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Analysis of the Region between Amino Acids 30 and 42 of Intact UmuD by a Monocysteine Approach

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On the basis of characterizations of a set of UmuD monocysteine derivatives, we had suggested that positions 24, 34, and 44 are closer to the intact UmuD homodimer interface than other positions tested (M. H. Lee, T. Ohta, and G. C. Walker, J. Bacteriol. 176:4825–4837, 1994). Because this region of UmuD also appeared to be important for interactions with RecA, we followed up on our previous study by constructing a second set of monocysteine UmuD derivatives with single cysteine substitutions at positions 30 to 42. We found that like the VC34 mutant, UmuD derivatives with monocysteine substitutions at positions 32 and 35 showed deficiencies in in vivo and in vitro RecA-mediated cleavage as well as in UV mutagenesis, suggesting that the position 32 to 35 region may be important for RecA-mediated cleavage of UmuD. Interestingly, UmuD with monocysteine substitutions at residues 33 and 40 showed a reduction in UV mutagenesis while retaining the ability to be cleaved by RecA in vivo, suggesting a deficiency in the subsequent role of the UmuD′ derivatives in mutagenesis. All of the UmuD monocysteine derivatives in the position 30 to 42 series purified indistinguishably from the wild-type protein. The observations that purified proteins of the UmuD derivatives RC37 and IC38 could be disulfide cross-linked quantitatively upon addition of iodine and yet were poorly modified with iodoacetate led us to suggest that the pairs of residues at positions 37 and 38 are extremely close to the $UmuD_2$ homodimer interface. These observations indicate that the structure of the $UmuD_2$ homodimer in solution is very different from the crystal structure of the $UmuD'_2$ homodimer reported by Peat et al. (T. S. Peat, E. G. Frank, J. P. McDonald, A. S. Levine, R. Woodgate, and W. A. Hendrickson, Nature [London] 380:727–730, 1996).

The process of UV and chemical mutagenesis in *Escherichia coli* requires the induction of cellular functions that facilitate translesion DNA synthesis, a process that results in the introduction of mutations at the site of the lesion (35). Genetically, this process was shown to require the expression of the products of three genes, *umuC*, *umuD*, and *recA* (17, 33), which are regulated as part of the SOS regulon (12). After exposure to a mutagen, RecA forms a nucleoprotein filament by binding to single-stranded DNA (ssDNA) generated during a cell's attempt to replicate its damaged DNA (30). This activates RecA for its role in SOS induction, and such activated RecA is referred to as RecA*. RecA* serves as a coprotease that facilitates the latent ability of LexA (23), λ cI (27), and UmuD (4) to autodigest. Cleavage of LexA is required for expression of the numerous genes under the control of the SOS regulon (12), including the *umuDC* operon. The 15-kDa UmuD protein is subsequently cleaved in a RecA-mediated fashion to yield the 12-kDa carboxy-terminal derivative, designated UmuD′ (4, 24, 31). Cleavage of UmuD to UmuD′ (24, 31) activates the protein for its role in UV and chemical mutagenesis (1, 24). A reconstituted in vitro bypass assay using an abasic site as a lesion showed that the proteins required for UV mutability included UmuD′, UmuC, RecA, and DNA polymerase III (26). UmuD was found to inhibit the process (26). UmuD and UmuD′ form homodimers as well as a heterodimer. The heterodimer was shown to be more stable in vitro and has been postulated to play a posttranslational role in negative regulation of UV mutagenesis which is perhaps particularly important during the shutdown of the SOS response (3).

Several hypotheses have been proposed for the mechanism of SOS mutagenesis, including (i) UmuD′ and UmuC affecting the processivity of DNA polymerase III (2, 34); (ii) UmuD′ and UmuC binding to the RecA-ssDNA complex, causing it to switch from a recombinational to a mutagenic bypass mode (32); and (iii) UmuD′ and UmuC inhibiting the ε (proofreading) subunit of DNA polymerase III (9). Several experiments have indicated an interaction between UmuD′ (10) or UmuC (11) and a RecA–single-stranded DNA complex. Furthermore, interactions between UmuC and UmuD or UmuC and UmuD′ have also been noted (16, 38). The different interactions between UmuD (or UmuD′), UmuC, and a RecA-ssDNA complex are consistent with the suggestion that these proteins are targeted to the lesion.

In order to learn about the protein-protein interactions of UmuD, we have initiated an approach based on the construction of a series of monocysteine derivatives of UmuD. The mutant derivative of UmuD in which an alanine is substituted for the single cysteine (designated CA24) is identical to wild-type UmuD in all properties that have been assessed. A series of UmuD monocysteine mutants was then constructed from CA24 that spanned the linear sequence of the protein (positions 19, 24, 34, 44, 57, 60, 67, 81, 89, 100, 112, and 126), and several of their genetic and biochemical properties were characterized (19). Oxidation of the purified monocysteine UmuD proteins with iodine revealed that derivatives having a single cysteine at position 24, 34, or 44 are cross-linked into the homodimer to a higher extent than derivatives having cysteines at the other positions tested. This conclusion is further supported by the *p*-azidoiodoacetanilide cross-linking results described in one of the accompanying papers (20). UmuD shares homology with LexA, λ cI, and other phage repressors as well

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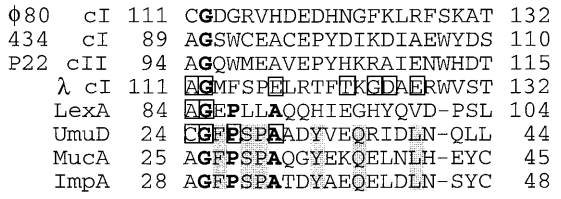


FIG. 1. Amino acid alignment of proteins that are homologous to UmuD. Shown is the region between amino acids 24 and 44 of UmuD. This figure is modified from reference 3. Positions of λ *c*I (13), LexA (21, 22), and UmuD (3, 24) at which amino acid substitutions have been shown to yield stable proteins that are defective in RecA-mediated cleavage are indicated by squares. Amino acids that are identical in the three mutagenesis proteins but are not shared with LexA or the three bacteriophage repressors are indicated by shading. Amino acids that are identical in four or more members of the set are indicated by boldfaced lettering.

