A Monocysteine Approach for Analyzing the Structure and Interactions of the UmuD Protein involved in SOS Mutagenesis

by

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BACHELOR OF SCIENCE IN BIOCHEMISTRY
UNIVERSITY OF CALIFORNIA, BERKELEY, 1989

Submitted to the Department of Biology in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY IN BIOLOGY
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

FEBRUARY 1996

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ABSTRACT

A series of monocysteine derivatives of UmuD was constructed and characterized and experiments were performed exploring the chemistry of the unique thiol group in each derivative. Based on these results several inferences were made regarding the relative proximity of certain residues to the UmuD dimer interface. In particular, we propose that the region including the Cys24-Gly25 cleavage site, Val34, and Leu44 are closer to the interface than the other positions tested.

We have also used the cysteine-specific photoactive crosslinker, *p*-azidoiodoacetanilide (AIA) to explore UmuD interactions with RecA. VC34 and SC81 crosslinked most efficiently to RecA indicating that these residues are closer to the RecA-UmuD interface than the other derivatives tested. SC57, SC67, and SC112 crosslinked moderately efficiently with RecA. Neither C24, the UmuD derivative with a cysteine located at the Cys24-Gly25 cleavage site, nor SC60, the UmuD derivative with a cysteine substitution at the position of the putative active site residue, was able to crosslink to RecA suggesting that RecA need not directly interact with residues involved in the cleavage reaction. SC19, located in the N-terminal fragment of UmuD that is cleaved, and LC44 also did not crosslink efficiently with RecA.

To follow up the previous study, a second set of monocysteine UmuD derivatives with single cysteine substitutions at positions 30 to 42 was constructed. The observation that purified proteins of the UmuD derivatives RC37 and IC38 could be disulfide crosslinked quantitatively upon addition of iodine and yet be poorly modified with iodoacetate led us to suggest that the pairs of residues at 37 and 38 are at the UmuD homodimer interface.

Finally, selected UmuD derivatives were disulfide crosslinked in dimers by exposure to oxidizing conditions and then incubated with activated RecA to determine whether such crosslinked dimers could undergo RecA-mediated cleavage. Generally, we found that the crosslinked $UmuD_2$ derivatives were cleaved very poorly upon incubation with activated RecA compared to a UmuD derivative lacking cysteines that had been treated identically. However, if these disulfide crosslinked derivatives were incubated with DTT prior to incubation with RecA, reducing the disulfide bonds, then the resulting extent of cleavage dramatically increased for each derivatives. This result suggests that the monomeric form of UmuD is a better substrate for the RecA-mediated cleavage reaction than the dimeric form.

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Acknowledgments

I would like to express my deepest gratitude to Graham Walker for his constant support, patience, wisdom, and guidance. I am especially grateful for his commitment to "believe in me until I believed in myself" and his investments of time and effort in my professional and personal growth.

During the more challenging times of this project, Peter Kim and H. Gobind Khorana have been a constant support of this effort. I'm thankful for their stimulating discussions and their encouragement. I also greatly appreciate Paul Schimmel and Gerhard Wagner for their willingness to be on my defense committee on such short notice and for their scientific insights and expertise.

I'm grateful to the members of the laboratory both past and present and friends in the Biology department. I've learned so much from them and I have really appreciated their friendships. Special thanks to Toshihiro Ohta who generously "showed me the ropes" when I first joined the lab. I'm grateful to Jundong Zhang for the crash course he gave me in organic chemistry. I also want to thank Angelina Guzzo for her contributions to this project. Thanks to Thomas Barthel for his friendship and for letting me see M.I.T. from a "new perspective". I'm also deeply grateful to Marianne White for her helpfulness in EVERYTHING. Her jovial personality, her genuine care for each member of the laboratory, and her humming in the hallways make me happy to be in this lab.

I am truly grateful for my friends from the Boston Chinese Evangelical Church and from the M. I. T. Chinese Christian Fellowship who have been my family away from home. I shudder to imagine what graduate school life might have been like had I not known them. I am grateful to Philip Lin, Marilyn Chen, and Hsi Jung Wu, my fellow sojourners in graduate school, for understanding the joys and woes of research, for sympathizing with and encouraging me to press on, for companionship and friendship, and for their prayers. I thank Charles Owyang for his prayers, his hugs and smiles, his friendship, and his computer. I'm grateful to Scott Tang for good memories, for accepting my weaknesses and highlighting my strengths, and for "keeping me in". To Angela Lih, my "kindred spirit" - your friendship has been one of the great blessings of my being in Boston. Thanks for being there through the laughter and the tears.

It would have been very difficult to persevere through and finish this program had it not been for the constant love and support of my family, Mom and Dad, Richard and Erica. To them I am forever grateful.

Lastly, I would like to thank God, Creator of all things, for being my source of life, strength, and joy.

Introduction

The ability of an organism to respond favorably to challenges to its genetic material is critical for its survival. Introduction of DNA lesions poses the threat of decreased fidelity eventually leading to mutations, or interference with replication leading to cell death. The cellular responses that cope with DNA lesions, best understood in *Escherichia coli*, consist of those that result in accurate repair of damaged DNA and those that result in the introduction of mutations. The strategies that result in the accurate repair of damaged DNA by the direct reversal of or by excision repair of the damage include photoreactivation of UV-induced pyrimidine dimers, excision repair, post-replication repair, and mismatch repair (35). Cells have also evolved other systems to cope with lesions introduced into their DNA which are not repaired by other mechanisms. These damage tolerance mechanisms include processes that repair single-stranded or double-stranded breaks in the DNA as well as a process which appears to involve polymerization of DNA past a lesion (translesion DNA synthesis) (35). In contrast to other mechanisms of coping with damaged DNA, this cellular mechanism can be highly mutagenic, and in fact, is required for most UV radiation and chemical mutagenesis (for extensive review, see ref.(26, 35, 80)). Mutagenesis in *E. coli* resulting from exposure to UV light and various chemicals is not a passive process but rather requires the participation of the products of three genes - *umuD*, *umuC*, and *recA* - which are regulated as part of the *recA*$^+$*lexA*$^+$-dependent SOS network. Thus, DNA lesions are not necessarily mutagenic by themselves but must be processed by an induced cellular mechanism in such a way that results in the introductions of mutations into the DNA.

Current model for the mechanism of the SOS regulation.

The *recA*$^+$*lexA*$^+$-dependent SOS network regulates the expression of more than 20 genes in response to lesions which are introduced into the DNA upon exposure of *E. coli* to UV radiation and various chemicals (35). The increased level of expression of these genes results in a complex set of physiological responses known as the SOS response (53, 64, 80, 81, 82). In the uninduced state, the expression of these genes, including the *recA* and *lexA* genes, are repressed by the LexA protein. Exposure of *E. coli* to UV radiation or various chemicals causing damage to its DNA or interference with replication of its DNA results in the generation of an intracellular distress signal. Evidence suggests this signal to be

regions of single-stranded DNA generated when the cell attempts to replicate past the damaged DNA or when its normal DNA replication process is interrupted (70). RecA, in the presence of a nucleoside triphosphate cofactor (ATP) becomes activated upon binding to these regions of single-stranded DNA and forming a nucleoprotein filament. The activated form of RecA (RecA*) then mediates the cleavage of LexA repressor (52) by facilitating the latent capacity of LexA to autodigest (51). This cleavage, which occurs at a specific Ala84-Gly85 bond in a hinge region between the amino and carboxy terminal domains of LexA (43), inactivates LexA as a repressor thereby allowing an increased level of expression of these genes. Several lines of evidence suggest that the mechanism of LexA autodigestion at alkaline pH occurs by a mechanism related to that of serine proteases in which Ser119 acts as the putative nucleophile in the cleavage reaction and Lys156 functions as the activator (74). Thus, since the functional groups that participate in the cleavage reaction are found in LexA, RecA* serves not as a traditional protease but rather as a coprotease in the cleavage reaction.

Regulation of SOS mutagenesis as part of the SOS response

A correlation of increased survival and increased mutagenesis after exposure to a DNA-damaging agent was first demonstrated by the analysis of the phenomena of Weigle reactivation and Weigle mutagenesis. Both the survival of irradiated bacteriophage λ and its mutation frequency were shown to increase identically as a function of increasing UV dosage to the infected bacteria. This evidence suggested that the same inducible mechanism is responsible for both increased survival and mutagenesis (67, 85). It was further shown that the irradiation of the bacteriophage had no effect on its mutation frequency unless the bacterial host had also been pre-irradiated (Weigle mutagenesis). These phenomena were later recognized to be regulated as part of the *recA*$^+$*lexA*$^+$-dependent SOS regulatory network when it was demonstrated that neither phenomena was observed in strains which carried either *recA(Def)* or *lexA(Ind*$^-$*)* mutations which prevent induction of the SOS response (20).

Requirement of the *umuD*, *umuC*, and *recA* gene products in mutagenesis

umuDC and naturally occurring umuDC analogs

Screening for *E. coli* mutants which were defective in SOS mutagenesis induced by UV light and various chemicals led to the identification of the *umuC*

locus (46, 76) which was later found to consist of two genes, *umuD* and *umuC*, organized in an operon (29, 47, 62). The *umuDC* operon is under the control of *recA*$^+$*lexA*$^+$-dependent regulatory network (4). Many *umuDC* analogs have also been found in a variety of enterobacteria (60, 72), and on several naturally occurring wide host-range plasmids (39, 80, 87). Considerable effort has been devoted to the study of the *mucAB* operon, an evolutionarily diverged analog of the *umuDC* operon, borne on the naturally occurring plasmid pKM101 (63). Plasmid pKM101 has played an important role in the Ames test for detecting carcinogens as mutagens, enhancing the sensitivity of the test by increasing the capacities for the survival and mutagenesis in the tester strains after exposure to a variety of mutagenic agents. Products of the *mucAB* operon suppress deficiencies of *umuDC* mutants and this suppression is similarly under SOS regulation (28, 83). The *umuDC* and *mucAB* operons have been cloned and their gene products identified. Both operons encode proteins of molecular weights 15 kDa and 45 kDa. The deduced sequences of MucA and UmuD are 41% identical at the amino acid level, while the MucB and UmuC proteins are 55% identical (62). The structural and functional similarities of MucAB and UmuDC suggest that they share a common evolutionary origin (62). However, despite these similarities, it has been shown that the MucAB system is much more efficient at mutagenesis, and in a *recA430* strain which is deficient in coprotease activity, MucAB but not UmuDC can mediate UV mutagenesis (10). In addition, though both systems are involved in functionally similar roles, interactions between the components of the two systems do not reconstitute activity in vivo. MucA cannot substitute for UmuD in a *umuD*$^-$ *umuC*$^+$ host and MucB cannot suppress the deficiency of a *umuD*$^+$*umuC*$^-$ host. This observation led to the suggestion that there might be a specific physical interaction between the two gene products of each operon and that the two pairs have diverged significantly so that interactions between UmuD and MucB, or between MucA and UmuC are not productive (62).

RecA-mediated posttranslational cleavage of UmuD

Sequencing of UmuD revealed that UmuD shares homology with the carboxy-terminal region of many bacteriophage repressors including the repressors of λ , ϕ80, 434, and P22, the LexA repressor, and the analogous proteins MucA and ImpA (9, 27, 62, 71) (please see Fig. 1). This homology has been shown to have functional significance in that cleavage of all of these proteins occurs at a specific Ala-Gly or Cys-Gly bond, most probably by similar mechanisms . Lin and Little

demonstrated that RecA actually facilitates the otherwise latent capacity of LexA to autodigest; incubation of LexA in alkaline pH results in its autodigestion to yield identical cleavage products to those obtained upon incubation with RecA* at neutral pH (51, 52). The cleavage reaction has been proposed to occur by a mechanism similar to that of serine proteases in which a nucleophile, apparently a serine residue conserved in all members of the family (Ser119), is activated by a lysine residue (Lys156) (74). The putative nucleophile and activator corresponding to Ser60 and Lys97 in UmuD respectively, have been shown genetically to be important in the cleavage reaction as well (59).

Through a series of genetic experiments it was demonstrated that RecA-mediated cleavage activates UmuD for it's role in mutagenesis (59). Using site directed mutagenesis, a set of UmuD mutant derivatives were constructed which were analogous to those in LexA and λ repressor mutations that blocked both autodigestion and RecA-mediated cleavage. These mutations largely abolished the mutability of the strains (59), suggesting a correlation between the cleavage of UmuD and its ability to function in mutagenesis. In addition, a *umuD* mutant was constructed with overlapping termination and initiation codons introduced into the *umuD* sequence at the site which corresponded to the putative cleavage site. The plasmid carrying this engineered form of UmuD encodes two polypeptides which are virtually the same as those that would result from cleavage of UmuD at the Cys24-Gly25 bond. It was observed that the introduction of this plasmid to a *umuD44* nonmutable strain restored the UV mutability of the cell to that of the wild type strain (59). This result ruled out the possibility that cleavage inactivates UmuD for its role in mutagenesis. It was subsequently shown that a plasmid encoding only the amino-terminal region of UmuD (amino acids 1 through 24) was not able to suppress the nonmutability of a *umuD44* strain but that a plasmid encoding only the carboxy-terminal region of UmuD (amino acids 25 through 139) was able to complement the UV nonmutability of the *umuD44* strain (59). These experiments indicated that RecA-mediated cleavage activates UmuD and that the carboxy-terminal fragment (termed UmuD') is necessary and sufficient for its role in mutagenesis. Thus, RecA plays a subsequent role in SOS mutagenesis by mediating the cleavage of UmuD.

Another genetic study that offered insight into the regulation of UmuD activity involved the isolation of a set of missense mutants of UmuD which were shown to be defective in UV mutagenesis and in RecA-mediated cleavage *in vivo*. Most of these mutants were dominant to the wildtype UmuD with respect to UV

mutagenesis but did not interfere with SOS induction (9). Cross-linking studies using glutaraldehyde suggested that UmuD and UmuD' formed homodimers as well as UmuD·UmuD' heterodimers but that at equilibrium, the UmuD·UmuD' heterodimers were more stable (9). Taken together, these results suggest that intact UmuD might play an additional role in modulating the activity of UmuD' by sequestering UmuD' and affecting the pool of available UmuD' in the cell. This might serve as another level of regulation in helping the cell return to the uninduced state (9).

Requirement of other gene products

RecA in a third role in mutagenesis

The possibility that RecA plays a third role in mutagenesis besides mediating the cleavage of LexA repressor and UmuD was raised by the observation that introduction of a plasmid encoding UmuD' (the active form of UmuD) did not suppress the UV nonmutability of a *lexA(Def)Δ(recA-srlR)306*::Tn*10* (59) and was subsequently supported by other studies (6, 24, 25, 30, 78). Insights concerning the nature of the third role of RecA in mutagenesis have been gained through the study of partial-loss-of -function *recA* mutants. The production of UmuD' and UmuC cannot restore the mutability phenotype of a *recA1730* strain, which is dominant for UV nonmutability even when the other SOS genes are expressed, indicating this strain to be defective in this third role of RecA (5, 25). RecA1730 has a mutation within a pocket formed between two RecA monomers which has been suggested to be the potential repressor and mutagenesis protein binding site (25, 77). This mutation causes the RecA protein to be deficient in nucleoprotein filament formation (24). In DNA mobility-shift assays, it was observed that the RecA1730 nucleoprotein filament formed less stable interactions with UmuD than the wild type RecA nucleoprotein filament (34). It was proposed from these results that the third, direct role for RecA in mutagenesis is to target the UmuD/C protein complex to the DNA and that this role requires the formation of RecA nucleoprotein filaments (34). Other experiments have led to the suggestion that the interaction of UmuD' and UmuC with the growing end of a RecA nucleofilament inhibits recombination and switches the RecA-coated DNA from being a substrate for recombination to being a substrate for bypass mutagenesis (75). This suggestion was primarily based on the observations that the overproduction of UmuD' and UmuC proteins in a Hfr x F^- conjugal cross inhibits recombination but that this recombination can be substantially suppressed by overproducing RecA.

DNA polymerase III

It appears that some form of DNA polymerase III holoenzyme, the major replicative DNA polymerase of *E. coli* , is the polymerase involved in mutagenesis by UV and various chemicals. SOS mutagenesis does not require DNA polymerase I or DNA polymerase II since cells carrying a deletion of the gene encoding DNA polymerase I (*polA*) (7) or a gene encoding a defective DNA polymerase II (*polB*) (44, 48) were still able to perform in mutagenesis induced by UV light and various chemicals. However, these experiments leave open the formal possibility that these polymerases could participate in SOS mutagenesis if they were present. Limited evidence has been provided for the involvement of DNA polymerase III in SOS mutagenesis. Support for this hypothesis comes from genetic experiments involving *E. coli* strains carrying a temperature-sensitive mutation in the *dnaE* gene encoding the α subunit of DNA polymerase III (13), as well as experiments involving strains carrying a temperature-sensitive mutation in *dnaE* and the *pcbA1* mutation which allows DNA polymerase I-dependent replication of the bacterial genome without a functional DNA polymerase III α subunit (41). The *pcbA1 dnaE* (Ts) strains are able to survive at the restrictive temperature (43 °C) for DNA polymerase III, but are non-mutable by UV light or certain chemicals at the restrictive temperature even though the SOS genes are expressed (12, 16, 40). The mutable phenotype is restored upon the introduction of a plasmid encoding the wildtype DNA polymerase III (41). The interpretation of these results was that DNA polymerase III was required for SOS mutagenesis. However, subsequent studies revealed that a *pcbA1 dnaE* (Ts) strain which also carried a mutation in the gene encoding the protein responsible for excision repair (*uvrA*) are mutable by EMS (58) at the restrictive temperature suggesting that the nonmutable phenotype observed previously could be explained by an alternative mechanism. The alternative interpretation is that the replication complex of a *pcbA1dnaE* mutant pauses at the DNA lesions for a longer period of time as compared to that of a wildtype cell allowing the excision repair function to remove lesion more completely (58). Biochemical evidence for the ability of DNA polymerase III to function in SOS mutagenesis is described below.

