives which contain sulfhydryls in the dimer interface, they did cross-link the most efficiently with this reagent. Both

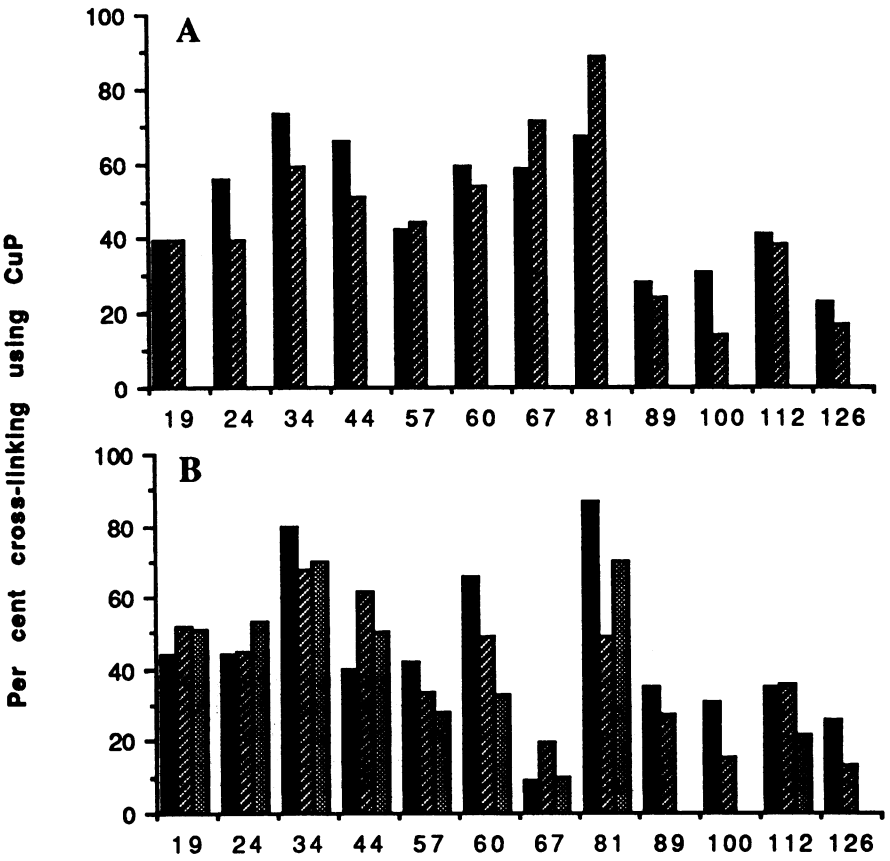

FIG. 5. Dependence of CuP cross-linking on UmuD concentration. Oxidations with O_2 catalyzed by CuP were conducted by reacting UmuD at 0.1 μM (dotted bars), 1 μM (hatched bars), or 10 μM (solid bars) with 0.48 mM Cu^{2+} and 0.65 mM phenanthroline for 5 min at 0°C as described in Materials and Methods. Reactions were conducted in 50 mM HEPES–100 mM NaCl, pH 8.1 (A) or 10 mM Na phosphate–100 mM NaCl, pH 7.3 (B). No detectable cross-linking was observed for derivatives AC89, QC100, and DC126 at a 0.1 μM concentration of UmuD (B).

SC67 and SC81, which have high reactivities to iodoacetate, cross-link poorly with I_2, and cross-link well with CuP, were cross-linked to different extents on treatment with BMH; SC67 cross-linked rather well (33%), while SC81 cross-linked poorly (6%). Mutants with sulfhydryls in several other locations, including SC57, which is quite reactive with iodoacetate, cross-linked poorly with BMH. The mutant with a sulfhydryl at position 60, the site of the putative nucleophile implicated in the cleavage reaction, also cross-linked very poorly with BMH. As expected, those mutants with sulfhydryls at positions deduced from the iodoacetate reactivity studies to be buried (AC89, QC100, and DC126) cross-linked poorly with this reagent.

DISCUSSION

In the present study, we analyzed a set of monocysteine derivatives of *E. coli* UmuD with the chosen sites of substitutions spanning the entire length of UmuD. Our hope was to gain information about the function and physical relationship of different regions along the entire length of UmuD. In an attempt to generate monocysteine derivatives that were biologically active, we made cysteine substitutions at sites that either (i) were not conserved in related proteins (UmuD analogs and repressors subject to RecA-mediated cleavage) or (ii) represent conservative substitutions. This strategy was largely successful; nevertheless, certain of the monocysteine derivatives had biological or biochemical characteristics that shed additional light on the functional elements of UmuD. We also have taken advantage of the chemical properties of the unique thiol group in each of the derivatives to gain information about the local environment around each unique cysteine.