as with various UmuD-like proteins, including the region corresponding to amino acids 24 to 44 of UmuD (Fig. 1). Interestingly, mutations in the corresponding region in λ *c*I (positions 111 to 132) were found to abolish RecA-mediated cleavage but not self-cleavage (13), suggesting that this region may be involved in interactions with RecA. Thus, we followed up our previous study by constructing a second set of monocysteine UmuD derivatives, each containing a single cysteine from amino acid 30 to 42. In this paper, we report our analyses of the effects of the single cysteine changes on biological activity by assay of UV mutagenesis and the effects on UmuD cleavage by RecA both in vivo and in vitro. We have also assessed the relative proximity of the cysteines to each other in the homodimer by measuring the ability of the cysteines to be cross-linked after oxidation with iodine or copper phenanthroline. Moreover, the ability to spontaneously cross-link the cysteines into a dimer after dialysis in a buffer lacking dithiothreitol (DTT) was assessed, and the results obtained by the different methods of cross-linking were compared. Inferences about the structure of intact UmuD made from these experiments are discussed in light of the crystal structure of UmuD′ recently reported by Peat et al. (25).

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains and plasmids used in this study are listed in Table 1. The antibiotics used (at the indicated concentrations) were ampicillin (100 μg/ml), chloramphenicol (30 μg/ml), kanamycin (50 μg/ml), and tetracycline (12.5 μg/ml).

Construction of monocysteine *umuD* mutant plasmids and overproduction and purification of UmuD. Construction of monocysteine *umuD* mutant plasmids was described elsewhere (19). All of the *umuD* mutants are under the control of the T7 promoter. *E. coli* SG1611 was used for the overproduction of the UmuD derivatives EC35, QC36, DC39, and LC40, and strain GW8400 was used for the overproduction of the UmuD derivatives AC30, AC31, DC32, YC33, RC37, IC38, NC41, and QC42. The monocysteine mutant proteins were purified to homogeneity as previously described (19) except that the buffer of the UmuD-containing fractions eluted from the Mono Q column was not exchanged. All the monocysteine UmuD derivatives purified indistinguishably from the wild-type protein. UmuD protein concentrations were determined with respect to the monomeric species.

UV mutability and RecA cleavage assays. UV mutagenesis was carried out according to the procedure of Elledge and Walker (6) with strain GW3200. In vivo RecA-mediated cleavage was performed with strain GW8017 by the following procedure. A saturated culture in minimal M9-glucose medium (28) supplemented with 0.1 mM $CaCl_2$, 0.1 mM $FeCl_3$, 0.1 mM $ZnSO_4$, 0.4% glucose, 5 μg of thiamine per ml, and antibiotics was diluted 1:10 into Luria broth containing the appropriate antibiotics. At an A_{600} of 0.4 to 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. After 1 h of incubation at 37°C, the culture was washed twice with an equal volume of 0.85% saline. The cells were UV irradiated at 50 J/m^2, centrifuged at 4,600 × *g* for 10 min, resuspended in an equal volume of Luria broth containing antibiotics, and incubated for 45 min at 37°C. UmuD cleavage was assessed by centrifuging the cells and resolving the protein from 0.05 A_{600} U of cells by electrophoresis on a sodium dodecyl sulfate (SDS)-polyacrylamide gel, transferring the protein to a polyvinylidene difluoride transfer membrane (Immobilon-P; Millipore, Bedford, Mass.), and blotting with a 1:10,000 dilution of affinity-purified antibodies raised against UmuD′. Cross-reacting material was visualized by chemiluminescence (Tropix, Bedford, Mass.) which was quantitated with an LKB Bromma 2202 Ultroscan laser densitometer.

In vitro RecA-mediated cleavage was carried out according to the protocol of Lee et al. (19) with some modifications. Reactions were conducted in 40 mM Tris-HCl (pH 8.0)–6.8 mM $MgCl_2$–30 mM NaCl–0.3 mM DTT with 42 ng of a 20-mer oligonucleotide per 20-μl sample volume and 0.68 mM ATPγS. UmuD at a concentration of 10 μM was incubated with 3.15 μM RecA at 37°C for 1 h.

Reactivity of mutant UmuD proteins to [^{3}H]iodoacetate and cross-linking of UmuD mutant derivatives with iodine and copper phenanthroline. Reactivity to

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Reference or source
Strains		
AB1157	*argE3*	
GW3200	Same as AB1157 but *umuD44*	24
SG1161	JM101 derivative; Δ(*lac-pro*) Δ*gal* Δ*lon510 supE thi*/(F′ traD36 *proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*)	14
RW82	Δ(*umuDC*)595::*cat*	37
GW8017	Same as AB1157 but Δ(*umuDC*)::*cat*	AB1157XP1(RW82)
GW4212	*recA938*::*cat*	36
GW8400	Same as SG1161 but *recA*938::*cat*	SG1161XP1(GW4212)
Plasmids		
pGW6070	UmuD expressed from T7 promoter	19
pGW6100	70-TGT to GCC; Cys-24 to Ala; pGW6070 derivative; *umuD131*	19
pGW7041	103-GAA to TGT; Glu-35 to Cys; pGW6070 derivative; *umuD142*	This work
pGW7051	106-CAG to TGT; Gln-36 to Cys; pGW6070 derivative; *umuD143*	This work
pGW7061	109-CGC to TGC; Arg-37 to Cys; pGW6070 derivative; *umuD144*	This work
pGW7071	112-ATC to TGC; Ile-38 to Cys; pGW6070 derivative; *umuD145*	This work
pGW7081	115-GAT to TGT; Asp-39 to Cys; pGW6070 derivative; *umuD146*	This work
pGW7091	118-CTG to TGC; Leu-40 to Cys; pGW6070 derivative; *umuD147*	This work
pGW7101	121-AAT to TGT; Asn-41 to Cys; pGW6070 derivative; *umuD148*	This work
pGW7111	124-CAA to TGT; Gln-42 to Cys; pGW6070 derivative; *umuD149*	This work
pGW7131	88-GCA to TGC; Ala-30 to Cys; pGW6070 derivative; *umuD150*	This work
pGW7141	91-GCA to TGC; Ala-31 to Cys; pGW6070 derivative; *umuD151*	This work
pGW7151	94-GAT to TGT; Asp-32 to Cys; pGW6070 derivative; *umuD152*	This work
pGW7161	97-TAC to TGT; Tyr-33 to Cys; pGW6070 derivative; *umuD153*	This work