GroEL and GroES

E. coli strains which overexpress the UmuD and UmuC proteins becomes cold sensitive for growth, i.e. they grow at 42 °C but not at 30 °C. The finding that mutations in *groES* and *groEL* suppress cold sensitivity stimulated further

experiments that led to the discovery that the GroES and GroEL proteins of *E. coli* are normally required for mutagenesis (22). GroES and GroEL are *E. coli* heat shock proteins which are homologous to members of the eukaryotic Hsp60 protein family (36) and are thought to be molecular chaperone proteins involved in the macromolecular assembly of protein complexes. *groE* mutants are defective in UV mutagenesis and this deficiency can be partially alleviated by the increased expression of *umuDC* (23). Coimmunoprecipitation of UmuC with GroEL with anti-GroEL antibodies suggests a physical interaction of these proteins *in vivo* (23). In addition, the half-life of the UmuC protein, expressed in the absence of UmuD or UmuD', is shorter in a *groE* mutant (6 min compared to around 17 min for the wildtype) suggesting that one possible role of the GroE proteins involves the stability and proper folding of the UmuC protein (22). The finding that inactive UmuC treated with Hsp70 and Hsp60 regains DNA binding activity and ability to promote translesion synthesis is consistent with this role of such molecular chaperones in their interactions with UmuC (65). Coexpression of *umuD'* with *umuC* results in a reduced requirement for GroE proteins in UV mutagenesis and also a significant increase in the half life of UmuC (23) suggesting that the GroE proteins stabilize UmuC until it is able to complex with UmuD or UmuD'. Interestingly, this requirement of UmuC for GroE function is not shared by its analog, MucB, for UV mutagenesis, UV resistance, phage reactivation and cold sensitivity (21).

Model for the molecular mechanism of SOS mutagenesis

Genetic and biochemical evidence support the following model for the mechanism of SOS mutagenesis (Please see Fig. 2). When DNA lesions are introduced by UV light or various chemicals, RecA is activated to RecA* in the presence of a nucleotide triphosphate (ATP) and an inducing signal (single-stranded DNA generated by the cell's attempt to replicate damaged DNA) (70). RecA* mediates the cleavage of the major cellular repressor, LexA (52), thereby allowing the increased transcription of various SOS genes including *umuD*, *umuC*, and *recA*. RecA* then mediates the posttranslational cleavage of UmuD at its Cys24-Gly25 in a manner mechanistically similar to the cleavage of LexA (17, 73). This cleavage has been shown genetically to activate UmuD, the resulting carboxy-terminal fragment (termed UmuD') being necessary and sufficient for its role in mutagenesis (59). UmuC is unstable by itself but is stabilized by GroEL and GroES until it can interact with UmuD or UmuD' (22, 23). Only the complex of

UmuC with $UmuD'_2$ is active in mutagenesis. The dimer $UmuD'_2$ associates with UmuC and RecA* and this complex interacts with DNA polymerase III in such a way that DNA lesions are bypassed at the expense of the introduction of mutations (35, 68). SOS mutagenesis appears to be due to a process of translesion synthesis in which the replicative machinery, involving UmuD', UmuC, RecA, and DNA polymerase III, encounters a non-coding or miscoding lesion, inserts an incorrect nucleotide across from the lesion and then continues elongation (35). Experiments of UV irradiation and delayed photoreversal of pyrimidine dimers in *E. coli* cells which are excision repair-deficient support a model for UV mutagenesis which involves a two step mechanism: (i) a misincorporation step opposite the photoproduct, and (ii) the bypass of the lesion which requires the UmuD, UmuC, and RecA proteins (11, 14, 15). The finding that most mutations resulting from SOS mutagenesis by UV light and various chemicals are targeted to the site of the lesion is consistent with this model of translesion synthesis (55). That the mutational spectra resulting from the exposure of cells to a specific mutagenic agent depend on the type of mutagen is also supportive of a mechanism of translesion synthesis in which the type of lesion that is present determines the base that is most likely incorporated opposite the lesion (55, 56); i.e., that the replicative assembly operating under SOS condition attempts to extract the available coding information for the lesions it encounters. The roles of UmuD' and UmuC in this process are still unclear. One possibility is that UmuD and UmuC function in altering the behavior of DNA polymerase III on damaged DNA by altering the molecular mechanism responsible for its processivity (8). This suggestion is based on the observations of limited amino acid similarities of UmuD and UmuC to gp45, gp44, and gp62, the DNA polymerase accessory proteins of bacteriophage T4. These T4 accessory proteins act together to make T4 DNA polymerase more processive. Other possibilities include modulating the action of the 3' -> 5' proofreading subunit (45, 49) or altering some other action of the polymerase that becomes limiting on damaged DNA templates (35).

Biochemical approaches for the study of the mechanistic process of SOS mutagenesis have recently been developed. Rajagopalan et al. (68) have reconstituted limited replicative bypass in an *in vitro* system with purified UmuD', renatured UmuC, RecA and DNA polymerase III proteins and a DNA substrate with a single abasic lesion. In another approach, Cohen-Fix and Livneh (19) have reported the development of a crude cell-free system made from SOS-induced cells

that is capable of processing UV-irradiated plasmid DNA to yield mutated DNA in a fashion that requires the *umuD*, *umuC*, and *recA* gene products.

Interactions of UmuD

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 homodimers and heterodimers (86), and the interactions of the UmuD·D' heterodimers are more stable than either of the homodimers (9). It seems likely that all three forms of the dimers interact with UmuC (86) and may interact with one or more components of DNA polymerase III holoenzyme (15, 42, 45, 54, 68). In addition, intact UmuD interacts with RecA* in a fashion that results in cleavage of its Cys24-Gly25 bond, and it is capable of autodigestion of the same bond if incubated at alkaline pH (17).

The role of RecA* in mediating the cleavage of repressor and mutagenesis proteins implies that a direct interaction between these proteins and RecA must occur which leads to the cleavage of the protein. This class of interaction has been visualized by electron microscopy for the complex of LexA with the RecA filament (88). In the study, the LexA repressor was found to bind within the deep helical groove of the activated RecA filament. The strikingly different effects of certain RecA mutations on its ability to mediate the cleavage of different repressor and mutagenesis proteins suggest that some contacts between the repressor or mutagenesis proteins and RecA might be specific for a particular protein. This view is supported by the observation that the RecA430 mutant (which has a glycine to serine substitution at position 204) is deficient in mediating the cleavage of LexA, very deficient in mediating the cleavage of UmuD (73) and λ repressor (69) but is proficient in mediating the cleavage of ϕ80 repressor (27).

Other evidence suggests that direct physical interactions also occur between RecA and the cleavage product of a mutagenesis protein (i.e., UmuD' or MucA'). In DNA mobility shift assays, UmuD' or MucA' as well as UmuD could be crosslinked by glutaraldehyde to a RecA filament (34). In addition, it has been observed that the overproduction of UmuD' and UmuC proteins in a Hfr x F^- conjugal cross inhibits recombination but that this recombination can be substantially suppressed by overproducing RecA. This experiment has led to the suggestion that the interaction of UmuD' and UmuC with the growing end of a RecA nucleofilament inhibits recombination and switches the RecA-coated DNA from being a substrate for recombination to being a substrate for bypass

mutagenesis (75). It is not yet understood whether the nature of the UmuD·RecA interactions and UmuD'·RecA interactions are similar or different. One observation which suggests that the interactions might be different is the finding that RecA430 fails to mediate the cleavage of UmuD, but is functional for mutagenesis when UmuD' is directly produced (59).

Structural information for intact 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 intact UmuD, we have initiated an approach for investigating the structure and interactions of UmuD that is based on the construction of a set of derivatives differing only in the position of the unique cysteine residue. With any missense mutant, one can carry out a 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 the single thiol group in each of the mutant proteins. These have the potential to yield insights into such issues as accessibility of particular amino acids to solvent, conformational changes undergone by the protein, and the nature of subunit interactions in multiprotein complexes (1, 2, 3, 31, 32, 33, 57, 61, 79, 84). Since UmuD and UmuD' appear to be involved in many protein interactions in this complex process of mutagenesis, knowledge of its interactions with other proteins would be necessary for a detailed understanding of its mechanism. This type of experimentation is not intended to replace direct physical examinations of structure; moreover, if the three-dimensional structure 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.

This thesis describes the construction and characterization of several UmuD monocysteine derivatives and the inferences made regarding the closeness of certain residues of UmuD to the UmuD homodimer interface. These inferences were primarily based on the reactivities of the unique cysteines to the alkylating agent, iodoacetate, and to different cysteine-specific crosslinkers: the zero-length crosslinkers (which promote disulfide bond formation), iodine and oxygen in a

reaction catalyzed by copper/phenanthroline, and a 13 Å homobifunctional crosslinker, *bis*-maleimidohexane (Chapter 2).

In extending these investigations of UmuD interactions to analyze interactions of UmuD with other proteins during mutagenesis using the monocysteine derivatives, we have employed the use of a cysteine-specific photoactive crosslinking reagent. Such a strategy does not require prior knowledge of the interacting sites of adjacent proteins or a cysteine residue at the site of interaction or mutagenesis of the interacting protein. Chapter 3 and Chapter 4 describe the synthesis of a 9 Å heterobifunctional cysteine-specific photoactive crosslinker, *p*-azidoiodoacetanilide, and its use in further elucidating $UmuD_2$ homodimer interactions and in investigating UmuD-RecA interactions.

Some evidence supports the thought that the region near the amino terminus of intact UmuD is involved in RecA-UmuD interactions and $UmuD_2$ homodimer interactions. This suggested to us that a closer analysis of the region between amino acids 30 to 42 of UmuD by studying mutants with unique cysteines substituted in this region might yield interesting insights into these interactions. The characterizations of this new set of derivatives and inferences drawn from these results are reported in Chapter 5.

Because both UmuD and UmuD' form homodimers and heterodimers (86) it is unclear whether the substrate for the RecA-mediated cleavage reaction (which converts UmuD to UmuD') is the monomeric form or dimeric form of UmuD. In the case of λ repressor which shares homology with UmuD, evidence has been presented which suggests that the repressor monomer is the preferred substrate (18, 38, 66). We have devised a strategy using the monocysteine approach to address the issue of whether UmuD is more efficiently cleaved as a monomer or a dimer. These findings are discussed in Chapter 6.

References

1. **Aggeler, R. and R. A. Capaldi**. 1993. ATP hydrolysis-linked structural changes in the N-terminal part of the γ subunit of *Escherichia coli* F_1 -ATPase examined by cross-linking studies. J. Biol. Chem. **268:**14576-14579.

2. **Aggeler, R., K. Chicas-Cruz, S. X. Cai, J. F. W. Keana and R. A. Capaldi**. 1992. Introduction of reactive cysteine residues in the ε subunit of *Escherichia coli* F_1 ATPase, modification of these sites with tetrafluorophenyl azide-maleimides, and examination of changes in the binding of the ε subunit when different nucleotides are in catalytic sites. Biochemistry **31:**2956-2961.

3. **Altenbach, C., S. L. Flitsch, H. G. Khorana and W. L. Hubbell**. 1989. Structural studies on transmembrane proteins. 2. Spin labeling of bacteriorhodopsin mutants at unique cysteines. Biochemistry **28:**7806-7812.

4. **Bagg, A., C. J. Kenyon and G. C. Walker**. 1981. Inducibility of a gene product required for UV and chemical mutagenesis in *Escherichia coli.* Proc. Natl. Acad. Sci. U.S.A. **78:**5749-5753.

5. **Bailone, A., S. Sommer, J. Knezevic, M. Dutreix and R. Devoret**. 1991. A RecA protein deficient in its interaction with the UmuDC complex. Biochimie **73:**479-484.

6. **Bates, H. and B. A. Bridges**. 1991. Mutagenic DNA repair in *Escherichia coli. XIX.* On the roles of RecA protein in ultraviolet light mutagenesis. Biochimie **73:**485-489.

7. **Bates, H., S. K. Randall, C. Rayssiguier, B. A. Bridges, M. F. Goodman and M. Radman**. 1989. Spontaneous and UV-induced mutations in *Escherichia coli* K-12 strains with altered or absent DNA polymerase I. J. Bacteriol. **171:**2480-2484.

8. **Battista, J. R., T. Nohmi, C. E. Donnelly and G. C. Walker.** 1988. Role of UmuD and UmuC in UV and chemical mutagenesis., 455-459. *In* E. C. Friedberg and P. C. Hanawalt (ed.), Mechanisms and consequences of DNA damage processing. Alan R. Liss, Inc., New York.

9. **Battista, J. R., T. Ohta, T. Nohmi, W. Sun and G. C. Walker**. 1990. Dominant negative *umuD* mutations decreasing RecA-mediated cleavage suggest roles for intact UmuD in modulation of SOS mutagenesis. Proc. Natl. Acad. Sci. U.S.A. **87:**7190-7194.

10. **Blanco, M., G. Herrera and V. Aleixandre**. 1986. Different efficiency of UmuDC and MucAB proteins in UV light induced mutagenesis in *Escherichia coli.* Mol. Gen. Genet. **205:**234-239.

11. **Bridges, B. A.** 1992. Mutagenesis after exposure of bacteria to ultraviolet light and delayed photoreversal. Mol. Gen. Genet. **233:**331-336.

12. **Bridges, B. A. and H. Bates**. 1990. Mutagenic DNA repair in *Escherichia coli* XVIII. Involvement of DNA polymerase III α-subunit (DnaE) in mutagenesis after exposure to UV light. Mutagenesis **5:**35-38.

13. **Bridges, B. A., R. P. Mottershead and S. G. Sedgwick**. 1976. Mutagenic DNA repair in *Escherichia coli*. Mol. Gen. Genet. **144:**53-58.

14. **Bridges, B. A. and R. Woodgate**. 1984. Mutagenic repair in *Escherichia coli*: The *umuC* gene product may be required for replication past pyrimidine dimers but not for the coding error in UV-mutagenesis. Mol. Gen. Genet. **196:**364-366.

15. **Bridges, B. A. and R. Woodgate**. 1985. Mutagenic repair in *Escherichia coli*: Products of the *recA* gene and of the *umuD* and *umuC* genes act at different steps in UV-induced mutagenesis. Proc. Natl. Acad. Sci. U.S.A. **82:**4193-4197.

16. **Bryan, S. K., M. Hagensee and R. E. Moses**. 1990. Holoenzyme DNA polymerase III fixes mutations. Mutat. Res. **243:**313-318.

17. **Burckhardt, S. E., R. Woodgate, R. H. Scheuermann and H. Echols**. 1988. UmuD mutagenesis protein of *Escherichia coli*: Overproduction, purification, and cleavage by RecA. Proc. Natl. Acad. Sci. U.S.A. **85:**1811-1815.

18. **Cohen, S., B. J. Knoll, J. W. Little and D. W. Mount**. 1981. Preferential cleavage of phage lambda repressor monomers by *recA* protease. Nature (London) **294:**182-184.

19. **Cohen-Fix, O. and Z. Livneh**. 1992. Biochemical analysis of UV mutagenesis in *Escherichia coli* by using a cell-free reaction coupled to a bioassay: Identification of a DNA repair-dependent pathway. Proc. Natl. Acad. Sci. U.S.A. **89:**3300-3304.

20. **Defais, M., P. Fauquet, M. Radman and M. Errera**. 1971. Ultraviolet reactivation and ultraviolet mutagenesis of lambda in different genetic systems. Virology **43:**495-503.

21. **Donnelly, C. E., S. Murli and G. C. Walker**. 1994. The *groE* gene products of *Escherichia coli* are dispensable for $mucA^{+}B^{+}$-dependent UV mutagenesis. Mutat. Res. **309:**225-233.

22. **Donnelly, C. E. and G. C. Walker**. 1989. *groE* mutants of *Escherichia coli* are defective in *umuDC*-dependent UV mutagenesis. J. Bacteriol. **171:**6117-6125.

23. **Donnelly, C. E. and G. C. Walker**. 1992. Coexpression of UmuD' with UmuC suppresses the UV mutagenesis deficiency of *groE* mutants. J. Bacteriol. **174:**3133-3139.

24. **Dutreix, M., B. Burnett, A. Bailone, C. M. Radding and R. Devoret**. 1992. A partially deficient mutant, *recA1730*, that fails to form normal nucleoprotein filaments. Mol. Gen. Genet. **232:**489-497.

25. **Dutreix, M., P. L. Moreau, A. Bailone, F. Galibert, J. R. Battista, G. C. Walker and R. Devoret**. 1989. New *recA* mutations that dissociate the various

RecA protein activities in *Escherichia coli* provide evidence for an additional role for RecA protein in UV mutagenesis. J. Bacteriol. **171:**2415-2423.

26. **Echols, H. and M. F. Goodman**. 1991. Fidelity mechanisms in DNA replication. Annu. Rev. Biochem. **60:**477-511.

27. **Eguchi, Y., T. Ogawa and H. Ogawa**. 1988. Cleavage of bacteriophage ϕ80 *c*I repressor by RecA protein. J. Mol. Biol. **202:**565-573.

28. **Elledge, S. J. and G. C. Walker**. 1983. The *muc* genes of pKM101 are induced by DNA damage. J. Bacteriol. **155:**1306-1315.

29. **Elledge, S. J. and G. C. Walker**. 1983. Proteins required for ultraviolet light and chemical mutagenesis: Identification of the products of the *umuC* locus of *Escherichia coli.* J. Mol. Biol. **164:**175-192.

30. **Ennis, D. G., N. Ossanna and D. W. Mount**. 1989. Genetic separation of *Escherichia coli recA* functions for SOS mutagenesis and repressor cleavage. J. Bacteriol. **171:**2533-2541.

31. **Falke, J. J., A. F. Dernburg, D. A. Sternberg, N. Zalkin, D. L. Milligan and D. E. Koshland, Jr.** 1988. Structure of a bacterial sensory receptor. A site-directed sulfhydryl study. J. Biol. Chem. **263:**14850-14858.