Although this type of experimentation has certain inherent ambiguities of interpretation, we have used simple interpretations of the results to make inferences concerning the three-dimensional structure of the UmuD protein. The assumptions we have made in interpreting our data are (i) that the proteins are in conformations similar to that of $UmuD^+$; (ii) that the reactivity of the sulfhydryl group with iodoacetate is predominantly influenced by its accessibility to iodoacetate (except in the case of SC60, the putative nucleophile, which might be more reactive to iodoacetate because of its local environment); and (iii) that the cross-linking results primarily reflect interresidue distances in proteins with the same conformation.

Whether the information obtained in these studies pertains to the most stable form of the dimer is unclear. Since UmuD is posttranslationally modified and interacts with many different proteins, it is possible that the structure of UmuD in solution is not static. Certainly, for the LexA-λcI-UmuD family, it would be reasonable to suggest that upon interacting with activated RecA, these proteins might undergo conformation shifts which are important for their proper functions within the cell. Thus, the lowest-energy conformation of a protein in a particular crystal form may not necessarily repre-

sent the biologically most important, or even dominant, form in solution. Two illustrative examples that are closely related to the field of DNA repair are topoisomerase I (31) and the carboxyl-terminal domain of Ada (39). In both these cases, the protein crystallized in a form that does not allow a direct explanation of its biological function and crystallographers have had to postulate that the protein adopts one or more alternative conformations that are different from that observed in the crystal. Furthermore, even if a protein can be successfully crystallized and the structure can be solved, there may be regions that are flexible. An example that is highly relevant to this discussion is the disordered loops in the RecA crystal structure (58). If one were to make monocysteine derivatives in one of those loops of RecA, as we have done with UmuD, then one might expect the results to be influenced by the flexibility of that region of the protein. Information on such interactions would be valuable in addressing questions of structure and function. Since UmuD is by no means the only interesting protein which has not been crystallized, the development of this strategy for studying UmuD may be of some use for the complementing of structural studies of proteins in other systems.

Cys-24, Val-34, and Leu-44: residues suggested by I_2 cross-linking to be closer to the interface than the other residues tested. Our results suggest that amino acids at positions 24, 34, and 44 are located closer to the interface of the $UmuD_2$ homodimer than the other residues we tested. This conclusion is based principally on the relative ease, in comparison with all other monocysteine derivatives, with which homodimers of $UmuD^+$ (i.e., C-24), VC34, and LC44 could be cross-linked by disulfide bridges under mild oxidizing conditions. The fact that they could also be cross-linked by the cysteine-specific homobifunctional reagent BMH much better than could certain other derivatives that were equally reactive with iodoacetate is consistent with this conclusion. All three positions appear to be reasonably accessible to the solvent as judged by the reactivity of the corresponding monocysteine derivatives with iodoacetate. Position 34 appears to be somewhat less exposed than the other two, possibly because it is partially buried in the $UmuD_2$ homodimer interface. A location of the Cys-24–Gly-25 cleavage site near the surface of the protein would be consistent with the recent observation of Kim and Little (29) that LexA can be cleaved in *trans*, implying that the corresponding cleavage site in LexA (Ala-84–Gly-85) is near the surface of the protein. A consensus Chou-Fasman secondary structure prediction based on the analyses of the UmuD, MucA, LexA, and λcI proteins suggests that residues 28 to 37 of UmuD might form an alpha helix.

The suggestion that the region from residues 24 to 44 is near the dimer interface is consistent with the results obtained from studies of both λ repressor and LexA. In their study of λ repressor, Pabo et al. (42) found that a carboxyl-terminal fragment from a papain partial digestion of λ repressor containing just a portion of this region (fragment b, including residues 122 to 236) did not form dimers, while the carboxyl-terminal fragment resulting from digestion at the normal cleavage site, or a papain digestion removing this region altogether (fragment c, including residues 132 to 236), resulted in subunits that can dimerize. They postulated that the residues in this region (residues 122 to 131) are not folded in fragment b as they would be in the native repressor and that this interferes with dimer formation. Our results provide additional evidence for the involvement of the residues in this region in dimerization.