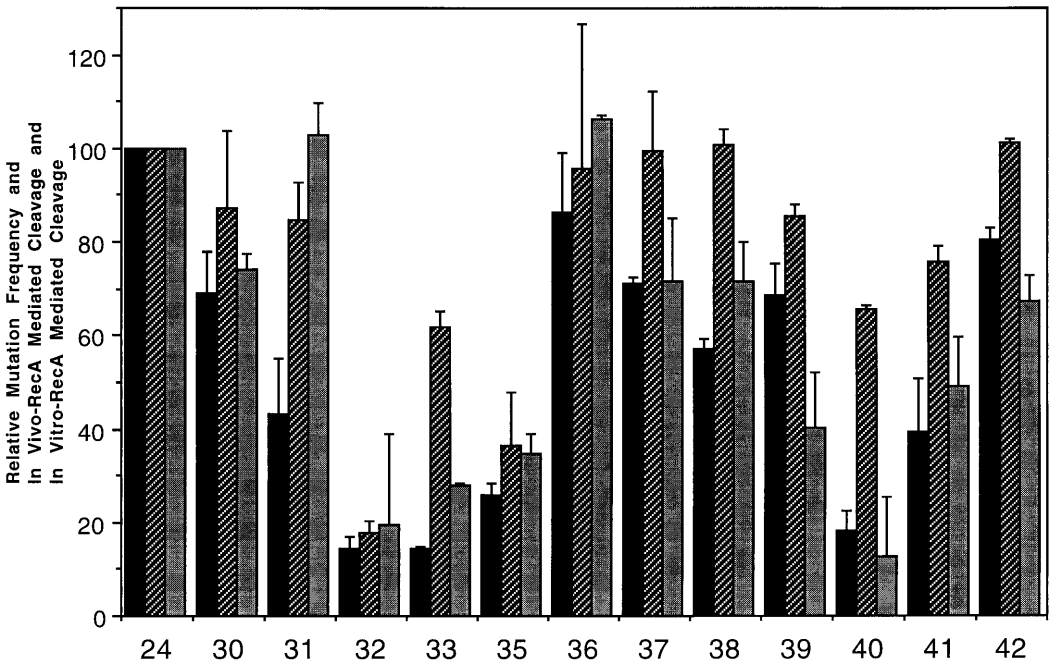


FIG. 2. Relative mutation frequency and in vivo and in vitro RecA-mediated cleavage. Mutagenesis was determined for cells irradiated with a UV dose of 20 J/m^2. In vivo and in vitro RecA-mediated cleavage assays were conducted as described in Materials and Methods. Black bars, relative mutation frequency; hatched bars, relative in vivo RecA-mediated cleavage; gray bars, relative in vitro RecA-mediated cleavage (all percentages of the wild-type level). The mean extent of in vivo RecA-mediated cleavage of UmuD$^+$ ± the standard deviation was 74.5% ± 5.5%. The extent of in vitro RecA-mediated cleavage of UmuD$^+$ was 60.5% ± 3.0%. The error bars signify the standard deviations. The numbers along the *x* axis correspond to the cysteine of the UmuD monocysteine derivative.

[^{3}H]iodoacetate was conducted as previously described (19) except that 0.6 mM DTT was present in the reaction mixture. Reactions with iodine and copper phenanthroline were performed as previously described (19) with the following exceptions: reactions with iodine were initiated by the addition of 1 mM aqueous iodine to 10 μM UmuD in 50 mM HEPES (pH 8.1)–100 mM NaCl–0.3 mM DTT. Oxidations with O_2 catalyzed by copper phenanthroline were done by reacting 10 μM UmuD with 1 mM Cu^{2+} (as $CuSO_4$) and 1.3 mM phenanthroline for 10 min at 0°C in 50 mM HEPES (pH 8.1)–100 mM NaCl–0.3 mM DTT.

Removal of reducing agent from UmuD solvent by dialysis. UmuD at a concentration of 13 μM in 10 mM sodium phosphate (pH 6.8)–100 mM NaCl–0.4 mM DTT was dialyzed against 10 mM sodium phosphate (pH 6.8)–100 mM NaCl–5 mM EDTA with a System 100 microdialyzer (Pierce, Rockford, Ill.) at 4°C for 2 h. SDS sample buffer was added after dialysis, and the proteins were resolved by electrophoresis on an SDS–13% polyacrylamide gel. The Coomassie blue-stained bands corresponding to the monomeric and dimeric forms of UmuD were quantitated with an LKB Bromma 2202 Ultroscan laser densitometer.