32. **Falke, J. J. and D. E. Koshland, Jr.** 1987. Global flexibility in a sensory receptor: a site-directed cross-linking approach. Science **237:**1596-1600.

33. **Flitsch, S. L. and H. G. Khorana**. 1989. Structural studies on transmembrane proteins. 1. Model study using bacteriorhodopsin mutants containing single cysteine residues. Biochemistry **28:**7800-7805.

34. **Frank, E. G., J. Hauser, A. S. Levine and R. Woodgate**. 1993. Targeting of the UmuD, UmuD' and MucA' mutagenesis proteins to DNA by the RecA protein. Proc. Natl. Acad. Sci. U.S.A. **90:**8169-8173.

35. **Friedberg, E. C., G. C. Walker and W. Siede.** 1995. DNA Repair and Mutagenesis. The American Society for Microbiology, Washington, DC.

36. **Frydman, J. and F. U. Hartl.** 1994. Molecular chaperone functions of hsp70 and hsp60 in protein folding, 251-283. *In* R. I. Morimoto, A. Tissieres and C. Georgopoulos (ed.), The biology of heat shock proteins and molecular chaperones. Cold Spring Harbor Press,

37. **Gimble, F. S. and R. T. Sauer**. 1986. λ repressor inactivation: properties of purified ind- proteins in the autodigestion and RecA-mediated cleavage reactions. J. Mol. Biol. **192:**39-47.

38. **Gimble, F. S. and R. T. Sauer**. 1989. Lambda repressor mutants that are better substrates for RecA-mediated cleavage. J. Mol. Biol. **206:**29-39.

39. **Glazebrook, J. A., K. K. Grewal and P. Strike**. 1986. Molecular analysis of the UV protection and mutation genes carried by the I incompatibility group plasmid TP110. J. Bacteriol. **168:**251-256.

40. **Hagensee, M. E., S. K. Bryan and R. E. Moses**. 1987. DNA polymerase III requirement for repair of DNA damage caused by methyl methanesulfonate and hydrogen peroxide. J. Bacteriol. **169:**4608-4613.

41. **Hagensee, M. E., T. L. Timme, S. Bryan and R. E. Moses**. 1987. DNA polymerase III of *Escherichia coli* is required for UV and ethyl methanesulfonate mutagenesis. Proc. Natl. Acad. Sci. U.S.A. **84:**4195-4199.

42. **Hevroni, D. and Z. Livneh**. 1988. Bypass and termination at apurinic sites during replication of single-stranded DNA *in vitro*: a model for apurinic site mutagenesis. Proc. Natl. Acad. Sci. USA **85:**5046-5050.

43. **Horii, T., T. Ogawa, T. Nakatani, T. Hase, H. Matsubara and H. Ogawa**. 1981. Regulation of SOS functions: Purification of *E. coli* LexA protein and determination of its specific site cleaved by the RecA protein. Cell **27:**515-522.

44. **Iwasaki, H., A. Nakata, G. C. Walker and H. Shinagawa**. 1990. The *Escherichia coli polB* gene, which encodes DNA polymerase II, is regulated by the SOS system. J. Bacteriol. **172:**6268-6273.

45. **Jonczyk, P., I. Fijalkowski and Z. Ciesla**. 1988. Overproduction of of the epsilon subunit of DNA polymerase III counteracts the SOS mutagenic responses of *Escherichia coli.* Proc. Natl. Acad. Sci. U.S.A. **85:**9124-9127.

46. **Kato, T. and Y. Shinoura**. 1977. Isolation and characterization of mutants of *Escherichia coli* deficient in induction of mutations by ultraviolet light. Mol. Gen. Genet. **156:**121-131.

47. **Kitagawa, Y., E. Akaboshi, H. Shinagawa, T. Horii, H. Ogawa and T. Kato**. 1985. Structural analysis of the *umu* operon required for inducible mutagenesis in *Escherichia coli.* Proc. Natl. Acad. Sci. U.S.A. **82:**4336-4340.

48. **Kow, Y. W., G. Fuandez, S. Hays, C. Bonner, M. F. Goodman and S. S. Wallace**. 1993. Absence of a role for DNA polymerase II in SOS-induced translesion bypass of ϕX174. J. Bacteriol. **175:**561-564.

49. **Lifsics, M. R., E. D. Lancy Jr and R. Maurer**. 1992. DNA replication defect in *Salmonella typhimurium* mutants lacking the editing (ε) subunit of DNA polymerase III. J. Bacteriol. **174:**6965-6973.

50. **Lin, L. L. and J. W. Little**. 1988. Isolation and characterization of noncleavable (Ind-) mutants of the LexA repressor of *Escherichia coli* K-12. J. Bacteriol. **170:**2163-2173.

51. **Little, J. W.** 1984. Autodigestion of *lexA* and phage λ repressors. Proc. Natl. Acad. Sci. U.S.A. **81:**1375-1379.

52. **Little, J. W., S. H. Edmiston, Z. Pacelli and D. W. Mount**. 1980. Cleavage of the *Escherichia coli lexA* protein by the *recA* protease. Proc. Natl. Acad. Sci. U.S.A. **77:**3225-3229.

53. **Little, J. W. and D. W. Mount**. 1982. The SOS regulatory system of *Escherichia coli.* Cell **29:**11-22.

54. **Livneh, Z.** 1986. Mechanism of replication of ultraviolet-irradiated single-stranded DNA by DNA polymerase III holoenzyme of *Escherichia coli*: Implications for SOS mutagenesis. J. Biol. Chem. **261:**9526-9533.

55. **Miller, J. H.** 1983. Mutational specificity in bacteria. Annu. Rev. Genet. **17:**215-238.

56. **Miller, J. H. and K. B. Low**. 1984. Specificity of mutagenesis resulting from the induction of the SOS system in the absence of mutagenic treatment. Cell **37:**675-682.

57. **Milligan, D. L. and D. E. Koshland, Jr.** 1988. Site-directed cross-linking. Establishing the dimeric structure of the aspartate receptor of bacterial chemotaxis. J. Biol. Chem. **263:**6268-6275.

58. **Moses, R. E., A. Byford and J. A. Hejna**. 1992. Replisome pausing in mutagenesis. Chromosoma **102:**S157-S160.

59. **Nohmi, T., J. R. Battista, L. A. Dodson and G. C. Walker**. 1988. RecA-mediated cleavage activates UmuD for mutagenesis: Mechanistic relationship between transcriptional derepression and posttranslational activation. Proc. Natl. Acad. Sci. U.S.A. **85:**1816-1820.

60. **Nohmi, T., M. Yamada, M. Watanabe, S. Murayama and T. Sofuni**. 1992. The roles of *umuDC*$_{ST}$ and *samAB* in UV mutagenesis and UV sensitivity of *Salmonella typhimurium.* J. Bacteriol. **174:**6948-6955.

61. **Pakula, A. A. and M. I. Simon**. 1992. Determination of transmembrane protein structure by disulfide cross-linking: The *Escherichia coli* Tar receptor. Proc. Natl. Acad. Sci. U.S.A. **89:**4144-4148.

62. **Perry, K. L., S. J. Elledge, B. Mitchell, L. Marsh and G. C. Walker**. 1985. *umuDC* and *mucAB* operons whose products are required for UV light- and chemical-induced mutagenesis: UmuD, MucA, and LexA proteins share homology. Proc. Natl. Acad. Sci. U.S.A. **82:**4331-4335.

63. **Perry, K. L. and G. C. Walker**. 1982. Identification of plasmid(pKM101)-coded proteins involved in mutagenesis and UV resistance. Nature (London) **300:**278-281.

64. **Peterson, K. R., N. Ossanna, A. T. Thliveris, D. G. Ennis and D. W. Mount**. 1988. Derepression of specific genes promotes DNA repair and mutagenesis in *Escherichia coli.* J. Bacteriol **170:**1-4.

65. **Petit, M.-A., W. Bedale, J. Osipiuk, C. Lu, M. Rajagopalan, P. McInerney, M. F. Goodman and E. Echols**. 1994. Sequential folding of UmuC

by the Hsp70 and Hsp60 chaperone complexes of *Escherichia coli*. J. Biol. Chem. **269:**23824-23829.

66. **Phizicky, E. M. and J. W. Roberts**. 1980. Kinetics of RecA protein-directed inactivation of repressors of phage lambda and phage P22. J. Mol. Biol. **139:**319-328.

67. **Radman, M.** 1975. SOS repair hypothesis: Phenomenology of an inducible DNA repair which is accompanied by mutagenesis., 355-367. *In* P. Hanawalt and R. B. Setlow (ed.), Molecular mechanisms for repair of DNA, Part A. Plenum Publishing Corp., New York.

68. **Rajagopalan, M., C. Lu, R. Woodgate, M. O'Donnell, M. F. Goodman and H. Echols**. 1992. Activity of the purified mutagenesis proteins UmuC, UmuD', and RecA in replicative bypass of an abasic DNA lesion by DNA polymerase III. Proc. Natl. Acad. Sci. U.S.A. **89:**10777-10781.

69. **Roberts, J. W. and C. W. Roberts**. 1981. Two mutations that alter the regulatory activity of *E. coli recA* protein. Nature (London) **290:**422-424.

70. **Sassanfar, M. and J. W. Roberts**. 1990. Nature of the SOS-inducing signal in *Escherichia coli*: The involvement of DNA replication. J. Mol. Biol. **212:**79-96.

71. **Sauer, R. T., R. R. Yocum, R. F. Doolittle, M. Lewis and C. O. Pabo**. 1982. Homology among DNA-binding proteins suggests use of a conserved super-secondary structure. Nature (London) **298:**447-451.

72. **Sedgwick, S. G., C. Ho and R. Woodgate**. 1991. Mutagenic DNA repair in Enterobacteria. J. Bacteriol. **173:**5604-5611.

73. **Shinagawa, H., H. Iwasaki, T. Kato and A. Nakata**. 1988. RecA protein-dependent cleavage of UmuD protein and SOS mutagenesis. Proc. Natl. Acad. Sci. U.S.A. **85:**1806-1810.

74. **Slilaty, S. N. and J. W. Little**. 1987. Lysine-156 and serine-119 are required for LexA repressor cleavage: A possible mechanism. Proc. Natl. Acad. Sci. U.S.A. **84:**3987-3991.

75. **Sommer, S., A. Bailone and R. Devoret**. 1993. The appearance of the UmuD'C protein complex in *Escherichia coli* switches repair from homologous recombination to SOS mutagenesis. Mol. Microbiol. **10:**963-971.

76. **Steinborn, G.** 1978. Uvm mutants of *Escherichia coli* K12 deficient in UV mutagenesis. I. Isolation of *uvm* mutants and their phenotypical characterization in DNA repair and mutagenesis. Mol. Gen. Genet. **165:**87-93.

77. **Story, R. M., I. T. Weber and T. A. Steitz**. 1992. The structure of the *E. coli recA* protein monomer and polymer. Nature (London) **355:**318-325.

78. **Sweasy, J. B., E. M. Witkin, N. Sinha and V. Roegner-Maniscalco**. 1990. RecA protein of *Escherichia coli* has a third essential role in SOS mutator activity. J. Bacteriol. **172:**3030-3036.

79. **Tao, T., C. J. Scheiner and M. Lamkin**. 1986. Site-specific photo-cross-linking studies on interactions between troponin and tropomyosin and between subunits of troponin. Biochemistry **25:**7633-7639.

80. **Walker, G. C.** 1984. Mutagenesis and inducible responses to deoxyribonucleic acid damage in *Escherichia coli*. Microbiol. Rev. **48:**60-93.

81. **Walker, G. C.** 1985. Inducible DNA repair systems. Annu. Rev. Biochem. **54:**425-457.

82. **Walker, G. C.** 1987. The SOS response of *Escherichia coli*., 1346-1357. *In* F. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaecter and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*, cellular and molecular biology. American Society for Microbiology, Washington, DC.

83. **Walker, G. C. and P. P. Dobson**. 1979. Mutagenesis and repair deficiencies of *Escherichia coli umuC* mutants are suppressed by the plasmid pKM101. Mol. Gen. Genet. **172:**17-24.

84. **Wang, Z., J. Gergely and T. Tao**. 1992. Characterization of the Ca^{2+} -triggered conformational transition in troponin C. Proc. Natl. Acad. Sci. U.S.A. **89:**11814-11817.

85. **Weigle, J. J.** 1953. Induction of mutation in a bacterial virus. Proc. Natl. Acad. Sci. U.S.A. **39:**628-636.

86. **Woodgate, R., M. Rajagopalan, C. Lu and H. Echols**. 1989. UmuC mutagenesis protein of *Escherichia coli:* Purification and interaction with UmuD and UmuD'. Proc. Natl. Acad. Sci. U.S.A. **86:**7301-7305.

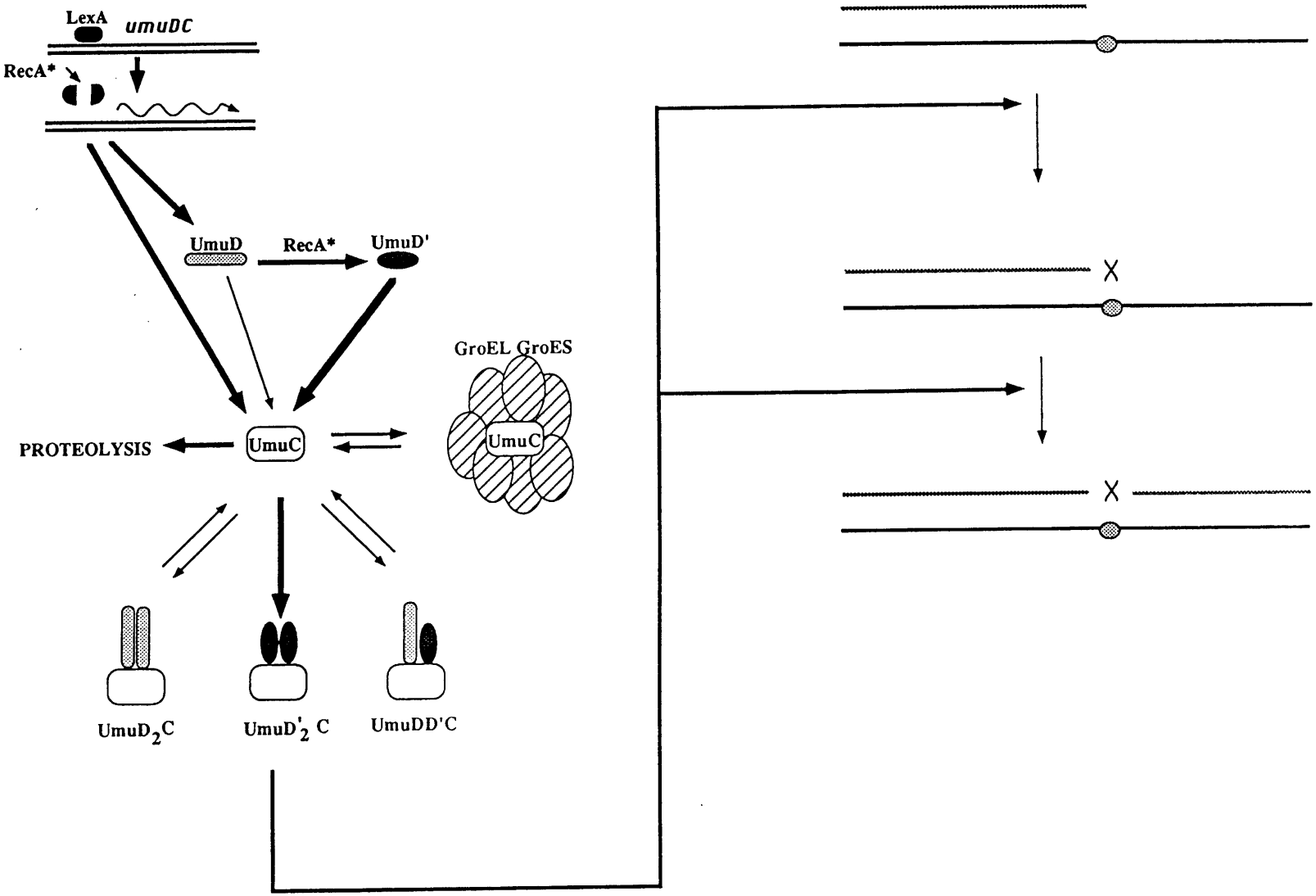
87. **Woodgate, R. and S. Sedgwick**. 1992. Mutagenesis induced by bacterial UmuDC proteins and their plasmid homologues. Mol. Microbiol. **6:**2213-2218.

88. **Yu, X. and E. H. Egelman**. 1993. The LexA repressor binds within the deep helical groove of the activated RecA filament. J. Mol. Biol. **231:**29-40.

Fig. 1. Homology among the bacteriophage ϕ80, 434, P22, and λ repressors, LexA, and the mutagenesis proteins UmuD, MucA, and ImpA. Amino acids that are identical in four or more members of the set are shaded. Positions of λ (37), LexA (50), and UmuD (9, 59) where amino acids substitutions have been shown to yield stable proteins that are defective in RecA-mediated cleavage are indicated by squares. Positions of λ repressor where an amino acid substitution has been shown to interfere with dimer formation are indicated by circles (38). Amino acids that are identical in the three mutagenesis proteins but are not shared with LexA or the bacteriophage repressors are indicated by bold lettering. The cleavage site is indicated by an arrowhead. This figure is reproduced from ref. (9). Used by permission.