Interestingly, Sauer and Gimble found mutations within a region in λ repressor that interfered with RecA-mediated cleavage but not with autodigestion at alkaline pH and suggested that this region is involved in interactions with RecA (22). Assuming the amino acid alignment for UmuD and λ repressor discussed by Battista et al. (6), the mutations would map to sites corresponding to A-30, E-35, R-37, I-38, and L-40 of UmuD within the region possibly involved in dimer interactions. Battista et al. (6) also reported other mutations in this region of UmuD (PS27 and AT30) that impair RecA-mediated cleavage. A simple interpretation of these observations is that there may be elements that are very close to the interface of the UmuD dimer that might also be involved in the interactions between UmuD and RecA that lead to UmuD cleavage. The observations that the VC34 mutant, which cross-linked efficiently as a dimer, was the most severely deficient of all the monocysteine derivatives in RecA-mediated cleavage is consistent with the idea that residues in the region involved in dimer interactions might also be involved in UmuD-RecA interactions. Both LexA and λ (10) repressor appear to be in their monomeric form while they are undergoing the interaction with activated RecA that leads to proteolytic cleavage. One possible explanation for these observations is that the rate of UmuD-RecA-mediated cleavage is controlled by the protection of elements of the RecA interaction site by dimerization. It will be interesting to see whether studies directly investigating the interactions of UmuD and RecA will be consistent with such a hypothesis.

In their studies of the cleavage of LexA repressor, Roland et al. (49) reported mutations in LexA which resulted in hypercleavable repressors, presumably by causing a conformation that is competent for cleavage. They proposed that RecA favors this conformation and thus increases the rate of reaction. The site of these mutations also lies within the region corresponding to that in UmuD which we propose to be in the dimer interface. It is possible that dimerization locks UmuD in a form which is unable to be cleaved and that dissociation to the monomeric form relaxes the stringency of conformation, thereby allowing a conformational change that brings the cleavage site to the active site, thus allowing cleavage to occur.

Ser-60, the putative nucleophile for the cleavage of the Cys-24–Gly-25 bond. Others (32–34, 48, 54, 55) have assembled evidence supporting the hypothesis that Ser-119 of LexA (which corresponds to Ser-60 of UmuD) acts as the nucleophile both in the RecA-mediated cleavage reaction and in the RecA-independent alkaline cleavage reaction, as well as in the recently discovered cleavage in *trans* reaction (29). We have previously discussed experiments that support the hypothesis that Ser-60 of UmuD functions analogously as the nucleophile in the cleavage of the UmuD Cys-24–Gly-25 bond, and that Ser-60 is not critical for the subsequent role of UmuD′ in SOS mutagenesis (41). Although the thiol group of SC60 reacts with iodoacetate to approximately the same extent as the thiol of C-24, which we have concluded is close to the dimer interface, SC60 was not cross-linked efficiently by disulfide bridges under mild oxidizing conditions (I_2) or by the thiol-specific homobifunctional reagent BMH. These results suggest that the sulfhydryls at position 60 either (i) are too far apart to be disulfide cross-linked or spanned by a BMH-derived cross-link; (ii) are sterically hindered for cross-linking with this reagent; or (iii) are located within a cleft region and are not accessible for cross-linking.

For the related proteins LexA and λ repressor, the third possibility is particularly likely. Roland et al. (49) and Slilaty and Little (54) hypothesized that the region containing the nucleophilic serine in LexA, Ser-119, is probably not well exposed to solvent. This conclusion is based on the finding that previous attempts to inhibit LexA autodigestion with the serine

protease inhibitor diisopropyl fluorophosphate were unsuccessful (54) and that a much higher concentration of diisopropyl fluorophosphate (20 mM compared with 1 mM previously tried) is required to modify 50% of the LexA in the 10-min incubation period and result in an inhibitory effect on autodigestion (48). In addition, Sussman and Alexander (59), in their analysis of the carboxyl terminus of λ repressor by antipeptide antibodies, also suggested that the region in λ repressor including the putative nucleophilic serine is not fully exposed to external reagents. Using antipeptide antibodies specific for a peptide containing the primary sequence of this region, they observed that this region is less accessible to the antibodies in the native state and does not become totally exposed even after treatment in denaturing conditions. These results, in conjunction with the secondary-structure prediction for this region consisting of a high turn index and high hydrophobicity, led them to propose an internal structure for this region.