RESULTS

Activity of the UmuD mutant proteins in UV mutagenesis and RecA-mediated cleavage studies. In this study, our strategy for choosing the sites for the single substitutions in this set of derivatives differed from that described previously (19). In that case we chose sites which would maximize the probability of obtaining biologically active molecules spanning the entire length of the UmuD protein (19), while in this study we chose a particularly interesting region of UmuD to make successive single cysteine substitutions. Thus, some derivatives have cysteine substitutions at sites which are conserved throughout the set of analogous mutagenesis proteins. In addition, some derivatives have cysteine substitutions that do not necessarily represent conservative substitutions; i.e., a cysteine may be substituted for residues other than serine or alanine in many cases. The effects that these mutations have on the ability of UmuD to perform in various capacities, such as UV mutagenesis and RecA-mediated cleavage, have the potential to yield insights into the significance of the residues in this particular region of the UmuD protein. The ability of the mutant UmuD proteins to participate in UV mutagenesis was determined by expressing them in a *umuD44* strain and measuring the reversion of an *argE3* mutant to Arg$^+$. Most of the UmuD monocysteine derivatives tested retained a substantial ability to participate in UV mutagenesis (40 to 90% of wild-type activity). The UmuD monocysteine derivatives which were most substantially impaired by the cysteine substitution were DC32, YC33, EC35, and LC40, which retained less than 30% of wild-type activity (Fig. 2), suggesting that the residues in these positions are important, either directly or indirectly, for UmuD to be able to participate in UV mutagenesis.

The ability of these monocysteine UmuD derivatives to productively interact with RecA in a manner which leads to UmuD cleavage, both in vitro and in vivo, was assessed. The UmuD monocysteine derivatives were purified to homogeneity, and their ability to be cleaved by RecA in vitro was measured. As mentioned in Materials and Methods, all the derivatives purified indistinguishably from wild-type UmuD. We found that wild-type UmuD was cleaved to an extent of around 60% in 1 h under the conditions described in Materials and Methods. Although cleavage of UmuD and related proteins has been studied extensively in an in vitro reaction consisting of RecA, ssDNA, and ATP (or a nonhydrolyzable analog) (5, 27), it is possible that there is an additional factor that functions in the in vivo reaction which is not present in our in vitro reaction. To determine the extent of RecA-mediated cleavage in vivo, a Δ*umuDC* strain carrying a UmuD mutant plasmid was 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 determined by Western blotting (immunoblotting) with affinity-purified UmuD antiserum (3) and was found to be around 75% for wild-type UmuD under these conditions.

We found that for many of the monocysteine derivatives, RecA-mediated cleavage roughly correlated with UV mutability. Since RecA-mediated cleavage is required to activate UmuD for mutagenesis, it is not surprising that those derivatives which were defective in RecA-mediated cleavage should also be defective in mutagenesis (3). One exception was the AC31 derivative, which displayed a partial reduction in UV mutability but a nearly wild-type ability to be cleaved by RecA, both in vivo and in vitro. This observation suggests that this mutant could be partially defective in a role in UV mutagenesis that occurs after cleavage of UmuD to UmuD′, such as in the interaction of UmuD′ with other proteins. Other exceptions were YC33 and LC40, which, although defective in UV mutagenesis and in vitro RecA-mediated cleavage, were able to be cleaved efficiently by RecA in vivo. There may be a factor required in vivo that is not present in our in vitro reaction. Additionally, these mutants may be like AC31 in that they are partially impaired in their ability to perform in some capacity in UV mutagenesis that occurs after the cleavage of UmuD to UmuD′.

Solvent accessibility of the UmuD 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. The results are expressed as the number of nanomoles of [^{3}H]iodoacetate that reacted with 0.20 nmol of UmuD in 1 h, a time period in which a fully modified population of UmuD would have incorporated 0.20 nmol of [^{3}H] iodoacetate (Fig. 3A). Generally, the extent of reactivity for each thiol group depends primarily on its exposure to solvent and also on its particular local electrostatic environment (7). Most of the mutants showed a moderate to high level of solvent accessibility. Two exceptions were RC37 and IC38, which reacted poorly with [^{3}H]iodoacetate. Moderate or high reactivity suggests that the thiol group of the UmuD derivative is accessible to solvent, whereas low reactivity suggests that the thiol group is buried within the interior of the protein or possibly within the dimer interface.

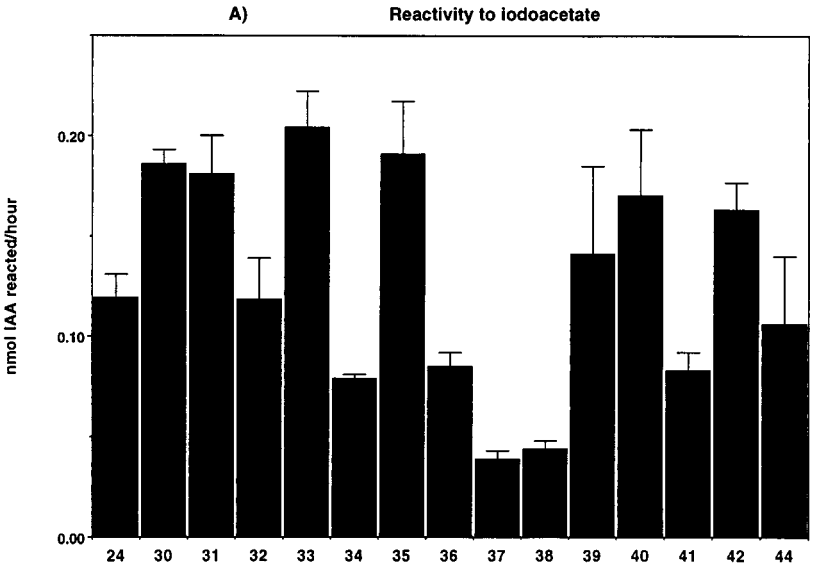
Cross-linking of the UmuD monocysteine derivatives with iodine or copper phenanthroline. In order to gain information concerning the positions of the various monocysteine substitutions relative to the dimer interface, we examined the susceptibilities of the homodimers of the UmuD monocysteine derivatives to cross-linkage by disulfide bond formation. In order for the UmuD monocysteine derivatives to cross-link, the two cysteines must be proximal to each other and in the correct orientation in the homodimer. This cross-linking was carried out by the addition of iodine (Fig. 3B) or copper phenanthroline (Fig. 3C). During the course of the purification of these monocysteine derivatives, we found that a significant proportion of the UmuD proteins (in particular, RC37 and IC38) spontaneously cross-linked in 0.1 mM DTT in the absence of any oxidizing agents. When we increased the DTT concentration to 1 mM, however, no disulfide bond formation was detectable. We therefore increased the final concentration of DTT in the buffer of the stocks of all of the UmuD mutant proteins to 1 mM in order to be consistent throughout the study and subsequently diluted the protein stocks into the appropriate reaction buffers. Thus, reaction buffers for this study contained higher DTT concentrations (0.3 mM final concentration) than previously published reaction conditions (<0.1 mM) (19). For this reason, the experiments using the UmuD monocysteine derivatives VC34 and LC44 were repeated and included with the new series of monocysteine mutant derivatives.