φ80 cI 84TVDAWDKNPTLPDDEVEVPFLKDIEFACGDGRVHDEDH
434 cI 63GTSDSNVRFVGHVEPKGKYPLIS-MVRAGSWCEACEPY
P22 cII 68DLSQTNVAYHSRHEPRGSYPLIS-WVSAGQWMEAVEPY
λcI 85YEMYEAVSMQPSLRSEYEYPVFS-HVQAGMFSPELRTF
LexA 58IVSGASRGIRLLQEEEEGLPLVG-RVAAGEPLLAQQHI
UmuD 1MLFIKPADL----REIVTFPLFSDLVQCGFPSPADDYV
MucA 1MKVDIFESSG---ASVHSIPFYLQRISAGFPSPAQGYE
ImpA 1MSTVYHRPADPSGDDSYVRPLFADRCQAGFPSPATDYA
NGFKLRFSKATLRRVGANSDGSGVLCFPASGDSME-PV-----IPDG
DIKDIAEWYDSD----VNLLGNG-FWLKVEGDSMTSPVGQ--SIPEG
HKRAIENWHDTT----VDCSEDS-FWLDVQGDSMTAPAG--LSIPEG
TKGDAERWVSTTKKASAD-----AFWLEVEGNSMTAPTGSKPSFPDG
EGHYQVD-PSLF-KPNAD------FLIRVSGMSMKDIG-----IMDG
EQRIDLN-QLLIQHPSAT------YFVKASGDSMIDGG-----ISDG
KQELNLH-EYCVRHPSAT------YFLRVSGSSMEDGR-----IHDG
EQELDLN-SYCISRPAAT------FFLRASGESMNQAG-----VQNG
ATVAVDTGNKRNIDGELYAI-----NQGDLKRIKQ---QLYRKPGGI
HMVLVDTGREP-VNGSLVVAKLTDANEATFKKLVIDGGQKYLKGLNK
MIILVDPEVEPR-NGKLVVAKVEGENEATFKKLVMDAGRKFLKPLNP
MLILVDPEQAVEP-GDFCIARIGG-DEETFKKLIRDSGQVFLQPINP
DLLAVHKTQDVR-NGQVVVARI--DDEVTMERLKKQGNKVELLPENS
DLLIVDSAITAS-HGDIVIAAV--DGEFTVKKLQLRPTV-ITIPMNS
DVLVVDRSLTAS-HGSIVVACI--HNEFTVKRLLLRPRPQCLMPMNK
DLLVVDRAEKPQ-HGDIVIAEI--DGEFTVKRLLLRPRPA-LEPV-S
LIR-------SINRDYDDEEADEADVEIIGFVFWYSVLRYRR
PSWPMT----PINGN----------CKIIGVVVEARVKFV
QY-PMI----EINGN----------CKIIGVVVDAKLANLP
QY-PMI----PCNES----------CSVVGKVIASQWPEETFG
EFKPIV----VDLRQ--------QSFTIEGLAVGVIRNGDWL
AYSPIT----ISSED---------TLDVFGVVIHVVKAMR
DF-PVYYID-PDNES----------VEIWGVVTHSLIEHPVCLR
DS-PEFRTLYPEN------------ICIFGVVTHVIHRTRLR

Fig. 2. When DNA lesions are introduced by UV light or various chemicals, RecA is activated to RecA* in the presence of a nucleotide triphosphate (ATP) and an inducing signal (single-stranded DNA generated by the cell's attempt to replicate damaged DNA) (70). RecA* mediates the cleavage of the major cellular repressor, LexA (52), thereby allowing the increased transcription of various SOS genes including *umuD*, *umuC*, and *recA*. RecA* then mediates the posttranslational cleavage of UmuD at its Cys24-Gly25 in a manner mechanistically similar to the cleavage of LexA (17, 73). This cleavage has been shown genetically to activate UmuD, the resulting carboxy-terminal fragment (termed UmuD') being necessary and sufficient for its role in mutagenesis (59). UmuC is unstable by itself but is stabilized by GroEL and GroES until it can interact with UmuD or UmuD' (22, 23). Only the complex of UmuC with $UmuD'_2$ is active in mutagenesis. The dimer $UmuD'_2$ associates with UmuC and RecA* and this complex interacts with DNA polymerase III in such a way that DNA lesions are bypassed at the expense of the introduction of mutations (35, 68). SOS mutagenesis appears to be due to a process of translesion synthesis in which the replicative machinery, involving UmuD', UmuC, RecA, and DNA polymerase III, encounters a non-coding or miscoding lesion, inserts an incorrect nucleotide across from the lesion and then continues elongation (35).


LexA
umuDC
RecA*
UmuD
RecA*
UmuD'
GroEL GroES
UmuC
PROTEOLYSIS
UmuC
UmuD2C
UmuD'2 C
UmuDD'C
X
X

Chapter 2

A monocysteine approach for probing the structure and interactions of the UmuD protein.

Published in the *Journal of Bacteriology* 176: 4825-4837 (1994).

JOURNAL OF BACTERIOLOGY, Aug. 1994, p. 4825–4837
0021-9193/94/$04.00+0

Vol. 176, No. 16

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	Referen or sour
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*ΔM15)	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 w
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 w
pGW6060	pGW6050 derivative with *umuC* deleted	This w
pGW6070	pGW6060 derivative with M13 ori. M13 ori from pZ152	This w
pGW6100	70TGT to GCC; Cys-24 to Ala; pGW6070 derivative; *umuD131*	This w
pGW6111	178TCT to TGT; Ser-60 to Cys; pGW6100 derivative; *umuD132*	This w
pGW6121	100GTT to TGT; Val-34 to Cys; pGW6100 derivative; *umuD133*	This w
pGW6131	130TTG to TGT; Leu-44 to Cys; pGW6100 derivative; *umuD134*	This w
pGW6141	376GAT to TGT; Asp-126 to Cys; pGW6100 derivative; *umuD137*	This w
pGW6151	241AGC to TGC; Ser-81 to Cys; pGW6100 derivative; *umuD136*	This w
pGW6161	55AGC to TGC; Ser-19 to Cys; pGW6100 derivative; *umuD138*	This w
pGW6171	169AGT to TGT; Ser-57 to Cys; pGW6100 derivative; *umuD139*	This w
pGW6181	199AGT to TGT; Ser-67 to Cys; pGW6100 derivative; *umuD135*	This w
pGW6191	265GCT to TGT; Ala-89 to Cys; pGW6100 derivative; *umuD140*	This w
pGW6211	298CAA to TGT; Gln-100 to Cys; pGW6100 derivative; *umuD141*	This w
pGW6221	334AGC to TGC; Ser-112 to Cys; pGW6100 derivative; *umuD142*	This w

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 char terizations of the mutant proteins. However, the power of monocysteine approach comes from the fact that one can a carry out an additional set of chemical investigations that t advantage of the presence of a single thiol group in each of mutant proteins (1–3, 16, 17, 19, 38, 44, 62, 65). These have potential to yield insights into such issues as the accessibilit particular amino acids to solvent, conformational chan undergone by the protein, and the nature of subunit inter tions in multiprotein complexes. This type of experimentat is not intended to replace direct physical examinations structure; moreover, if the three-dimensional structure UmuD is eventually solved by crystallographic or nucl magnetic resonance techniques, it will be possible to use res obtained in these studies to evaluate the proposed struct and to develop additional models concerning the nature UmuD's interactions with various proteins. In the meanti since such a structural model for UmuD is not prese available, the results obtained from studies of monocyste derivatives can be used to make significant inferences ab the nature of UmuD's three-dimensional structure in solu and about the nature of its intermolecular interactions. In paper, we describe the construction and characterizatio these monocysteine derivatives and discuss the qualita structural inferences made from this type of experimentati

MATERIALS AND METHODS

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

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.

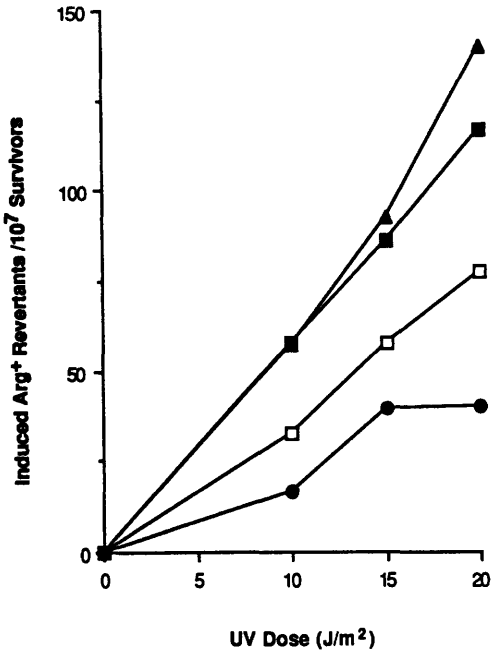

FIG. 1. Effect of plasmids encoding UmuD mutant proteins on UV mutagenesis in an AB1157 *umuD44* strain (GW3200). Assays were conducted in the absence of IPTG. Open squares, pGW2020 ($UmuD^+$, under LexA control); solid squares, pGW6070, pAC-T7 ($UmuD^+$, under T7 control); solid triangles, pGW6100,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

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.

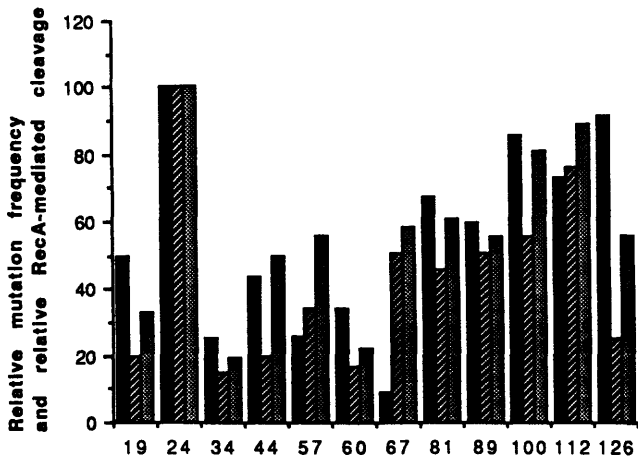

FIG. 2. Relative mutation frequency and in vivo and in vitro RecA-mediated cleavage. Mutagenesis was determined for cells irradiated with a UV dose of 20 J/m^2. In vivo and in vitro RecA-mediated cleavage assays were conducted as described in Materials and Methods. Solid bars, relative mutation frequency (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.

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 their

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).

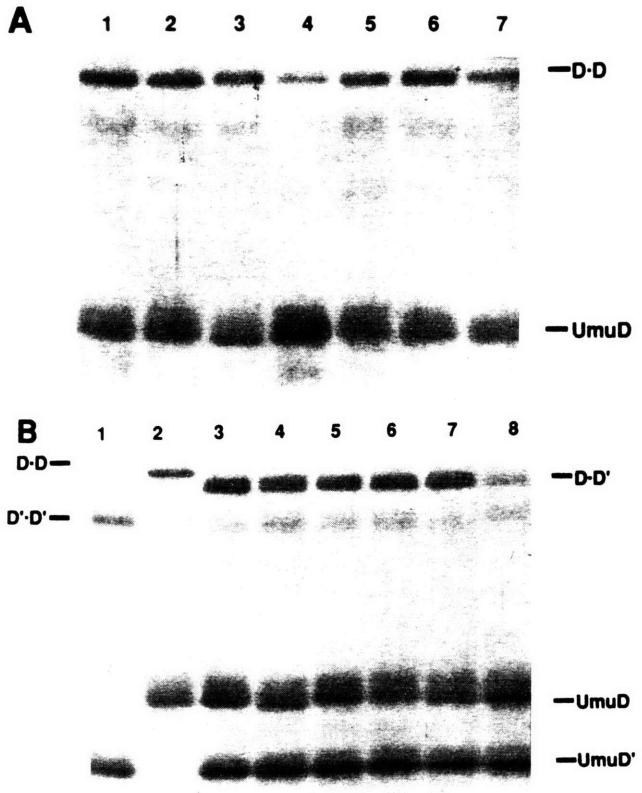

FIG. 3. Glutaraldehyde cross-linking of UmuD monocysteine mutant homodimers and UmuD · UmuD′ heterodimers. (A) Glutaraldehyde cross-linking of $UmuD_2$ was carried out by adding a final concentration of 0.05% glutaraldehyde to 10 μM UmuD and incubating for 5 min. Lanes: 1, CA24; 2, $UmuD^+$; 3, VC34; 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)

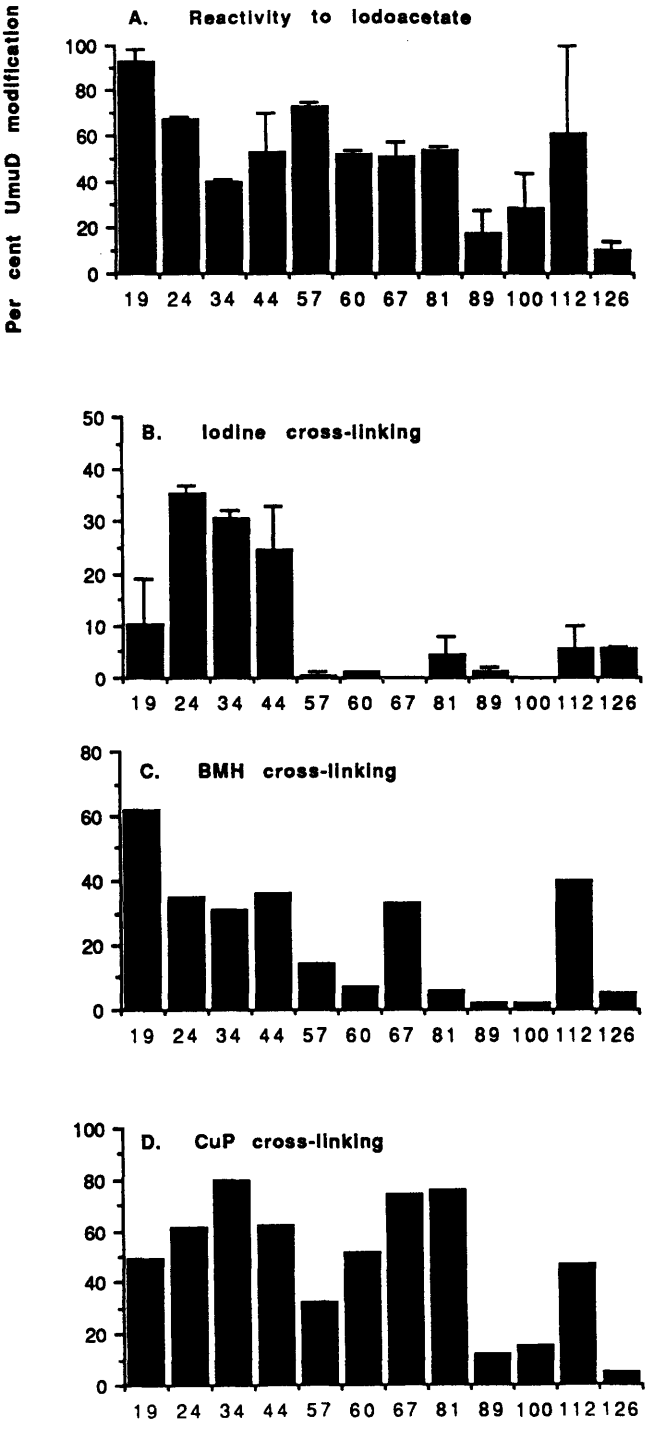

FIG. 4. (A) Reactivity of UmuD monocysteine mutant proteins with [^{3}H]iodoacetate. The percentage of total protein modified by iodoacetate in 60 min was measured. UmuD at a concentration of 20 μM was incubated with a 40-fold molar excess of [^{3}H]iodoacetate in 50 mM HEPES (pH 8.1)–500 mM NaCl for 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–10 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

rotein that is cleaved. The high-level reactivity of SC19 in this ragment suggests that it is in a well-exposed region. Those erivatives that have low reactivities, AC89, QC100, and)C126, are most likely buried within the interior of the protein r at least minimally exposed to the exterior environment. ulfhydryls located at any of the other locations on the protein, e., positions 24, 34, 44, 57, 60, 67, 81, and 112, had reactivities anging from 40 to 80% modification in 60 min. We interpret his as meaning that these sulfhydryls are quite exposed to the olvent. The reductions in the reactivities of these sulfhydryls ompared with that of SC19 may be explained by one or more f the following: (i) the sulfhydryl may not be fully exposed ecause of the folding of the protein, (ii) the reactivity of the ulfhydryl with iodoacetate could be slightly influenced by the)cal electrostatic environment, or (iii) the sulfhydryl could be artially protected from reaction with iodoacetate by the imerization of UmuD.

Disulfide cross-linking of UmuD monocysteine derivatives. n order to gain information concerning the positions of the arious monocysteine substitutions relative to the dimer inter- ıce, we examined the susceptibilities of the homodimers of ıe UmuD monocysteine derivatives to becoming cross-linked y disulfide bonds. This cross-linking reaction can be carried ut by the addition of iodine (I_2) (43) or CuP (8, 16–18, 38). 'he formation of disulfide-linked dimers of UmuD monocys- :ine derivatives occurs much more readily on the addition of 'uP than on addition of I_2, and this difference is reflected in ıe results shown in Fig. 4B and D. For reactions catalyzed by 'uP, mixtures were incubated at 0°C for 10 min before uenching with EDTA and NEM, while reactions catalyzed by)dine were carried out at 22°C for 20 min before quenching ith NEM. Fig. 4B shows that disulfide formation upon iodine eatment of monocysteine mutant homodimers occurs effi- ently for C-24, VC34, and LC44 (~30% cross-linked); mod- rately for SC19 (10%); and appreciably less for the other ıutants (0 to 5%). The fact that the susceptibilities of the ırious monocysteine derivatives to cross-linking upon iodine eatment did not correlate at all with the susceptibilities of the ıme proteins to reaction with iodoacetate strongly suggests ıat the susceptibilities of the various derivatives to disulfide oss-linking are a result of the differences in the positions of ıe sulfhydryl pairs in the homodimers of the monocysteine muD derivatives rather than of their accessibility to reagents ı solution. Thus, the observation that UmuD derivatives with steine at positions 24, 34, and 44 were most efficiently oss-linked suggests that the regions of these positions are oser to the dimer interface than the other positions tested. he less efficient cross-linking of SC19 compared with that of ıe derivatives, C-24, VC34, and LC44, suggests that the pair sulfhydryls in the homodimer of this mutant might be ırther apart than those of C-24, VC34, or LC44.