Our data are consistent with these interpretations; however, the reasonable ability of the thiol groups in the UmuD derivative SC60 to react with the smaller reagent, iodoacetate, and to form disulfide bonds on addition of the stronger oxidizing agent, CuP, shows that the sulfhydryls at position 60 in the dimer are not totally inaccessible. This inference is consistent with Kim and Little's result that the corresponding Ser-119 of LexA is able to catalyze peptide cleavage in *trans* (29), an observation that implies that the residue is not entirely buried in the protein structure. The ability of the sulfhydryls in this position to cross-link in the presence of CuP indicates that this region may have flexibility which, because of structural fluctuations, allows the formation of disulfide bonds (17). We have argued above that the cleavage site itself must be close to the interface of the $UmuD_2$ homodimer. It will be interesting to see whether Ser-60 is close to the Cys-24–Gly-25 cleavage site or whether Ser-60 is brought into closer proximity to the Cys-24–Gly-25 bond by a RecA-mediated conformational change of UmuD.

Ser-19, a residue in the N-terminal domain of UmuD. Ser-19 is located in the N-terminal domain of UmuD that is removed by RecA-mediated cleavage. The SC19 derivative was the most reactive with iodoacetate of all the derivatives we tested, suggesting that position 19 is very well exposed to solvent. Although the SC19 $UmuD_2$ homodimer was cross-linked by disulfide bonds less well than the $UmuD^+$ (C-24) protein and the VC34 and LC44 monocysteine derivatives under mild oxidizing conditions, it was the monocysteine derivative most efficiently cross-linked by the cysteine-specific homobifunctional reagent BMH. One reasonable interpretation of this observation would be that, in the UmuD homodimer, the serines at position 19 are farther apart than the amino acids at positions 24, 34, and 44 but that they are close enough together that their monocysteine derivatives can be bridged by a cross-link created by reaction with BMH. (This interpretation would suggest that the serines at position 19 are less than 13.9 Å (1.39 nm) apart in the UmuD homodimer.) It was interesting that the relatively conservative substitution of cysteine for serine at position 19 resulted in a significant (70 to 80%) reduction in RecA-mediated cleavage, indicating that alterations that affect RecA-mediated cleavage can be located in the amino-terminal side of the cleavage site as well as in the carboxyl-terminal side (6). This is also consistent with the finding of Lin and Little that mutations in the corresponding residue in LexA also caused a severe impairment of the ability of the mutant proteins to undergo RecA-mediated cleavage and autodigestion (32, 33).

Ser-57, Ser-67, and Ser-81. Ser-57, Ser-67, and Ser-81 are all located in the central region of the UmuD protein sequence, and their corresponding monocysteine derivatives were fairly reactive with iodoacetate, suggesting that they are reasonably exposed to the solvent. The monocysteine derivatives of the SC57, SC67, and SC81 $UmuD_2$ homodimers could not be efficiently cross-linked by disulfide bridges under mild oxidizing conditions. However, the SC67 derivative differed from the other two in that it could be cross-linked by BMH to the same extent as $UmuD^+$ (C-24) and the VC34 and LC44 monocysteine derivatives. As for Ser-19, we suggest that in the $UmuD_2$ homodimer, the serines at position 67 are too far apart for their monocysteine derivatives to be cross-linked by a disulfide bridge but are close enough for their monocysteine derivatives to be cross-linked by BMH. Interestingly, Ser-67 is located within the region of 11 amino acid residues, from positions 65 to 75, that is highly conserved within the family of UmuD analogs that play roles in mutagenesis but not in the family of related repressors (6). Our observation that the SC67 mutation affects the UV mutagenesis phenotype much more dramatically than it affects RecA-mediated cleavage suggests that it is important for the subsequent role of UmuD′ in SOS mutagenesis. One of the dominant negative mutations described by Battista et al. (6), GR65, which is located within this region, is defective in both RecA-mediated cleavage and mutagenesis. It is possible that the amino acid at this position is also important for a subsequent role of UmuD′ in mutagenesis; however, the radical substitution of arginine for glycine may cause such changes within the local environment of the site that distinguishing between these roles may not be possible.

In their screen for second-site suppressors which restore the ability of the λ *ind*$^-$ mutant repressor GR185 to undergo RecA-mediated cleavage, Gimble and Sauer (23) isolated three independent revertants. Two of the three second-site mutations (AT152 and PT158) were located in the C-terminal fragment of λ repressor in the corresponding region between residues D-63 and S-67 of UmuD. These mutants were described to be better substrates for RecA-mediated cleavage because of their reduced ability to form dimers. Since the structure of the C-terminal fragment of λ repressor is not known, it is not clear whether these mutations affect repressor dimerization directly by interfering with interactions in the interface or indirectly by causing conformational changes. Our observation that SC67 does not cross-link well on addition of iodine but does cross-link well on addition of BMH suggests that the residues in this local region are not as close to the dimer interface. However, efficient cross-linking with CuP suggests potential flexibility in the region. Such an interpretation supports the possibility of indirect rather than direct effects of the AT152 and PT158 of λ on dimerization. Further elucidation of the mechanism of these mutations affecting dimerization await direct physical studies of the interactions of UmuD.