When the monocysteine mutants were oxidized with iodine, both RC37 and IC38 showed nearly quantitative cross-linking. In contrast, oxidation of the remaining subset of monocysteine derivatives resulted in a considerably lower level of cross-linking, ranging from 4.5 to 45%. This is a striking result considering the fact that most of the derivatives (with the exception of RC37 and IC38) reacted quite well with iodoacetate, indicating that the sulfhydryl groups were reasonably exposed to solvent. Even within this small region from amino acid 30 to 42, the position of the cysteine substitution greatly affects the ability of the UmuD derivatives to be cross-linked in the homodimer upon oxidation with iodine. As a control, this set of monocysteine mutants was incubated in the presence of 0.3 mM DTT for 1 h at 37°C without any oxidizing agent, and no detectable disulfide bond formation occurred (data not shown), ruling out the possibility that the observed cross-linking was due to spontaneous disulfide bond formation.

As observed previously (19), the copper phenanthroline-catalyzed oxidation of the UmuD monocysteine mutant proteins in the homodimer resulted in cross-linking data that are consistent with those obtained with iodine oxidation, but the differences between the derivatives are less striking. RC37 and IC38 are still the most efficiently cross-linked; however, all of the other mutants were also able to cross-link with moderate efficiency when this reagent was used. A simple explanation for these observations is that during the more prolonged copper phenanthroline-catalyzed air oxidation of the thiol groups, local flexibility within the monomer and the separation of the homodimer into monomers allows two sulfhydryls to come close enough for cross-linking to occur. No cross-linking was observed when the UmuD derivative lacking cysteine, CA24, was exposed to iodine or copper phenanthroline (data not shown).

Spontaneous cross-linking of UmuD dimers after removal of DTT by dialysis. Because certain UmuD monocysteine derivatives spontaneously cross-link via disulfide bond formation in the presence of low concentrations of DTT, we thought it would be interesting to survey the ability of our entire set of UmuD monocysteine derivatives to spontaneously cross-link upon removal of DTT by dialysis. The monocysteine derivatives in a buffer containing 0.4 mM DTT were dialyzed for 2 h at 4°C, and the resulting percentage of disulfide-cross-linked dimers is plotted in Fig. 4. Dialysis of the derivatives with cysteine substitutions within the region between positions 37 and 41 resulted in a high degree of cross-linking. Other derivatives which resulted in efficient dimer cross-linking are C24 (wild-type UmuD), AC30, AC31, and LC44. SC19, SC60, SC112, DC126, and derivatives with cysteine substitutions

FIG. 3. Iodoacetate reactivities and cross-linking ability of UmuD monocysteine derivatives. (A) Reactivity of UmuD monocysteine mutant proteins to [^{3}H]iodoacetate. The amount of total protein modified by [^{3}H]iodoacetate (IAA) in 60 min was measured. UmuD at a concentration of 20 μM was incubated with a 65-fold molar excess of [^{3}H]iodoacetate in 50 mM HEPES (pH 8.1)–500 mM NaCl–0.6 mM DTT for 60 min in the dark at 37°C. The counts determined for CA24 (UmuD without a cysteine) were only slightly above background level and were subtracted as background. (B) Percentage of UmuD cross-linked by using iodine (I_2). UmuD (10 μM) was incubated with 1 mM iodine for 20 min at 22°C as described in Materials and Methods. (C) Percentage of UmuD cross-linked by using copper phenanthroline (CuP). Oxidations with O_2 catalyzed by CuP were conducted by reacting 10 μM UmuD with 1 mM Cu^{2+} and 1.3 mM phenanthroline for 10 min at 0°C in 50 mM HEPES (pH 8.1)–100 mM NaCl as described in Materials and Methods. The error bars in all three panels signify the standard deviations. The numbers along the *x* axis correspond to the cysteine of the UmuD monocysteine derivative.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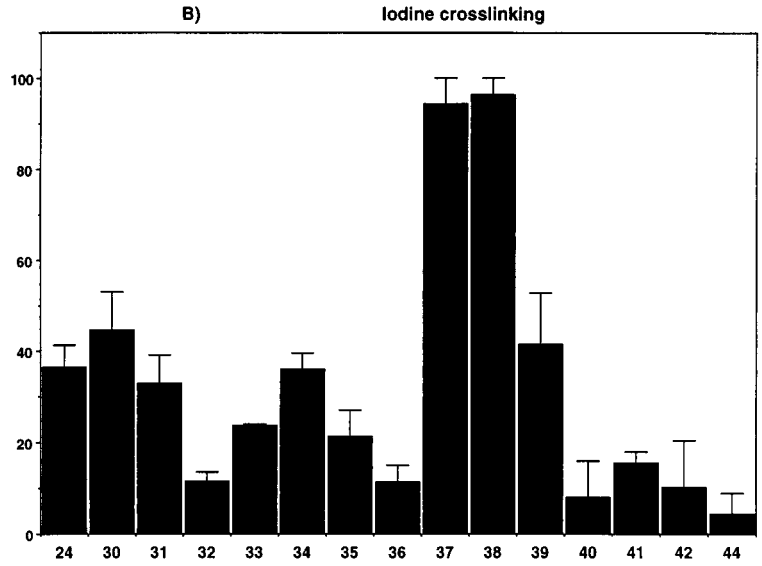
0.10
0.20
24 30 31 32 33 34 35 36 37 38 39 40 41 42 44