This interpretation is also supported by the data from CuP oss-linking. Again, cross-linking occurs very readily for muD derivatives having sulfhydryls at positions 24, 34, and 4, and in fact can be effectively driven to completion on crease of the temperature from 0 to 22°C in the same action time (data not shown). This high level of disulfide oss-linking efficiency is consistent with the assignment of ese positions to the dimer interface, and in light of these ıta, the modest reduction in iodoacetate reactivities of these sidues can be reasonably explained by hypothesizing that merization causes the sulfhydryls in these positions to be ırtially protected from reaction with iodoacetate. The inter- ediate efficiency of cross-linking for SC19 with CuP is also ınsistent 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

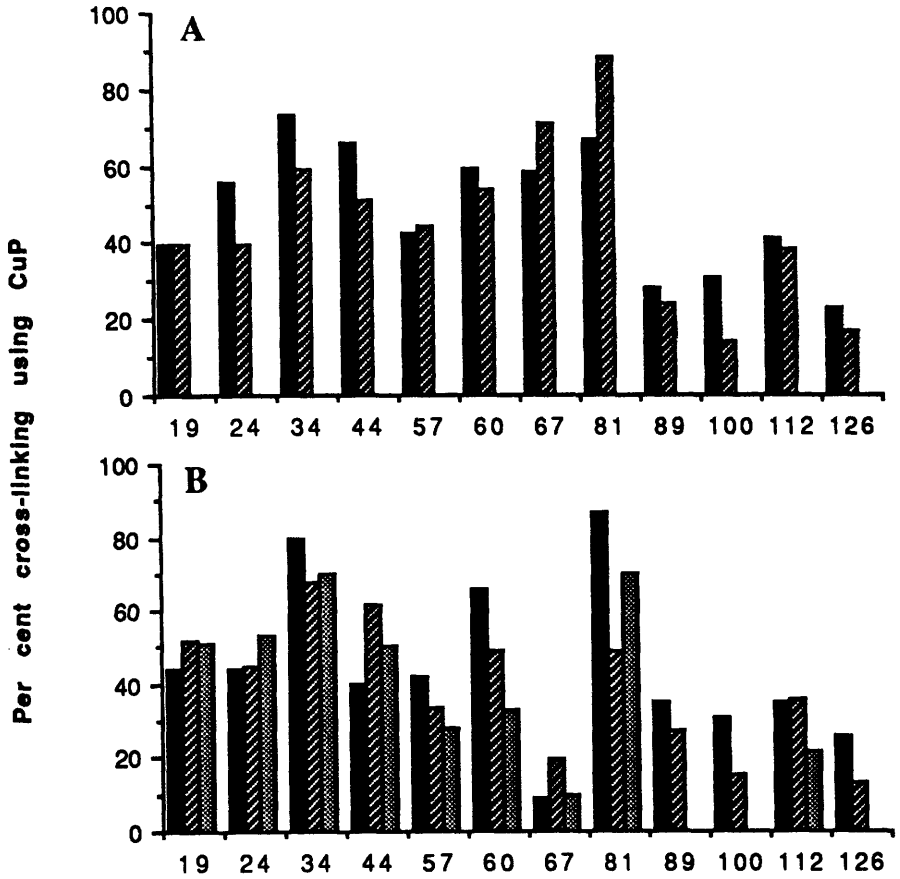

FIG. 5. Dependence of CuP cross-linking on UmuD concentration. Oxidations with O_2 catalyzed by CuP were conducted by reacting UmuD at 0.1 μM (dotted bars), 1 μM (hatched bars), or 10 μM (solid bars) with 0.48 mM Cu^{2+} and 0.65 mM phenanthroline for 5 min at 0°C as described in Materials and Methods. 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 inter-residue 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-

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

Cys-24, Val-34, and Leu-44: residues suggested by I_2 cross-nking to be closer to the interface than the other residues ested. Our results suggest that amino acids at positions 24, 34, nd 44 are located closer to the interface of the $UmuD_2$ omodimer than the other residues we tested. This conclusion based principally on the relative ease, in comparison with all ther monocysteine derivatives, with which homodimers of muD^+ (i.e., C-24), VC34, and LC44 could be cross-linked by isulfide bridges under mild oxidizing conditions. The fact that ey could also be cross-linked by the cysteine-specific homoifunctional reagent BMH much better than could certain ther derivatives that were equally reactive with iodoacetate is onsistent with this conclusion. All three positions appear to be easonably accessible to the solvent as judged by the reactivity f the corresponding monocysteine derivatives with iodoacetate. Position 34 appears to be somewhat less exposed than e other two, possibly because it is partially buried in the muD_2 homodimer interface. A location of the Cys-24–Gly-25 eavage site near the surface of the protein would be consisnt with the recent observation of Kim and Little (29) that exA can be cleaved in *trans*, implying that the corresponding eavage site in LexA (Ala-84–Gly-85) is near the surface of e protein. A consensus Chou-Fasman secondary structure rediction based on the analyses of the UmuD, MucA, LexA, d λcI proteins suggests that residues 28 to 37 of UmuD ight form an alpha helix.

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

Interestingly, Sauer and Gimble found mutations within a gion 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 fa reactive with iodoacetate, suggesting that they are reasona exposed to the solvent. The monocysteine derivatives of SC57, SC67, and SC81 $UmuD_2$ homodimers could not efficiently cross-linked by disulfide bridges under mild oxi ing conditions. However, the SC67 derivative differed from other two in that it could be cross-linked by BMH to the sa extent as $UmuD^+$ (C-24) and the VC34 and LC44 monoc teine derivatives. As for Ser-19, we suggest that in the Umu homodimer, the serines at position 67 are too far apart their monocysteine derivatives to be cross-linked by a disul bridge but are close enough for their monocysteine derivati to be cross-linked by BMH. Interestingly, Ser-67 is loca within the region of 11 amino acid residues, from positions to 75, that is highly conserved within the family of Um analogs that play roles in mutagenesis but not in the famil related repressors (6). Our observation that the SC67 mutat affects the UV mutagenesis phenotype much more dram cally than it affects RecA-mediated cleavage suggests that i important for the subsequent role of UmuD′ in SOS mutag esis. One of the dominant negative mutations described Battista et al. (6), GR65, which is located within this regior defective in both RecA-mediated cleavage and mutagenesi is possible that the amino acid at this position is also import for a subsequent role of UmuD′ in mutagenesis; however, radical substitution of arginine for glycine may cause s changes within the local environment of the site that dis guishing between these roles may not be possible.

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

Although neither the SC57 nor the SC81 monocyst derivative was significantly cross-linked by mild oxidizing tr ments or by exposure to BMH, and the SC57 derivative somewhat more reactive than the SC81 derivative to iod etate, the SC81 derivative was more efficiently cross-linke CuP treatment than the SC57 derivative. If the cross-lin caused by CuP treatment represents the trapping of trans intermediates of structurally fluctuating molecules (see abo then these observations could be explained by postulating position 57 is exposed to solvent but located within a pock cleft such that even transient movements bringing the sul dryls together occur very infrequently or that position 57 fact on the outer surfaces of the homodimer and is optimally positioned for disulfide bond formation within dimer. Position 81 is within a small region of amino acids

s 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, hese results suggest that the small region around Ser-81 is not ritical for either of these UmuD functions.

Ala-89, Gln-100, Ser-112, and Asp-126. The monocysteine lerivatives AC89, QC100, and SC112, which have alterations n the carboxyl-terminal one-third of the UmuD protein, were argely proficient in both RecA-mediated cleavage and SOS nutagenesis. The relatively low reactivities of the AC89 and QC100 monocysteine derivatives with iodoacetate and their ailure to be significantly cross-linked, even with CuP treatnent, are consistent with positions 89 and 100 not being very ccessible to the solvent. Both positions 89 and 100 flank the egion of conserved residues which include Lys-97, the UmuD ounterpart to the proposed proton acceptor Lys-156 of LexA, ut neither Ala-89 nor Gln-100 seems particularly important or the cleavage reaction.

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

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

The fact that UmuD, a 15-kDa protein, undergoes so many ifferent types of interactions (from the intramolecular autoigestion reaction to interactions in the $UmuD_2$ homodimer nd the UmuD · UmuD' heterodimer to interactions with ther proteins involved in mutagenesis such as UmuC, RecA, nd, possibly, components of DNA polymerase III) makes JmuD an attractive model system for the study of structurenction relationships. Using a monocysteine approach for the vestigation of the structure and interactions of UmuD, we ave developed a model for the topological arrangement of rtain residues in UmuD. Further elucidation of the properes 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.

ACKNOWLEDGMENTS

We are grateful to S. Karnik for providing pAC-T7 and pVSR, S. Gottesman for providing SG1611, and N. Freitag for providing RecA protein. We thank J. Resek and T. Nohmi for helpful discussions and members of the laboratory for helpful discussions, comments, and suggestions on the manuscript.

This work was supported by Public Health Service grant CA21615 awarded by the National Cancer Institute. M.H.L. was supported by Predoctoral Training Grant 5T32GM07287 from the National Institutes of Health.

REFERENCES

1. **Aggeler, R., and R. A. Capaldi.** 1993. ATP hydrolysis-linked structural changes in the N-terminal part of the γ subunit of *Escherichia coli* F_1-ATPase examined by cross-linking studies. J. Biol. Chem. **268:**14576–14579.
2. **Aggeler, R., K. Chicas-Cruz, S. X. Cai, J. F. W. Keana, and R. A. Capaldi.** 1992. Introduction of reactive cysteine residues in the ε subunit of *Escherichia coli* F_1 ATPase, modification of these sites with tetrafluorophenyl azide-maleimides, and examination of changes in the binding of the ε subunit when different nucleotides are in catalytic sites. Biochemistry **31:**2956–2961.
3. **Altenbach, C., S. L. Flitsch, H. G. Khorana, and W. L. Hubbell.** 1989. Structural studies on transmembrane proteins. 2. Spin labeling of bacteriorhodopsin mutants at unique cysteines. Biochemistry **28:**7806–7812.
4. **Bagg, A., C. J. Kenyon, and G. C. Walker.** 1981. Inducibility of a gene product required for UV and chemical mutagenesis in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **78:**5749–5753.
5. **Battista, J. R., T. Nohmi, C. E. Donnelly, and G. C. Walker.** 1988. Role of UmuD and UmuC in UV and chemical mutagenesis, p. 455–459. *In* E. C. Friedberg and P. C. Hanawalt (ed.), Mechanisms and consequences of DNA damage processing. Alan R. Liss, Inc., New York.
6. **Battista, J. R., T. Ohta, T. Nohmi, W. Sun, and G. C. Walker.** 1990. Dominant negative *umuD* mutations decreasing RecA-mediated cleavage suggest roles for intact UmuD in modulation of SOS mutagenesis. Proc. Natl. Acad. Sci. USA **87:**7190–7194.
7. **Bridges, B. A., and R. Woodgate.** 1985. Mutagenic repair in *Escherichia coli*: products of the *recA* gene and of the *umuD* and *umuC* genes act at different steps in UV-induced mutagenesis. Proc. Natl. Acad. Sci. USA **82:**4193–4197.
8. **Bubis, J., and H. G. Khorana.** 1990. Sites of interaction in the complex between β- and γ-subunits of transducin. J. Biol. Chem. **265:**12995–12999.
9. **Burckhardt, S. E., R. Woodgate, R. H. Scheuermann, and H. Echols.** 1988. UmuD mutagenesis protein of *Escherichia coli*: overproduction, purification, and cleavage by RecA. Proc. Natl. Acad. Sci. USA **85:**1811–1815.
10. **Cohen, S., B. J. Knoll, J. W. Little, and D. W. Mount.** 1981. Preferential cleavage of phage λ repressor monomers by *recA* protease. Nature (London) **294:**182–184.
11. **Cohen-Fix, O., and Z. Livneh.** 1992. Biochemical analysis of UV mutagenesis in *Escherichia coli* by using a cell-free reaction coupled to a bioassay: identification of a DNA repair-dependent pathway. Proc. Natl. Acad. Sci. USA **89:**3300–3304.
12. **Cox, M. M., K. McEntee, and I. R. Lehman.** 1981. A simple and rapid procedure for the large scale purification of the RecA protein of *Escherichia coli*. J. Biol. Chem. **256:**4676–4678.
13. **Dutreix, M., P. L. Moreau, A. Bailone, F. Galibert, J. R. Battista, G. C. Walker, and R. Devoret.** 1989. New *recA* mutations that dissociate the various RecA protein activities in *Escherichia coli* provide evidence for an additional role for RecA protein in UV mutagenesis. J. Bacteriol. **171:**2415–2423.
14. **Eguchi, Y., T. Ogawa, and H. Ogawa.** 1988. Cleavage of bacteriophage φ80 *cI* repressor by RecA protein. J. Mol. Biol. **202:**565–573.

15. **Elledge, S. J., and G. C. Walker.** 1983. Proteins required for ultraviolet light and chemical mutagenesis: identification of the products of the *umuC* locus of *Escherichia coli*. J. Mol. Biol. **164:**175–192.
16. **Falke, J. J., A. F. Dernburg, D. A. Sternberg, N. Zalkin, D. L. Milligan, and D. E. Koshland, Jr.** 1988. Structure of a bacterial sensory receptor. A site-directed sulfhydryl study. J. Biol. Chem. **263:**14850–14858.
17. **Falke, J. J., and D. E. Koshland, Jr.** 1987. Global flexibility in a sensory receptor: a site-directed cross-linking approach. Science **237:**1596–1600.
18. **First, E. A., J. Bubis, and S. S. Taylor.** 1988. Subunit interaction sites between the regulatory and catalytic subunits of cAMP-dependent protein kinase. J. Biol. Chem. **263:**5176–5182.
19. **Flitsch, S. L., and H. G. Khorana.** 1989. Structural studies on transmembrane proteins. 1. Model study using bacteriorhodopsin mutants containing single cysteine residues. Biochemistry **28:** 7800–7805.
20. **Frank, E. G., J. Hauser, A. S. Levine, and R. Woodgate.** 1993. Targeting of the UmuD, UmuD′ and MucA′ mutagenesis proteins to DNA by RecA protein. Proc. Natl. Acad. Sci. USA **90:**8169–8173.
21. **Freitag, N., and K. McEntee.** 1989. "Activated"-RecA protein affinity chromatography of LexA repressor and other SOS-regulated proteins. Proc. Natl. Acad. Sci. USA **86:**8363–8367.
22. **Gimble, F. S., and R. T. Sauer.** 1986. λ repressor inactivation: properties of purified ind⁻ proteins in the autodigestion and RecA-mediated cleavage reactions. J. Mol. Biol. **192:**39–47.
23. **Gimble, F. S., and R. T. Sauer.** 1989. Lambda repressor mutants that are better substrates for RecA-mediated cleavage. J. Mol. Biol. **206:**29–39.
24. **Gottesman, S.** 1990. Minimizing proteolysis in *Escherichia coli*: genetic solutions. Methods Enzymol. **185:**119–129.
25. **Hevroni, D., and Z. Livneh.** 1988. Bypass and termination at apurinic sites during replication of single-stranded DNA *in vitro*: a model for apurinic site mutagenesis. Proc. Natl. Acad. Sci. USA **85:**5046–5050.
26. **Jonczyk, P., I. Fijalkowska, and Z. Ciesla.** 1988. Overproduction of the ε subunit of DNA polymerase III counteracts the SOS mutagenic response of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **85:**9124–9127.
27. **Kato, T., and Y. Shinoura.** 1977. Isolation and characterization of mutants of *Escherichia coli* deficient in induction of mutations by ultraviolet light. Mol. Gen. Genet. **156:**121–131.
28. **Khorana, H. G., M. S. Braiman, B. H. Chao, T. Doi, S. L. Flitsch, M. A. Gilles-Gonzales, N. R. Hackett, S. S. Karnik, T. Mogi, M. M. Nassal, and L. J. Stern.** 1987. Site-specific mutagenesis in structure-function studies of bacteriorhodopsin. Chemica Scripta **27B:**137–147.
29. **Kim, B., and J. W. Little.** 1993. LexA and λ cI repressors as enzymes: specific cleavage in an intermolecular reaction. Cell **73:**1165–1173.
30. **Landschultz, W. H., P. F. Johnson, and S. L. McKnight.** 1989. The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. Science **243:**1681–1688.
31. **Lima, C. D., J. C. Wang, and A. Mondragon.** 1994. Three-dimensional structure of the 67K N-terminal fragment of *E. coli* DNA topoisomerase I. Nature (London) **367:**138–146.
32. **Lin, L. L., and J. W. Little.** 1988. Isolation and characterization of noncleavable (Ind⁻) mutants of the LexA repressor of *Escherichia coli* K-12. J. Bacteriol. **170:**2163–2173.
33. **Lin, L. L., and J. W. Little.** 1989. Autodigestion and RecA-dependent cleavage of Ind⁻ mutant LexA proteins. J. Mol. Biol. **210:**439–452.
34. **Little, J. W.** 1984. Autodigestion of LexA and phage λ repressors. Proc. Natl. Acad. Sci. USA **81:**1375–1379.
35. **Little, J. W., S. H. Edmiston, Z. Pacelli, and D. W. Mount.** 1980. Cleavage of the *Escherichia coli* LexA protein by the RecA protease. Proc. Natl. Acad. Sci. USA **77:**3225–3229.
36. **Little, J. W., and D. W. Mount.** 1982. The SOS regulatory system of *Escherichia coli*. Cell **29:**11–22.
37. **Livneh, Z.** 1986. Mechanism of replication of ultraviolet-irradiated single-stranded DNA by DNA polymerase III holoenzyme of *Escherichia coli*. J. Biol. Chem. **261:**9526–9533.
38. **Milligan, D. L., and D. E. Koshland, Jr.** 1988. Site-directed cross-linking. Establishing the dimeric structure of the aspartate receptor of bacterial chemotaxis. J. Biol. Chem. **263:**6268–6275.
39. **Moore, M. H., J. M. Gulbis, E. J. Dodson, B. Demple, and P. C. E. Moody.** 1994. Crystal structure of a suicidal DNA repair protein: the Ada O⁶methylguanine-DNA methyltransferase from *E. coli*. EMBO J. **13:**1495–1501.
40. **Murli, S., and G. Walker.** Unpublished data.
41. **Nohmi, T., J. R. Battista, L. A. Dodson, and G. C. Walker.** 1988. RecA-mediated cleavage activates UmuD for mutagenesis: mechanistic relationship between transcriptional derepression and post-translational activation. Proc. Natl. Acad. Sci. USA **85:**1816–1820.
42. **Pabo, C. O., R. T. Sauer, J. M. Sturtevant, and M. Ptashne.** 1979. The λ repressor contains two domains. Proc. Natl. Acad. Sci. USA **76:**1608–1612.
43. **Pakula, A. A., and M. I. Simon.** 1992. Determination of transmembrane protein structure by disulfide cross-linking: the *Escherichia coli* Tar receptor. Proc. Natl. Acad. Sci. USA **89:**4144–4148.
44. **Park, H. S., T. Tao, and P. D. Cantler.** 1991. Proximity relationships between sites on myosin and actin. Resonance energy transfer determination of the following distances using a hybrid myosin: those between Cys-55 on the *Mercenaria* regulatory light chain, SH-1 on the *Aequipecten* myosin heavy chain, and Cys-374 of actin. Biochemistry **30:**3189–3195.
45. **Perry, K. L., S. J. Elledge, B. Mitchell, L. Marsh, and G. C. Walker.** 1985. *umuDC* and *mucAB* operons whose products are required for UV light- and chemical-induced mutagenesis: UmuD, MucA, and LexA proteins share homology. Proc. Natl. Acad. Sci. USA **82:**4331–4335.
46. **Peterson, K. R., N. Ossanna, A. T. Thliveris, D. G. Ennis, and D. W. Mount.** 1988. Derepression of specific genes promotes DNA repair and mutagenesis in *Escherichia coli*. J. Bacteriol. **170:**1–4.
47. **Rajagopalan, M., C. Lu, R. Woodgate, M. O'Donnell, M. F. Goodman, and H. Echols.** 1992. Activity of the purified mutagenesis proteins UmuC, UmuD′, and RecA in replicative bypass of an abasic DNA lesion by DNA polymerase III. Proc. Natl. Acad. Sci. USA **89:**10777–10781.
48. **Roland, K. L., and J. W. Little.** 1990. Reaction of LexA repressor with diisopropylfluorophosphate: a test of the serine protease model. J. Biol. Chem. **265:**12828–12835.
49. **Roland, K. L., M. H. Smith, J. A. Rupley, and J. W. Little.** 1992. *In vitro* analysis of mutant LexA proteins with an increased rate of specific cleavage. J. Mol. Biol. **228:**395–408.
50. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning—a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
51. **Sauer, R. T., R. R. Yocum, R. F. Doolittle, M. Lewis, and C. O. Pabo.** 1982. Homology among DNA-binding proteins suggests use of a conserved super-secondary structure. Nature (London) **298:** 447–451.
52. **Shinagawa, H., H. Iwasaki, T. Kato, and A. Nakata.** 1988. RecA protein-dependent cleavage of UmuD protein and SOS mutagenesis. Proc. Natl. Acad. Sci. USA **85:**1806–1810.
53. **Shinagawa, H., T. Kato, T. Ise, K. Makino, and A. Nakata.** 1983. Cloning and characterization of the *umu* operon responsible for inducible mutagenesis in *Escherichia coli*. Gene **23:**167–174.
54. **Slilaty, S. N., and J. W. Little.** 1987. Lysine-156 and serine-119 are required for LexA repressor cleavage: a possible mechanism. Proc. Natl. Acad. Sci. USA **84:**3987–3991.
55. **Slilaty, S. N., J. A. Rupley, and J. W. Little.** 1986. Intramolecular cleavage of LexA and phage λ repressors: dependence of kinetics on repressor concentration, pH, temperature, and solvent. Biochemistry **25:**6866–6875.
56. **Sommer, S., A. Bailone, and R. Devoret.** 1993. The appearance of the UmuD′C protein complex in *Escherichia coli* switches repair from homologous recombination to SOS mutagenesis. Mol. Microbiol. **10:**963–971.
57. **Steinborn, G.** 1978. *uvm* mutants of *Escherichia coli* K12 deficient in UV mutagenesis. I. Isolation of *uvm* mutants and their phenotypical characterization in DNA repair and mutagenesis. Mol. Gen. Genet. **165:**87–93.
58. **Story, R. M., I. T. Weber, and T. A. Steitz.** 1992. The structure of the *E. coli recA* protein monomer and polymer. Nature (London) **355:**318–325.