Although neither the SC57 nor the SC81 monocysteine derivative was significantly cross-linked by mild oxidizing treatments or by exposure to BMH, and the SC57 derivative was somewhat more reactive than the SC81 derivative to iodoacetate, the SC81 derivative was more efficiently cross-linked by CuP treatment than the SC57 derivative. If the cross-linking caused by CuP treatment represents the trapping of transient intermediates of structurally fluctuating molecules (see above), then these observations could be explained by postulating that position 57 is exposed to solvent but located within a pocket or cleft such that even transient movements bringing the sulfhydryls together occur very infrequently or that position 57 is in fact on the outer surfaces of the homodimer and is not optimally positioned for disulfide bond formation within the dimer. Position 81 is within a small region of amino acids that

is not conserved within the UmuD-LexA family of proteins. Furthermore, the SC81 derivative was quite proficient in both RecA-mediated cleavage and mutagenesis. Taken together, these results suggest that the small region around Ser-81 is not critical for either of these UmuD functions.

Ala-89, Gln-100, Ser-112, and Asp-126. The monocysteine derivatives AC89, QC100, and SC112, which have alterations in the carboxyl-terminal one-third of the UmuD protein, were largely proficient in both RecA-mediated cleavage and SOS mutagenesis. The relatively low reactivities of the AC89 and QC100 monocysteine derivatives with iodoacetate and their failure to be significantly cross-linked, even with CuP treatment, are consistent with positions 89 and 100 not being very accessible to the solvent. Both positions 89 and 100 flank the region of conserved residues which include Lys-97, the UmuD counterpart to the proposed proton acceptor Lys-156 of LexA, but neither Ala-89 nor Gln-100 seems particularly important for the cleavage reaction.

Ser-112 resembles Ser-67 in that the SC112 $UmuD_2$ homodimer is reasonably reactive with iodoacetate and efficiently cross-linked upon exposure to either BMH or CuP but not by exposure to mild oxidizing conditions. As for Ser-67, a simple interpretation of these results would be that Ser-112 is reasonably exposed to the solvent and close enough to the $UmuD_2$ homodimer interface that the corresponding monocysteine derivatives can be cross-linked by BMH but not close enough to be cross-linked by a disulfide bridge.

The DC126 monocysteine derivative of UmuD was the least reactive to iodoacetate of all of the proteins we examined and failed to cross-link significantly under any of the conditions examined. We interpret this as meaning that position 126 is buried within the folded UmuD structure or is completely buried in the interface of the $UmuD_2$ homodimer. It is interesting that substitution of cysteine for Asp-126 had a modest effect on the stability of the $UmuD_2$ homodimer but had a major effect on the ability of UmuD to form a stable heterodimer with UmuD′. This impairment of DC126 in heterodimer formation might account for the relatively high UV mutability of a strain expressing the DC126 derivative (91% of the wild-type level) in spite of its reduced ability to undergo RecA-mediated cleavage (25% of the wild-type level). Heterodimer formation has been proposed as a possible mechanism for the shutoff of UV mutagenesis, with the intact UmuD protein behaving like an inhibitor of UV mutagenesis (6, 47). Decreased ability for heterodimer formation of DC126 would permit UV mutagenesis to proceed more efficiently at a lower extent of UmuD cleavage. It is not yet clear whether the effect on heterodimer formation caused by the DC126 mutation is due to the loss of a specific contact or to an effect on UmuD structure. Battista et al. (6) found that a mutation of the conserved glycine at position 129 to aspartate also affected RecA-mediated cleavage and hypothesized that this carboxyl-terminal region of conservation may play an important role in protein architecture.

The fact that UmuD, a 15-kDa protein, undergoes so many different types of interactions (from the intramolecular autodigestion reaction to interactions in the $UmuD_2$ homodimer and the UmuD · UmuD′ heterodimer to interactions with other proteins involved in mutagenesis such as UmuC, RecA, and, possibly, components of DNA polymerase III) makes UmuD an attractive model system for the study of structure-function relationships. Using a monocysteine approach for the investigation of the structure and interactions of UmuD, we have developed a model for the topological arrangement of certain residues in UmuD. Further elucidation of the properties of the UmuD monocysteine derivatives described here, for example, by probing the interactions with other proteins involved in UV and chemical mutagenesis, should yield interesting results and provide insights into possible mechanistic roles for UmuD in mutagenesis.

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