B)
Iodine crosslinking
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20
40
60
80
100
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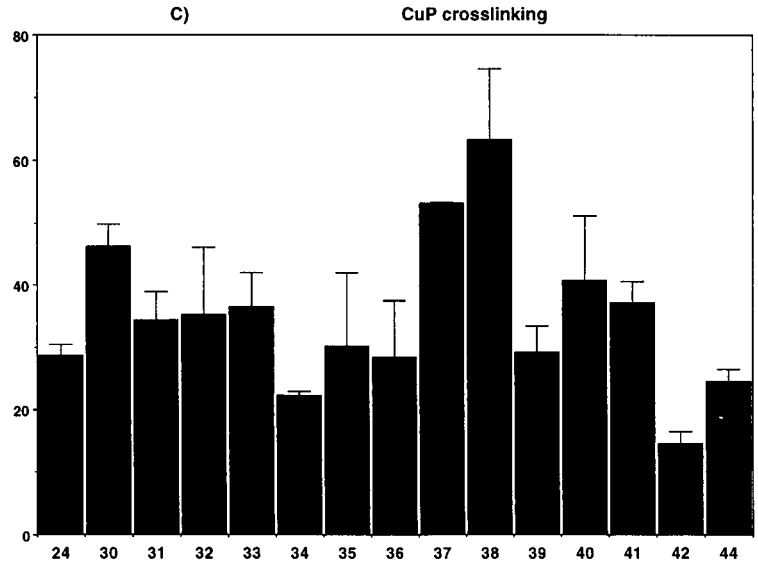
C)
CuP crosslinking
0
20
40
60
80
24 30 31 32 33 34 35 36 37 38 39 40 41 42 44

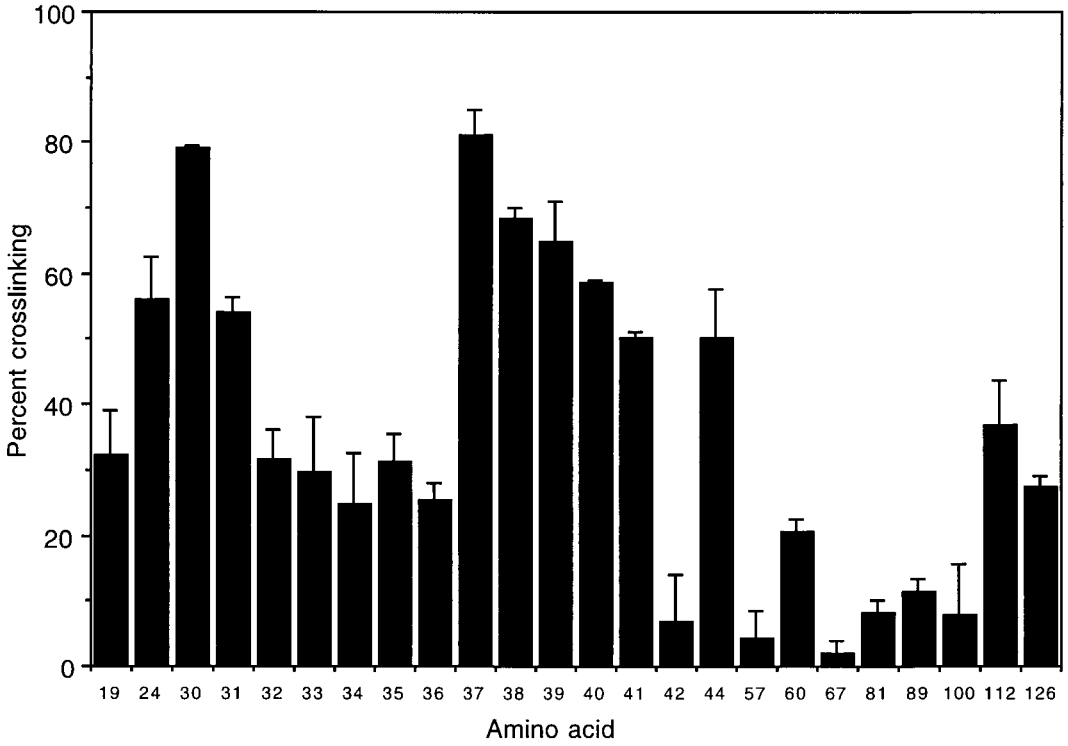

FIG. 4. Cross-linking of the monocysteine UmuD derivatives in the homodimer during dialysis. UmuD (13 μM) in a buffer containing 0.4 mM DTT was dialyzed for 2 h at 4°C against 10 mM sodium phosphate (pH 6.8)–100 mM NaCl–5 mM EDTA as described in Materials and Methods. The error bars signify the standard deviations.

within the region between amino acids 32 and 36 all have a moderate ability to spontaneously form disulfide cross-linkages in the dimer. Finally, QC42, SC57, SC67, SC81, AC89, and QC100 were able to cross-link poorly upon removal of the reducing agent.

DISCUSSION

On the basis of the relative abilities of several monocysteine derivatives of UmuD to cross-link via cysteine-specific cross-linking agents, we previously suggested that the region including the Cys-24–Gly-25 cleavage site, Val-34, and Leu-44 is closer to the UmuD homodimer interface than the other residues tested (19). Other evidence also suggested that this region is important for UmuD interactions with RecA (3, 13, 20). To further analyze the interactions in this region of UmuD, we constructed a set of UmuD monocysteine derivatives in which single cysteine substitutions were made in the region of UmuD between amino acids 30 and 42 (inclusive) and characterized them genetically and biochemically. Our studies of the structure of the homodimer of intact UmuD in solution strongly indicate that Arg-37 and Ile-38 are very close to the dimer interface of $UmuD_2$. This inference is based on the striking ease of disulfide cross-linking of the RC37 and IC38 monocysteine derivatives upon treatment with iodine. Nearly quantitative disulfide cross-linking of these derivatives occurred very rapidly upon oxidation with iodine, in contrast to the cross-linking efficiencies of the other monocysteine UmuD derivatives tested, which ranged from approximately 10 to 50%. Interestingly, when the ability of each of the mutant UmuD proteins to be modified by [^{3}H]iodoacetate was assessed, we found that all of the purified monocysteine derivatives except RC37 and IC38 were quite reactive with iodoacetate (Fig. 3A). A simple explanation for this observation is that these residues are buried within the dimer interface and thus are less accessible for reaction with iodoacetate. The AC30 and VC34 proteins can be cross-linked by iodine relatively efficiently, although not as well as RC37 or IC38, but appear to be more accessible to solvent as measured by reactivity to iodoacetate. Thus, positions 30 and 34 may be relatively near the $UmuD_2$ homodimer interface but are not as close to it as positions 37 and 38.