59. **Sussman, R., and H. B. Z. Alexander.** 1989. Structural analysis of the carboxy terminus of bacteriophage λ repressor determined by antipeptide antibodies. J. Bacteriol. **171:**1235–1244.
60. **Sweasy, J. B., E. M. Witkin, N. Sinha, and V. Roegner-Maniscalco.** 1990. RecA protein of *Escherichia coli* has a third essential role in SOS mutator activity. J. Bacteriol. **172:**3030–3036.
61. **Tadmor, Y., R. Ascarelli-Goell, R. Skaliter, and Z. Livneh.** 1992. Overproduction of the β subunit of DNA polymerase III holoenzyme reduces UV mutagenesis in *Escherichia coli*. J. Bacteriol. **174:**2517–2524.
62. **Tao, T., C. J. Scheiner, and M. Lamkin.** 1986. Site-specific photo-cross-linking studies on interactions between troponin and tropomyosin and between subunits of troponin. Biochemistry **25:**7633–7639.
63. **Walker, G. C.** 1984. Mutagenesis and inducible responses to deoxynucleotide damage in *Escherichia coli*. Microbiol. Rev. **48:** 60–93.
64. **Walker, G. C.** 1985. Inducible DNA repair systems. Annu. Rev. Biochem. **54:**425–457.
65. **Wang, Z., J. Gergely, and T. Tao.** 1992. Characterization of the Ca^{2+}-triggered conformational transition in troponin C. Proc. Natl. Acad. Sci. USA **89:**11814–11817.
66. **Witkin, E. M.** 1976. Ultraviolet mutagenesis and inducible DNA repair in *Escherichia coli*. Bacteriol. Rev. **40:**869–907.
67. **Wong, S. S.** 1993. CRC chemistry of protein conjugation and cross-linking. CRC Press, Inc., Boca Raton, Fla.
68. **Woodgate, R., M. Rajagopalan, C. Lu, and H. Echols.** 1989. UmuC mutagenesis protein of *Escherichia coli*: purification and interaction with UmuD and UmuD′. Proc. Natl. Acad. Sci. USA **86:**7301–7305.
69. **Zagursky, R. J., and M. L. Berman.** 1984. Cloning vectors that yield high levels of single-stranded DNA for rapid DNA sequencing. Gene **27:**183–191.

Chapter 3

p-Azidoiodoacetanilide, a new short photocrosslinker that has greater cysteine specificity than *p*-azidophenacyl bromide and *p*-azidobromoacetanilide

Published in the *Biochemical and Biophysical Research Communications* 217: 1177-1184 (1995).

Vol. 217, No. 3, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 26, 1995 Pages 1177–1184

P-AZIDOIODOACETANILIDE, A NEW SHORT PHOTOCROSSLINKER THAT HAS GREATER CYSTEINE SPECIFICITY THAN *P*-AZIDOPHENACYL BROMIDE AND *P*-AZIDOBROMOACETANILIDE

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Received November 15, 1995

An important criterion for a protein modifying agent is its residue selectivity. We report the synthesis of a new photocrosslinking agent, *p*-azidoiodoacetanilide (AIA), which has a greater specificity to modify cysteine residues than the widely used *p*-azidophenacyl bromide (APB). Crosslinking of UmuD protein, which only has one cysteine, in the homodimer using APB or AIA resulted in 39% and 30% crosslinking, respectively; however, crosslinking of UmuD/C24A, a derivative with no cysteines, resulted in 16% crosslinked dimer using APB but only 2% using AIA. In addition, incorporation of [2-^{14}C]APB into UmuD/C24A was 43% the amount of incorporation into wildtype UmuD, whereas incorporation of [2-^{14}C]AIA into UmuD/C24A was only 13% the amount incorporated into wildtype UmuD. We also examined the cysteine specificity of *p*-azidobromoacetanilide (ABA) and found it to be less cysteine specific than AIA.

The use of heterobifunctional photoreactivatable cross-linkers is proving to be an effective method for the investigation of both structural and functional properties of biological targets (1,2). The strategy used in many investigations involves chemically modifying a unique cysteine residue within the protein (3) or a phosphothioate residue in DNA (4) or 4-thiouridine in tRNA (5) with the crosslinker and using the derivatized residue as a probe of the local environment. The modified protein, DNA, or RNA is incubated with another protein or DNA of interest. Exposure of the resulting complex to UV light results in covalent cross-linking of the complex. For systems which take advantage of the reactivity of sulfhydryl groups, the commercially available crosslinker, *p*-azidophenacyl bromide (APB) has been the reagent of choice in several investigations of protein-protein (3) as well as protein-DNA (6-8) and protein-tRNA interactions (9). The reported advantages of this reagent are i) its short length (9 Å); ii) its reactivity; and iii) its specificity for sulfhydryl groups.

We have also tried APB in our investigations of protein interactions of the UmuD homodimer. UmuD plays important roles in the process of UV and chemical mutagenesis in *Escherichia coli* (10-12). The UmuD protein has only one cysteine in its amino acid sequence (13,14). Substitution of this cysteine by an alanine results in a derivative, UmuD/C24A, whose

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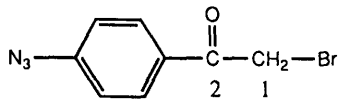
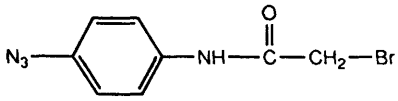
0006-291X/95 $12.00

function is indistinguishable from wildtype (15). UmuD exists as stable homodimers in solution (16,17). In the course of our investigations, however, we obtained evidence for some non-specific incorporation of APB into a protein lacking cysteines and for subsequent crosslinking after UV irradiation even when we employed conditions for modification and crosslinking reported by other investigators (3,6-8). In particular, the UmuD derivative with no cysteines, UmuD/C24A, was able to incorporate [2-^{14}C]APB at 43% the rate of incorporation for the wildtype protein. Furthermore, 16% of the modified UmuD/C24A protein became crosslinked in the homodimer form after UV irradiation. Such non-specific incorporation and crosslinking may not have been observed by other investigators since control experiments using a protein with no cysteines were not performed or not reported in their cross-linking studies and because nonradioactively labeled APB was employed (6-8).

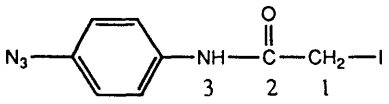
Various cysteine-specific labeling agents contain the iodoacetamido functional group. We therefore have designed and synthesized a new bifunctional photolabile reagent, *p*-azidoiodoacetanilide (AIA) (Figure 1), which has a cysteine-specific iodoacetamido functional group and an azido photolabile group. *p*-Azidoiodoacetanilide is comparable in length and reactivity but has greater cysteine-specificity than APB. We report in this paper the synthesis of AIA and the application of AIA in the crosslinking of UmuD homodimer.



p-Azidophenacyl bromide (APB)



p-Azidobromoacetanilide (ABA)



p-Azidoiodoacetanilide (AIA)

Fig. 1. Chemical structures of *p*-azidophenacyl bromide (APB), *p*-azidobromoacetanilide (ABA), and *p*-azidoiodoacetanilide (AIA).

Materials and Methods

UmuD and UmuD/C24A proteins were prepared as described (15). The unlabeled APB was purchased from Sigma and the ^{14}C-labeled APB was prepared by the method of Hixson and Hixson (18). *p*-Azidobromoacetanilide (ABA) was prepared by condensing bromoacetic acid with *p*-azidoaniline in the presence of dicyclohexylcarbodiimide (DCC) as reported (19).

Synthesis of *p*-azidoiodoacetanilide. *p*-Azidoaniline (20,21) 50 mg (0.373 mmole), iodoacetic acid 69.4 mg (0.373 mmole), and DCC 154 mg (0.746 mmole) were dissolved in 2 ml dry THF and stirred at room temperature for 5 hours. After the reaction was complete, the reaction solution was filtered. The filtrate was either dried by rotary evaporation or over a tream of nitrogen. The remainder was dissolved in 5 ml ethyl acetate; washed with 3 x 2 ml 2N HCl, 3 x 2 ml saturated $NaHCO_3$, and dried over $MgSO_4$. *p*-Azidoiodoacetanilide was purified by applying the crude product to a silica gel column and eluting with 1:1 hexane and ethyl acetate. We obtained 94 mg with a yield of 84%. ^{1}H NMR ($CDCl_3$): δ ppm 7.50 (d, 2H), 7.00 (d, 2H), 3.85 (s, 2H). IR (cm^{-1}): 3290, 2082, 1649, 1550, 1506, 1298, 1086, 834, 798, 729, 684. MS(EI) cald for $C_8H_7IN_4O$: 302. Found 302.

Synthesis of ^{14}C-labeled *p*-azidoiodoacetanilide. 100 μCi Iodo[2-^{14}C]acetic acid (53 mCi/mmol, Amersham), 3.3 mg unlabeled iodoacetic acid, 2.5 mg *p*-azidoaniline and 7.5 mg DCC were dissolved in 1 ml dry THF and stirred at room temperature for 5 hours. The reaction was filtered through a Pasteur pipet filled with a small piece of cotton. The filtrate was dried over a tream of nitrogen. The residue was dissolved in 2 ml ethyl acetate; washed with 3 x 1 ml 2 N HCl, 3 x 1 ml saturated $NaHCO_3$, and dried over $MgSO_4$. The radiolabeled *p*-azidoiodo[2-^{14}C]acetanilide was purified by applying the crude product to a silica gel column and eluting with 1:1 hexane and ethyl acetate. We obtained 3.1 mg with a specific activity of 4.8 mCi/mmol, a yield of 58%.

Incorporation of [2-^{14}C]*p*-azidophenacyl bromide or [2-^{14}C]*p*-azidoiodoacetanilide into the UmuD protein. UmuD at a 20 μM concentration in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) (pH 8.0) containing 500 mM NaCl was incubated with 20x molar excess of the appropriate reagent in the dark at 37°C for 10, 30, 60, and 120 minutes. To quench the reaction, an equal volume of SDS sample buffer containing 10% ß-mercaptoethanol was added to the reaction mixture. Reagents were separated from the 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 hours in 0.5 ml Solvable (DuPont-New England Nuclear)-0.5 ml H_2O. Subsequently, 10 ml of Formula 989 (Dupont-New England Nuclear) were added, samples were vigorously mixed, and ^{14}C disintegrations were counted with the Beckman LS 6000SC Liquid Scintillation counter.

Crosslinking of UmuD with *p*-azidoiodoacetanilide, *p*-azidobromoacetanilide, and *p*-azidophenacyl bromide. UmuD at a 40 μM concentration in 50 mM HEPES (pH 8.0)

containing 500 mM NaCl was incubated with a 40x molar excess of AIA, APB, or ABA in the dark at 37 °C for 1 hour. The reaction mixture was dialyzed in a microdialyzer for 30 minutes to remove excess reagent and then exposed to UV light at 320 nm with a power output of 90 $\mu W/cm^2$ on ice for 15 minutes to initiate the photolysis reaction. The reaction was quenched by addition of an equal volume of SDS sample buffer containing 10% ß-mercaptoethanol. Crosslinked species were resolved from non-crosslinked species by electrophoresis on a 13% polyacrylamide gel. The protein bands were visualized by staining with Coomassie blue.

Results

The synthesis of *p*-azidoaniline is relatively simple and well documented (20,21). *p*-Azidobromoacetanilide has been previously prepared by condensing *p*-azidoaniline with bromoacetic acid and has been used to modify nucleic acids (19,22). In a similar fashion, we synthesized *p*-azidoiodoacetanilide by condensing *p*-azidoaniline with iodoacetic acid in the presence of DCC at room temperature for a few hours. The labeled *p*-azidoiodoacetanilide was prepared by the same chemical procedures as used in the synthesis of unlabeled *p*-azidoiodoacetanilide except that radiolabeled iodoacetic acid was included in the reaction. We have prepared *p*-azidoiodo[2-^{14}C]acetanilide, but *p*-azidoiodo[1-^{14}C]acetanilide and *p*-azidoiodo[1-^{3}H]acetanilide can be prepared in a similar way.

APB has been previously used as a cysteine-specific photolabile crosslinker to study protein-protein and protein-nucleic acid interactions (3,6-8). We examined the specificity of APB, AIA and ABA by investigating the differences in their incorporation into UmuD and UmuD/C24A. We also examined differences in the ability of the modified protein to become crosslinked in the homodimer form after UV irradiation. UmuD has only one cysteine located at position 24 in its amino acid sequence (13,14). This cysteine has been substituted by an alanine in UmuD/C24A without affecting its biological activity (15). UmuD and UmuD/C24A were modified by APB, AIA and ABA in 40x molar excess in 50 mM HEPES/pH 8.0/0.5 M NaCl buffer at 37°C for 1 hour and subsequently UV irradiated at 320 nm for 15 minutes. As shown in Fig. 2, UmuD was crosslinked by each of the three crosslinkers, with APB giving the highest yield of crosslinking. UmuD/C24A, the derivative with no cysteine, can still be substantially crosslinked by APB (16.5%) and ABA (15.2%) indicating APB and ABA can attach to other nucleophilic residues besides cysteine. UmuD/C24A was crosslinked to a much lesser extent with AIA (2%) than the wildtype UmuD suggesting that AIA preferentially modified the protein at the cysteine residue with higher specificity than the other two reagents. This experiment clearly shows a greater specificity of AIA for cysteine residues in UmuD compared to APB and ABA. The high degree of crosslinking of UmuD after treatment with APB and UV irradiation evidently resulted not only from the modification of cysteine-24 with APB but also from the modification of other amino acid residues as well.

To explore this issue further, we directly measured the incorporation of APB and AIA into UmuD and UmuD/C24A by using radiolabeled [2-^{14}C]APB and [2-^{14}C]AIA. UmuD at a 20 µM concentration in 50 mM HEPES (pH 8.0) containing 500 mM NaCl was incubated with a

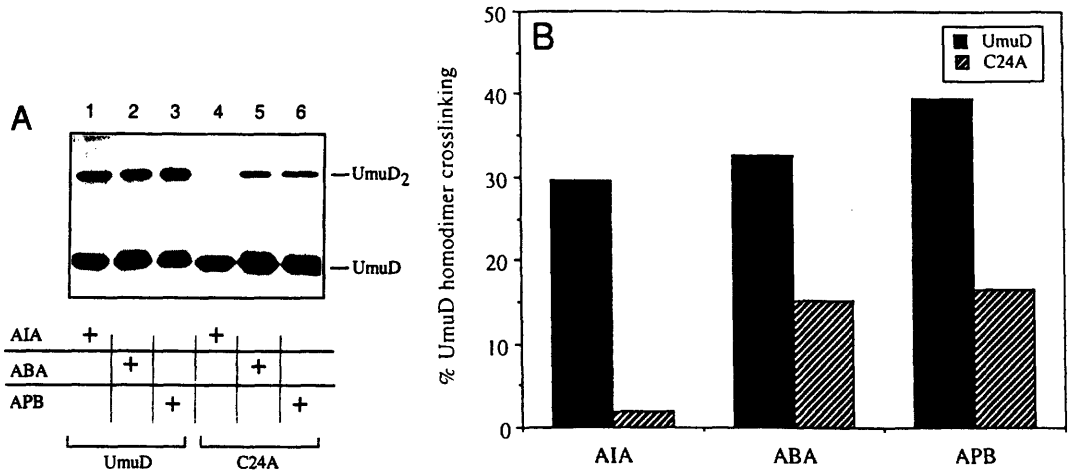


Fig. 2. Crosslinking of UmuD and UmuD/C24A by AIA, ABA and APB. UmuD and UmuD/C24A at 40 μM concentration were incubated with a 40x molar excess of AIA, ABA and APB at 37°C for 1 hour. The reaction mixtures were dialyzed, UV irradiated and subject to SDS-PAGE gel electrophoresis. Panel A, lane 1-3, UmuD crosslinking by AIA, ABA and APB; lane 4-6, UmuD/C24A crosslinking by AIA, ABA and APB. The extent of UmuD and UmuD/C24A crosslinked by AIA, ABA and APB quantitated by densitometry scanning of the above Coomassie-stained gel (Panel **B**).

20x molar excess of the appropriate reagent in the dark at 37 °C for 2 hours. Samples were quenched by the addition of an equal volume of SDS sample buffer containing 10% β-mercaptoethanol, and modified proteins were separated from excess reagent by electrophoresis on a polyacrylamide gel. After a 2 hour incubation, the molar incorporation of [2-^{14}C]AIA into UmuD was calculated to be about 0.42 mol AIA/mol UmuD, and the molar incorporation of [2-^{14}C]APB was calculated to be about 0.65 mol APB/mol UmuD. Results from the reaction of each reagent with UmuD/C24A indicate the extent of non-specific incorporation of each reagent. This incorporation was proportionally greater for the reaction using APB (approaching 43% the extent of the incorporation of APB into the wildtype protein) compared to the reaction using AIA which is about 13% the incorporation of AIA into the wildtype protein. This result suggests that [2-^{14}C]AIA has a much greater cysteine-specificity than [2-^{14}C]APB.

We examined the time course of the reactions of UmuD with both [2-^{14}C]AIA and [2-^{14}C]APB (Fig. 3). Each reagent was allowed to react with UmuD following the same procedure described above, and samples were taken and quenched at 10, 30, 60 and 120 minutes. The reaction curve for both of the reagents seem to consist of two phases, a rapid incorporation phase and a slower phase. Rapid incorporation of the radioactive reagent seems to occur within the first 10 minutes of reaction after which time the incorporation occurs much more slowly. The rapid phase of the reaction most likely corresponds to the reaction of the reagents to the cysteine within UmuD. The slower phase probably is due to the reaction of the reagents to other nucleophilic residues besides cysteine (perhaps lysine, arginine, serine, or tyrosine). The time course of the reaction of each of the reagents with UmuD/C24A is also shown. The shape of

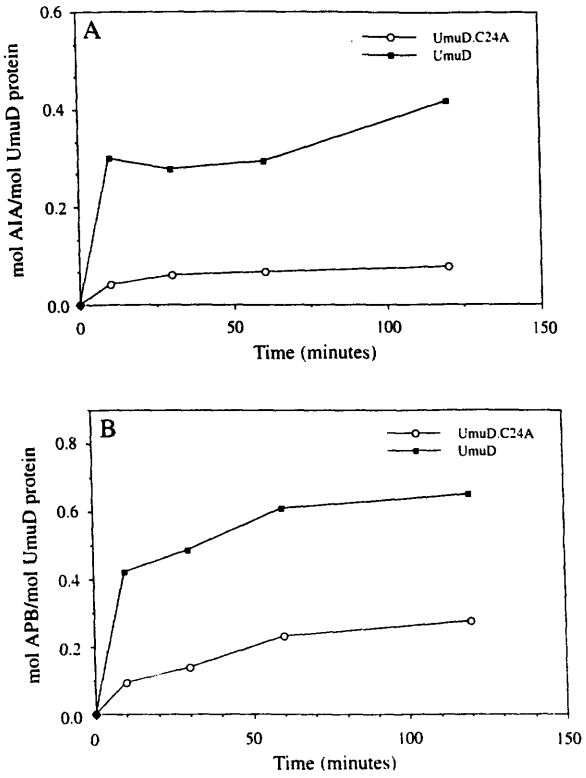


Fig. 3. Time course of incorporation of [2-^{14}C]AIA (**A**)and [2-^{14}C]APB (**B**) into UmuD and UmuD/C24A. UmuD and UmuD/C24A at 20 μM concentration were incubated at 37°C with a 20x molar excess of [2-^{14}C]AIA and [2-^{14}C]APB for 10, 30, 60, and 120 minutes in the dark. Samples were quenched by the addition of an equal volume of SDS sample buffer containing 10% β-mercaptoethanol, and modified proteins were separated from excess reagent by electrophoresis on a polyacrylamide gel. The radioactivity of the protein bands was determined by liquid scintillation counting.

these curves roughly correspond to the shape of the slower phase of the reaction to UmuD. This is consistent with the suggestion that the second phase of the reaction is due predominantly to the non-specific modification of UmuD. The graphs of these results indicate that the rate of non-specific incorporation for AIA is slower than that for APB, tapering off after the first 30 minutes of reaction, whereas the rate of non-specific incorporation for APB is greater and continues to steadily increase even after two hours of incubation.

From our crosslinking and incorporation experiments, we conclude that the use of a high molar excess of APB to protein results in significant non-specific incorporation of APB into UmuD. Under the same reaction conditions, AIA is a more highly cysteine specific photocrosslinker with a crosslinking efficiency comparable to APB.

Discussion

For many investigations exploring specific interactions, one of the most important criteria for a protein modifying agent is its selectivity. Applying a protein modifying agent without a

high selectivity for a specific residue introduces ambiguities into the data and complicates the interpretation of results. APB has been previously used as a cysteine-specific photocrosslinker to investigate protein-protein and protein-nucleic acid interactions (3,6-8). As we demonstrated in the Results section, APB does not react exclusively with cysteines in the UmuD protein. In their original paper on the synthesis and application of APB, Hixson and Hixson (18) reported that APB reacted rapidly with the active site of sulfhydryl groups and then reacted slowly with other nucleophilic groups on the enzyme. They strongly suggested that "in experiments where only one active site sulfhydryl group is to carry the photolabile group, an excess of the inhibitor (APB) should not be employed." In practice it is difficult to maintain an exact 1:1 molar ratio. Often a reducing agent like dithiolthreitol (DTT) or β-mercaptoethanol is present in the buffer to prevent the oxidation of the protein. The presence of a reducing agent in the buffer complicates the calculation of the amount of reagent that should be used. Use of an excess amount (up to 40x molar excess) modifying agent has been often reported in the literature (6-8). Non-specific modification will likely occur under conditions where an excess amount of APB is used.

We have synthesized a new short cysteine-specific photocrosslinker, *p*-azidoiodoacetanilide whose crosslinking ability is comparable to APB. We found that AIA, even when used in 40x molar excess to the protein being modified, resulted in negligible non-specific crosslinking. For applications which require a radiolabeled crosslinker, another advantage of AIA is that it can be easily labeled with ^{14}C or ^{3}H using the commercially available iodo[1-^{14}C]acetic acid, iodo[2-^{14}C]acetic acid, or iodo[1-^{3}H]acetic acid. The labeling reaction is a single step reaction that occurs at the last step of synthesis, and the subsequent workup and purification of the labeled AIA are relatively simple. The synthesis of radiolabeled APB is considerably more complicated.

ABA can be synthesized and labeled as easily as AIA, but we found ABA lacks the cysteine selectivity of AIA. We have used AIA successfully to modify and crosslink UmuD in the dimeric form. This reagent will prove useful in further studies of the interactions of UmuD with other proteins involved in UV mutagenesis and should prove useful in other systems as well.

Acknowledgments

We are grateful to Meng Chen and Qun Xue for running the Mass Spectrometry and IR Spectrometry for us. We thank the members of our laboratory for helpful discussions, comments, and suggestions on the manuscript.

This work was supported by Public Health Service grant GM28988 from the National Institute of General Medical Sciences and by Public Health Service grant CA21615 awarded by the National Cancer Institute. J. Z. was supported by the National Institute of Health postdoctoral fellowship grant GM17100-02.

References

1. Bayley, H. (1983) *Photogenerated reagents in biochemistry and molecular biology* Elsevier, New York
2. Brunner, J. (1993) *Annu. Rev. Biochem.* 62, 483-514
3. Hardwicke, P. M. D., and Szent-Gyorgyi, A. G. (1985) *J. Mol. Biol.* 183, 203-211

4. Yang, S.-W., and Nash, H. A. (1994) *Proc. Natl. Acad. Sci. USA* 91, 12183-12187
5. Wetzel, R., and Soll, D. (1977) *Nucleic Acids Res.* 4, 1681-1694
6. Pendergrast, P. S., Chen, Y., Ebright, Y. W., and Ebright, R. H. (1992) *Proc. Natl. Acad. Sci. USA* 89, 10287-10291
7. Dumoulin, P., Oertel-Buchheit, P., Granger-Schnarr, M., and Schnarr, M. (1993) *Proc. Natl. Acad. Sci. USA* 90, 2030-2034
8. Chen, Y., and Ebright, R. H. (1993) *J. Mol. Biol.* 230, 453-460
9. Schwartz, I., and Ofengand, J. (1974) *Proc. Natl. Acad. Sci. USA* 71, 3951-3955
10. Walker, G. C. (1995) *Trends in Biochem. Sci.* , in press
11. Walker, G. C. (1984) *Microbiol. Rev.* 48, 60-93
12. Friedberg, E. C., Walker, G. C., and Siede, W. (1995) *DNA repair and mutagenesis*, ASM Press
13. Perry, K. L., Elledge, S. J., Mitchell, B., Marsh, L., and Walker, G. C. (1985) *Proc. Natl. Acad. Sci. USA* 82, 4331-4335
14. Kitagawa, Y., Akaboshi, E., Shinagawa, H., Horii, T., Ogawa, H., and Kato, T. (1985) *Proc. Natl. Acad. Sci. USA* 82, 4336-4340
15. Lee, M. H., Ohta, T., and Walker, G. C. (1994) *J. Bacteriol.* 176, 4825-4837
16. Battista, J. R., Ohta, T., Nohmi, T., Sun, W., and Walker, G. C. (1990) *Proc. Natl. Acad. Sci. USA* 87, 7190-7194
17. Woodgate, R., Rajagopalan, M., Lu, C., and Echols, H. (1989) *Proc. Natl. Acad. Sci. USA* 86, 7301-7305
18. Hixson, S. H., and Hixson, S. S. (1975) *Biochem.* 14, 4251-4254
19. Budker, V. G., Knorre, D. G., Kravchenko, V. V., Lavrik, O. I., Nevinsky, G. A., and Teplova, N. M. (1974) *FEBS Lett.* 49, 159-162
20. Smith, P. A. S., Hall, J. H., and Kan, R. O. (1962) *J. Am. Chem. Soc.* 84, 485-489
21. Escher, E. H. F., Robert, H., and Guillemette, G. (1979) *Helvet. Chim. Acta* 62, 1217-1222
22. Eshaghpour, H., Soll, D., and Crothers, D. M. (1979) *Nucleic Acids Res.* 7, 1485-1495

Reprinted from Biochemical and Biophysical Research Communications 217, 1177–1184 (1995)

Chapter 4

Crosslinking of UmuD to RecA using the cysteine-specific photoactive crosslinker *p*-azidoiodoacetanilide

Abstract. SOS mutagenesis in *Escherichia coli* requires the participation of a specialized system involving the activated form of UmuD (UmuD'), UmuC, RecA and DNA polymerase III proteins. In an effort to understand the mechanism of UmuD in UV mutagenesis we have extended our investigations of UmuD interactions using the monocysteine approach to study not only interactions of UmuD in the homodimer but also of UmuD with RecA. We have used the cysteine-specific photoactive crosslinker, *p*-azidoiodoacetanilide (AIA). The extent of reaction of the UmuD derivatives with AIA was similar to the reaction of UmuD with iodoacetate reported previously. Results of crosslinking of the UmuD derivatives in the homodimer using AIA are also consistent with our previously reported results of the relative closeness of the residues to the dimer interface. With respect to the UmuD-RecA interface, VC34 and SC81 crosslinked most efficiently to RecA indicating that these residues are closer than the other derivatives tested. SC57, SC67, and SC112 crosslinked moderately efficiently with RecA. Neither C24, the UmuD derivative with a cysteine located at the Cys24-Gly25 cleavage site, nor SC60, the UmuD derivative with a cysteine substitution at the position of the putative active site residue, was able to crosslink to RecA suggesting that RecA need not directly interact with residues involved in the cleavage reaction. SC19, located in the N-terminal fragment of UmuD that is cleaved, and LC44 also did not crosslink efficiently with RecA.

Mutagenesis in *Escherichia coli* resulting from exposure to UV radiation and various chemicals is not a passive process but rather requires the participation of a specialized system involving the activated form of UmuD (UmuD'), UmuC, RecA and DNA polymerase III proteins (10). The production of the UmuD, UmuC and RecA proteins are regulated as part of the *recA*$^+$*lexA*$^+$-dependent SOS response (10) which is induced when RecA, activated by single-stranded DNA generated by the cell's attempt to replicate damaged DNA (27), mediates the proteolytic cleavage of LexA at the Ala-84-Gly-85 cleavage site (19), apparently by facilitating the otherwise latent capacity of LexA to autodigest (18). RecA*, the activated form of RecA, also mediates the post-translational cleavage of UmuD at its Cys24-Gly25 bond by a similar mechanism (4, 29), removing the first 24 amino acids and activating UmuD (designated UmuD') for its role in mutagenesis (22). UmuD shares homology with the C-terminal regions of LexA, the repressors of the bacteriophages λ, ϕ80, 434, and P22, and with UmuD analogs that play roles in mutagenesis such as MucA and ImpA (1, 8, 24, 28). 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 manner similar to that of serine proteases in which a nucleophile, apparently a serine residue conserved in all members of the family, is activated by a lysine residue (30). Various genetic experiments indicate that RecA plays a third direct role in mutagenesis beyond mediating the proteolytic cleavage of LexA and UmuD (7, 9, 22, 33).

The role of RecA* in mediating the cleavage of repressor and mutagenesis proteins implies that a direct interaction between these proteins and RecA must occur which leads to the cleavage of the protein. This class of interaction has been visualized by electron microscopy for the complex of LexA with the RecA filament (35). In the study, the LexA repressor was found to bind within the deep helical groove of the activated RecA filament. The strikingly different effects of certain RecA mutations on its ability to mediate the cleavage of different repressor and mutagenesis proteins suggest that some contacts between the repressor or mutagenesis proteins and RecA might be specific for a particular protein. This view is supported by the observation that the RecA430 mutant (which has a glycine to serine substitution at position 204) is deficient in mediating the cleavage of LexA, very deficient in mediating the cleavage of UmuD (29) and λ repressor (26), but is proficient in mediating the cleavage of ϕ80 repressor (8).

Other evidence suggests that direct physical interactions also occur between RecA and the cleavage product of a mutagenesis protein (i.e., UmuD' or MucA'). In DNA mobility shift assays, UmuD' or MucA' as well as UmuD could be crosslinked by glutaraldehyde to a RecA filament (9). In addition, it has been observed that the overproduction of UmuD' and UmuC proteins in a Hfr x F^- conjugal cross inhibits recombination but that this recombination can be substantially suppressed by overproducing RecA. This experiment has led to the suggestion that the interaction of UmuD' and UmuC with the growing end of a RecA nucleofilament inhibits recombination and switches the RecA-coated DNA from being a substrate for recombination to being a substrate for bypass mutagenesis (31). It is not yet understood whether the nature of the UmuD·RecA interactions and UmuD'·RecA interactions are similar or different. One observation which suggests that the interactions might be different is the finding that RecA430 fails to mediate the cleavage of UmuD, but is functional for mutagenesis when UmuD' is directly produced (22).

SOS mutagenesis appears to be due to a process of translesion synthesis in which the replicative machinery, involving UmuD', UmuC, RecA and DNA polymerase III, encounters a non-coding or miscoding lesion, inserts an incorrect nucleotide across from the lesion and then continues elongation (10). Biochemical approaches for the study of the mechanistic process of SOS mutagenesis have recently been developed. Rajagopalan et al. (25) have reconstituted limited replicative bypass in an *in vitro* system with purified UmuD', renatured UmuC, RecA and DNA polymerase III proteins and a DNA substrate with a single abasic lesion. In another approach, Cohen-Fix and Livneh (6) have reported the development of a crude cell-free system made from SOS-induced cells that is capable of processing UV-irradiated plasmid DNA to yield mutated DNA in a fashion that requires the *umuD*, *umuC*, and *recA* gene products.