All of our solution studies on the intact $UmuD_2$ homodimer were performed in the absence of any structural data. However, after our studies were completed, Peat et al. reported the crystal structure of the cleaved form of UmuD, UmuD′, to 2.5 Å (0.25 nm) (25). In the crystal structure of UmuD′, the amino-terminal tail (including amino acids 30 to 42) extends outward from a globular head. Residues Tyr-52, Val-54, Ile-87, Phe-94, and Phe-128 of each UmuD′ monomer participate in hydrophobic interactions at the $UmuD'_2$ homodimer interface. In addition, a salt bridge is formed between Glu-93 of one monomer and Lys-55 of the associating monomer. The amino-terminal tails in the $UmuD'_2$ homodimer protrude out in opposite directions and do not participate in dimer interactions. It seems possible that the region from amino acid 32 to 40 is seen as an extended terminal tail with a unique conformation in the crystal as a consequence of crystal packing forces. As shown in Fig. 5, residues 34, 37, and 38 are located in the amino-terminal tails in the $UmuD'_2$ homodimer, whereas our studies of the $UmuD_2$ homodimer in solution indicate that residues 30, 34, 37, and 38 (the region containing residue 30 is disordered in the crystal) are very close to the $UmuD_2$ dimer interface. Thus, our results strongly suggest that the structures of the $UmuD_2$ homodimer in solution and the $UmuD'_2$ ho-

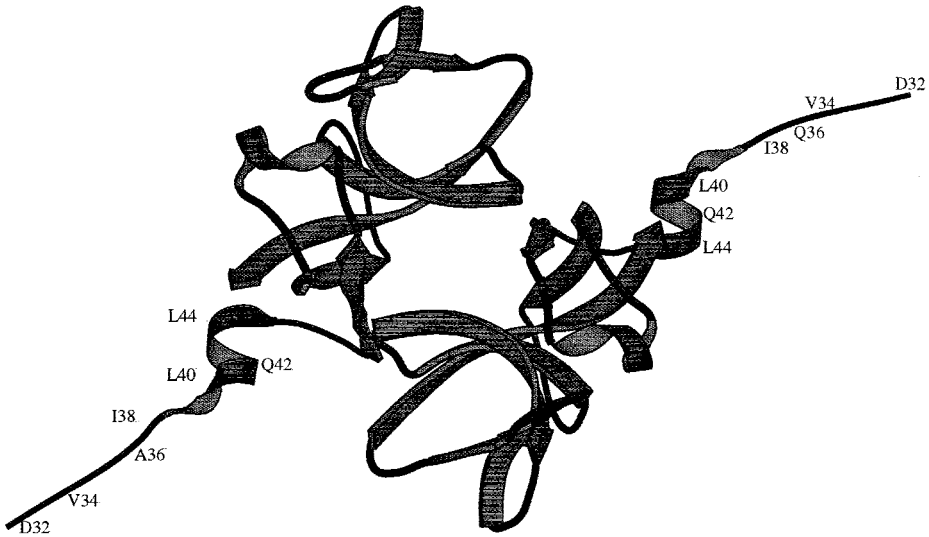

FIG. 5. Ribbon diagram of the UmuD′$_2$ homodimer as determined by Peat et al. (25). Indicated are some of the amino acids that were changed in our study of the intact UmuD protein. It is important to note that it is the structure of the UmuD′ protein which is depicted. Amino acids 30 to 42 are clearly not at the UmuD′ homodimer interface.

modimer (as determined from the crystal structure) are radically different.

To further study the structure and interactions of intact UmuD by analyzing the region between residues 30 through 42 (which seems important not only for $UmuD_2$ homodimer interactions but also for interactions with RecA [3, 13, 20]), our strategy involved choosing this particularly interesting region of UmuD as the site for successive single cysteine substitutions. When the abilities of the mutants to perform in UV mutagenesis and to be cleaved by RecA both in vivo and in vitro were assessed (Fig. 2), we observed that many of the monocysteine derivatives retained a significant ability to perform in these capacities. Although monocysteine derivatives DC32 and EC35 were substantially impaired in all of the tested functions, YC33 and LC40 were interesting because while they retained significant in vivo RecA-mediated cleavage, their ability to perform in UV mutagenesis was defective. We had previously reported that the VC34 UmuD derivative was also impaired in its ability to perform in these functions (19). In addition, as discussed in one of the accompanying papers (20), we were able to cross-link the VC34 derivative to RecA by using the cysteine-specific photoactive cross-linker *p*-azidoiodoacetanilide. Taken together, these results suggest that amino acids important for UmuD cleavage and its subsequent role in UV mutagenesis overlap but are not identical, and they support the theory that the amino acid 31 to 35 region is important for interactions with RecA*. Such a conclusion is supported by Gimble and Sauer's isolation of λ cI mutants that are deficient in RecA*-mediated cleavage but not in the RecA-independent cleavage under alkaline conditions (13). These mutations include EK117, TI122, GD124, DV125, DY125, DN125, and EK127 of λ *c*I, which correspond to Ala-30, Glu-35, Arg-37, Ile-38, and Leu-40 of UmuD (Fig. 1). It is possible that residues in the region of UmuD′ that correspond to amino acids 30 to 40 of intact UmuD could play a role in the interaction between UmuD′ and RecA* that has been reported (10), but further work will be required to address this point.

The abilities of the UmuD monocysteine derivatives to cross-link in the homodimer were determined by three different means: oxidation with iodine, oxidation with oxygen catalyzed by copper phenanthroline, and spontaneous oxidation with oxygen upon removal of the reducing agent by dialysis. Of the three methods for discerning the relative proximity of cysteine residues by disulfide cross-linking, iodine oxidation seemed to be the most discriminatory. Interestingly, the oxidation reaction involving iodine occurs so rapidly that we were unable to follow the kinetics of the cross-linking reaction. Although under our standard reaction conditions the proteins are exposed to iodine at 22°C for 20 min, we found no detectable difference in the amount of disulfide bond formation even when the reaction was carried out at 4°C for 1 min (data not shown). Furthermore, the amount of disulfide-cross-linked dimers cannot be further increased by a second addition of iodine. The explanation for this phenomenon lies in the mechanism of disulfide bond formation caused by iodine oxidation (35). Iodine oxidation of the thiol group of a UmuD monocysteine derivative results in a sulfenyl iodide intermediate (35), a reaction that apparently occurs very rapidly. A subsequent reaction of the sulfenyl iodide with the thiol group of the associating UmuD monomer is required to form a disulfide bond and to cross-link the dimer. The sulfenyl iodide intermediate is very labile (35), and thus this reaction requires a close proximal relationship between the sulfenyl iodide and the thiol group (35). However, if a water molecule attacks the sulfenyl iodide intermediate instead of an adjacent thiol group, a protein sulfenic acid is formed which is no longer available for cross-linking. The sulfenic acid is possibly further oxidized to a sulfinic or sulfonic acid (18). Thus, it seems likely that the reaction mechanism can be summarized as shown in Fig. 6. Because this reaction proceeds very rapidly (less than 1 min), the extent of $UmuD_2$ disulfide cross-linking promoted by iodine probably closely reflects the proportion of cysteines that are in close proximity in the dimer within a small window of time.

Our results suggest that iodine oxidation provides a good assessment of the proximity of the cysteines to one another in the homodimer interface. From the iodine cross-linking data it is evident that RC37 and IC38 clearly cross-linked the most efficiently of the derivatives that we tested. Oxidation with copper phenanthroline yielded the same results; however,

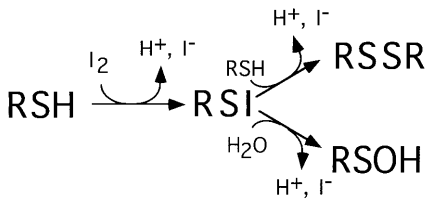

FIG. 6. Proposed reaction mechanism for disulfide bond formation via iodine oxidation.

UmuD derivatives with cysteines in other positions also formed disulfide bonds in the dimer to a moderate degree under these conditions. It is possible that this region (residues 30 to 42) is quite flexible in the intact UmuD proteins. If this is the case, then the copper phenanthroline-catalyzed oxidation of the derivatives could involve the capturing of transient intermediates in which the residues of associating monomers are brought close enough to each other to be disulfide cross-linked (8, 19). Thus, we would expect all the derivatives with cysteine substitutions in this region to react with about the same efficiency unless the positions of the pairs of cysteines in the dimer were particularly close in the native conformation, as we suggest is the case for Arg-37 and Ile-38. Surprisingly, we found that the spontaneous oxidation of thiols to form disulfide bonds during dialysis was not as discriminatory as the oxidation of thiols by iodine. Since spontaneous air oxidation of thiols appears to be a very mild method of oxidation, we had initially expected this procedure to be highly selective for only those pairs of cysteines which are optimally positioned in the native structure.

Oxidation by dialysis is not as discriminatory (Fig. 4), but this method has been used before in structure-function investigations of other proteins, such as the γδ resolvase protein (15), for which the crystal structure of the catalytic subunit is known (29). In the case of the γδ resolvase, inspection of the crystal structure and computer modeling were employed to identify positions for cysteine substitutions which would minimize distortion of the dimer interface in the disulfide-cross-linked dimer. Two of the mutants cross-linked completely in the dimer, and one mutant cross-linked to a level of 50% after 12 h of dialysis. These observed results were interpreted to imply that dialyzing away the reducing agent and then testing for disulfide bond formation is a good method for identifying those residues which are relatively close in the native conformation of the dimer. However, in our investigations of derivatives with cysteine substitutions within the region of amino acids 30 to 44, we found that in addition to RC37 and IC38, the UmuD derivatives AC30, AC31, DC39, LC40, NC41, and LC44 also disulfide cross-linked quite efficiently during dialysis (Fig. 4). In our experiments, we allowed dialysis to proceed for only 2 h, although even an additional 2 h of dialysis resulted in a higher yield of cross-linked dimers (data not shown). Moreover, we found that in addition to those derivatives in the first set of monocysteine proteins which were expected to cross-link efficiently (SC19, C24, VC34, and LC44), the UmuD derivatives SC60, SC112, and DC126 also appeared to cross-link relatively more efficiently during dialysis than upon treatment with iodine (determined previously [19]). These results suggest that inferences about the solution structure made from the spontaneous disulfide cross-linking of a single pair of cysteines after dialyzing away the reducing agent should be made with caution.

It will be interesting to evaluate the structure of UmuD with results obtained from this monocysteine approach. Furthermore, analysis of the UmuD′$_2$ structure by a monocysteine approach will also provide insights into interactions within the UmuD′$_2$ homodimer and into interactions of UmuD′ with other proteins involved in mutagenesis. A better understanding of the mechanism of activation of UmuD to UmuD′ upon RecA-mediated cleavage might also be gained by a comparison of the structures and interactions of UmuD and UmuD′.

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