In an effort to understand the mechanism of UmuD in UV mutagenesis by gaining insight into its structure/function relationship and its interactions with other proteins, we have initiated a monocysteine approach for studying the UmuD protein. UmuD has one cysteine in its amino acid sequence located at the Cys24-Gly25 cleavage site. The substitution of this cysteine with an alanine residue results in a derivative whose function is indistinguishable from the wild type (14). This observation has allowed us to construct a family of UmuD proteins differing only in the position of the unique cysteine residue. In designing this set of monocysteine derivatives, we attempted to maximize the probability of obtaining

biologically active molecules by making cysteine substitutions at sites which (i) represented conservative substitutions or (ii) were located in regions of the amino acid sequence which were not conserved in related proteins (UmuD analogs and repressors subject to RecA-mediated cleavage). The locations of the cysteine substitutions were also chosen to sample regions along the entire length of the UmuD protein. From our initial characterizations of the UmuD monocysteine derivatives, we had made several inferences concerning the relative topological arrangement of certain residues of UmuD in relation to the homodimer interface (14). The assignments were primarily based on the solvent accessibility of the cysteines at these positions as determined by iodoacetate reactivities, and the relative ease of homodimer crosslinking of the monocysteine derivatives by formation of disulfide bonds upon mild oxidation with iodine, or by reaction with the cysteine-specific crosslinker, *bis*-maleimidohexane. Specifically, we suggested that Cys24 (of the Cys24-Gly25 cleavage site), Val34, and Leu44 are closer to the homodimer interface than the other residues tested. We also suggested that Ser60, the site of the putative nucleophile in the cleavage reaction, is not as close to the dimer interface or located in a cleft region. Still others, such as Ser19, located in the N-terminal fragment of UmuD that is removed by RecA-mediated cleavage, as well as Ser57, Ser67, Ser81, and Ser112, in the central region of the UmuD protein sequence, were suggested to be further from the dimer interface.

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

sufficient amount of crosslinked complex is obtained, the site of crosslinking of the interacting protein can then be identified by enzymatically digesting the complex and isolating the crosslinked fragment. The identity of the crosslinked peptide in the interacting protein can be determined by mass spectrometry and the exact position of the crosslink determined by sequencing (3, 5, 15). In this paper, we have extended our study of UmuD interactions by probing interactions of the $UmuD_2$ homodimer as well as interactions of UmuD with RecA. We have used the cysteine-specific photoactive crosslinker, *p*-azidoiodoacetanilide, in these investigations of UmuD interactions.

Materials and Methods

UmuD mutant derivatives were produced and purified as described (14). Unlabeled *p*-azidoiodoacetanilide and [2-^{14}C]*p*-azidoiodoacetanilide were synthesized as described (36).

Incorporation of [2-^{14}C]*p*-azido-iodoacetanilide into the UmuD protein. UmuD mutant derivatives at a 20 μM concentration in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) (pH 8.0) containing 500 mM NaCl was incubated with a 10x molar excess of [2-^{14}C]azidoiodoacetanilide in the dark at 37°C for 1 h. To quench the reaction, an equal volume of SDS sample buffer containing 10% ß-mercaptoethanol was added to the reaction mixture. Reagents were separated from the 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 Solvable (DuPont-New England Nuclear)-0.5 ml H_2O. Subsequently, 10 ml of Formula 989 (Dupont-New England Nuclear) were added, samples were vigorously mixed, and ^{14}C disintegrations were counted with the Beckman LS 6000SC Liquid Scintillation counter.

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

Normalization of UmuD homodimer crosslinking data. UmuD crosslinking data was normalized to account for the differences in UmuD modification by AIA. The maximum value for the percentage of UmuD crosslinking for a given mutant derivative (based on 100% modification by AIA)

was obtained by dividing the actual percentage of crosslinked UmuD by the percent of UmuD modified as described by the following equation:

$$\%\ \text{UmuD crosslinked}_{max} = \frac{(\%\ \text{UmuD crosslinked}_{observed})}{\%\ \text{UmuD modified by AIA}} \cdot 100\%$$

This equation was derived as follows:

Given that the fraction of modified UmuD (designated UmuD*) of the total population is X, the fraction of unmodified UmuD is then, 1-X. Most of the UmuD proteins exist as dimers in solution (1, 34). A small percentage (< 1%) of the UmuD derivatives C24 (wildtype UmuD) and VC34 was found in dimers of dimers ($UmuD_4$). To simplify calculations, this small population of $UmuD_4$ for these derivatives was not included in the equation. The population of modified and unmodified dimers would therefore consist of the following: $UmuD^*_2$, $UmuD_2$, and UmuD*·UmuD. This population can be described by the following equation:

$$X^2 + (1-X)^2 + 2 \cdot X(1-X) = 1,$$

where X^2 is the fraction of $UmuD^*_2$, $(1-X)^2$ is the fraction of $UmuD_2$, and $2 \cdot X(1-X)$ is the fraction of UmuD*·UmuD in the population. Let Y = the maximum % UmuD crosslinked for 100% UmuD modification for a given UmuD mutant derivative. Because the two proteins in $UmuD^*_2$ each has an attached crosslinker and one protein in UmuD*·UmuD has an attached crosslinker, it is reasonable to assume that the probability of $UmuD^*_2$ crosslinking on exposure to UV light is 2 times the probability of UmuD*·UmuD crosslinking. For a given mutant UmuD derivative with a given degree of modification, the percentage of UmuD crosslinked would be,

$$\%\ \text{UmuD crosslinked} = Y \cdot X^2 + (Y/2) \cdot [2 \cdot X(1-X)], \text{ which simplifies to}$$

$$\%\ \text{UmuD crosslinked} = Y \cdot X.$$

Solving for Y, $Y = \frac{\%\ \text{UmuD crosslinked}}{\%\ \text{UmuD modified by AIA}} \cdot 100\%$

Crosslinking of UmuD derivatives to RecA using *p*-azido-iodoacetanilide. UmuD derivatives were modified with AIA as described above for UmuD homodimer crosslinking. After dialysis against 40 mM Tris buffer (pH 8.0), 100 mM NaCl, 0.1 mM EDTA, modified UmuD derivatives at a final concentration of

45 μM were incubated for 5 min at 37°C in the dark with 8 μM RecA activated in the presence of 180 μM ATPγS, 8.8 ng/μl $p(dT)_{27}$ (Pharmacia), and 18 mM $MgCl_2$. Reaction mixtures were then quickly transferred to a 96 well, tissue culture serocluster with U-bottom wells (Costar) and exposed to 320 nm UV light at a power output of 90 $\mu W/cm^2$ on ice for 15 min. The photolysis reaction was quenched by removal from light and addition of 15 μl SDS sample buffer containing 10 % β-mercaptoethanol. Different molecular weight species were resolved by electrophoresis on a 13% polyacrylamide gel containing sodium dodecyl sulfate (SDS) and visualized by Coomassie staining or by Western blotting by chemiluminescence. To visualize protein species by chemiluminescence, samples of crosslinked mixtures from above reactions were resolved by electrophoresis as described above, transferred to polyvinylidene difluoride transfer membrane (Immobilon-P), and blotting with affinity-purified antibodies raised against UmuD' or RecA. Cross-reacting material was visualized by chemiluminescence (Tropix).

Results

Modification of UmuD derivatives with [14C]*p*-azido-iodoacetanilide (AIA). We initially chose the commercially available photoactive crosslinker, *p*-azidophenacyl bromide (APB), for these investigations because of its length (only 9 Å) and the high reactivity of its photoactive end. However, crosslinking and incorporation studies of CA24, the UmuD derivative with no cysteine, using APB suggested that APB did not react exclusively with cysteines (36). We therefore synthesized and used a new reagent, *p*-azidoiodoacetanilide (AIA), for the following investigation (Fig.1). *p*-Azidoiodoacetanilide is comparable in length and reactivity but has greater cysteine-specificity than APB (36).

We continued our investigations of UmuD interactions using the subset of UmuD derivatives which reacted well with [3H]iodoacetate (14). Efficient reactivity with iodoacetate implies that the sulfhydryl group is exposed and can be readily modified by our crosslinking reagent. We used [14C]AIA to check the extent of incorporation into each derivative. Length of incubation and conditions were chosen to maximize specific incorporation and minimize non-specific incorporation (36). UmuD mutant derivatives at a 20 μM concentration were incubated with a 10x molar excess of [2-^{14}C]AIA in the dark at 37°C for 1 h. The results are shown in Fig. 2. Most of the derivatives reacted to an extent of 60% to 80%. The reactivity of SC19 and UmuD wildtype were only slightly lower, approximately 50% modification after 1 hour. The values for incorporation of AIA into SC19 and UmuD are slightly lower than the values for the extent of incorporation of iodoacetate; however, for the most part, AIA reactivities are comparable to the [3H]iodoacetate reactivities of the UmuD derivatives previously reported (14). This result is to be expected assuming that the cysteine-specific functional group of the photocrosslinker reacts in a manner, and with a reactivity, similar to iodoacetate. The small differences observed in reactivity between the two reagents might be due to the negative charge on iodoacetate or to the presence of the hydrophobic phenyl ring in AIA.

Crosslinking of UmuD derivatives in the homodimer using AIA. When a UmuD derivative that has been modified with AIA is allowed to form a complex with another protein (or proteins) and is UV irradiated, the photoactive end of the crosslinker will react with any nucleophilic group in the vicinity. The photoactivatable end of AIA is an azido group which, when activated on exposure to UV light, is only reactive for about 0.1 to 5 μsec (2). Therefore crosslinking of

two interacting proteins using this reagent suggests a close spatial relationship of the regions of the proteins which are crosslinked (within the 9Å length of the crosslinker). UmuD derivatives at a 80 μM concentration were incubated with a 10x molar excess of AIA in the dark at 37 °C for 1 h then dialyzed to remove excess reagent. UmuD derivatives at a final concentration of 60 μM were then exposed to UV light on ice for 15 min to initiate the photolysis reaction. The results of UmuD crosslinking in the homodimer using this reagent are shown in Fig. 3, Panel A. A small percentage (< 1%) of the UmuD derivatives C24 (wildtype UmuD) and VC34 migrated to a position on the gel consistent with a dimer of dimers ($UmuD_4$) (data not shown).

These results were normalized to account for the differences in degree of modification by [14C]AIA (Fig. 3, Panel B), since a population of UmuD with a greater degree of modification will have a higher probability of being crosslinked in the UmuD homodimer. The maximum percent crosslinking in the homodimer, for a fully modified population, calculated as described in the Methods section, of a given monocysteine derivative is proportional to the probability of crosslinking for that monocysteine derivative.

The derivatives that crosslinked most efficiently were C24(wildtype), VC34 and LC44, which crosslinked to the extents of 32%, 26% and 34% respectively. The efficient crosslinking of these derivatives is consistent with our previously reported crosslinking results (14). Other UmuD monocysteine derivatives that also crosslinked to a moderate extent were SC19 and SC57, which crosslinked 19% and 20% respectively. In the experiments examining crosslinking by disulfide bridges after oxidation with iodine, SC57 resulted in very little crosslinking (14). It is possible that the position of this substitution is in contact with the adjacent UmuD though the point of contact may be too far from position 57 of the adjacent protein to permit disulfide bond formation. The other mutant derivatives (SC60, SC67, SC81, and SC112) crosslinked to an extent of between 4% and 14%, just slightly higher than background levels (as determined by crosslinking of the CA24 derivative which lacks cysteines). These results are consistent with the previously reported results from iodine crosslinking. This demonstrates the usefulness of this reagent in identifying the points of protein interactions.

UmuD crosslinking to RecA* using AIA. For these experiments we wanted to optimize conditions for UmuD interactions with RecA and as a result, maximize their probability for crosslinking. Preliminary experiments examining

UmuD-RecA interactions under the conditions routinely used for RecA-mediated cleavage (4) suggested that under these conditions at equilibrium, the UmuD-RecA complex was not present in abundance. In studies of UmuD reactivities to [3H]iodoacetate, addition of RecA* did not protect any of the UmuD monocysteine derivatives from reacting with [3H]iodoacetate (data not shown). In addition, we were not able to obtain significant crosslinking using either of two commercially available homobifunctional crosslinkers, *bis*-maleimidohexane, which crosslinks at cysteine residues, or glutaraldehyde, which crosslinks at lysine residues. We therefore tried to optimize conditions by taking into account the following factors: i) the molar ratio of UmuD to RecA in solution; ii) the concentration of cofactor used for RecA activation; iii) length of time for incubation of UmuD with activated RecA; iv) the temperature for the photolysis reaction.

On the basis of their electron microscopic studies of RecA-LexA complexes, Yu and Egelman (35) suggested that LexA does not bind RecA with a stoichiometry of 1:1 but rather it binds with some cooperativity at random locations along the RecA filament, saturating at about 40% occupancy. In their image analysis of negatively stained filaments, they observed i) no binding with 3.4 μM LexA fragment (with 6 μM RecA) and ii) nearly saturating binding at 6 μM intact LexA (with 1.5 μM RecA), assuming that the binding parameters are the same for the intact LexA and the fragment. Their model of cooperative binding predicted that in the first case they would have 18% occupancy of LexA binding sites and 36% occupancy in the second case. Since a variety of lines of evidence indicate that RecA mediates the cleavage of UmuD in a manner similar to the manner by which it mediates LexA cleavage, we thought it possible that this cooperative model for binding might also apply to the interaction of UmuD with RecA. After unsuccessful attempts to crosslink UmuD to RecA using molar ratios of UmuD:RecA less than 4:1, we found that we were able to obtain the most successful crosslinking of these complexes using molar ratios of UmuD:RecA greater than or equal to 4:1.

RecA requires the presence of single-stranded DNA (ssDNA) and a nucleotide cofactor to be active for cleavage. We encountered two problems when utilizing the commonly used cofactor, ATPγS, to activate RecA. First, the photoactive azido group of AIA is very sensitive to, and can be quenched by, reducing agents such as β-mercaptoethanol or dithiothreitol (DTT) (2). We found that an excess amount of ATPγS introduced a trace amount of reducing agent which seemed to

quench the azido group of the crosslinker during UV irradiation. Also, in comparison to other cofactors, ATPγS seemed to be most efficient at activating RecA for mediating proteolytic cleavage. We wanted to avoid excessive conversion of UmuD to UmuD'. In an attempt to circumvent both of these difficulties, we tried various other cofactors including dATP. In addition we tried forming a RecA-ssDNA-ADP-AlF_4^- complex (20) with the hope of achieving a more stable association of UmuD with the RecA nucleoprotein filament. However, these methods did not seem to be as effective in promoting UmuD-RecA interactions, as evidenced by a substantial decrease in the rate of RecA-mediated cleavage and low yield of UmuD-RecA crosslinked complexes. We therefore decided to use ATPγS, but at significantly reduced concentrations as compared to the concentrations routinely employed in RecA-mediated cleavage reactions (10 fold less), and to incubate the UmuD with activated RecA at 37°C for a brief time period (5 min) to initiate formation of UmuD-RecA complexes yet minimize cleavage.

We carried out a preliminary screen of the ability of the various UmuD monocysteine derivatives modified with AIA to crosslink to activated RecA and found VC34 to crosslink most efficiently. We therefore focused first on the VC34 derivative to test crosslinking conditions and to compare its crosslinking ability to that of the UmuD derivative lacking cysteines, CA24. These two UmuD derivatives at a concentration of 80 μM were incubated with a 10x molar excess of AIA for 1 hr at 37°C in the dark. Reaction mixtures were dialyzed to remove excess reagent. Modified UmuD derivatives at a final concentration of 40 μM were then incubated at 37 °C for 5 min with 8 μM RecA activated in the presence of ssDNA and ATPγS. The mixture was then exposed to UV light (320 nm) for 15 min on ice. Fig. 4, Panel A shows representative crosslinking data of the mutants VC34 and CA24. Photolysis of derivatized VC34 with activated RecA (shown in lane 1) resulted in the appearance on the gel of several new higher molecular weight species. The most prominent species had an approximate molecular weight of 72 kD and comprised about 1% of the total protein in the reaction. A crosslinked complex containing RecA (38 kD) and $UmuD_2$ (30 kD) would migrate to a position corresponding to this approximate molecular weight. Other species which appear very faint on the Coomassie stained gel migrate to positions corresponding to molecular weights of 64 kD, 97 kD, and greater than 100 kD respectively. It is unclear whether the different species represent complexes differing in the number of UmuD monomers per UmuD·RecA

complex, different conformational isomers of the same UmuD·RecA complex, or different complexes containing UmuD and UmuD' crosslinked to RecA. Western analysis of the crosslinking of the UmuD derivative, VC34, to RecA is shown in Fig. 4, Panel B, lanes 1 and 3. The higher molecular weight species migrating to positions described above crossreacted with both αUmuD (lane 1) and αRecA (lane 3) antibodies supporting the suggestion that these complexes contain both UmuD and RecA.

In Fig. 4, Panel A, lane 2, the VC34 derivative modified with AIA is photoactivated for crosslinking in the absence of RecA. The absence of the appearance of the same pattern of higher molecular weight species in this reaction indicates that the formation of these complexes is dependent on the presence of activated RecA. This result suggests that the presence of RecA* causes a complex change in the ability of VC34 modified with AIA to react, possibly by allowing UmuD to react with another UmuD in a new way, by allowing the attachment of multiple UmuD monomers onto a single RecA monomer, or by allowing a combination of these two events to occur. It seems unlikely that the UmuD·RecA complexes also contain DNA since the single crosslinker present on VC34 must crosslink to another VC34 or RecA in order to become attached to the complex. In addition, crosslinking of a UmuD derivative in the presence of DNA and the absence of RecA did not result in new species which might be consistent with UmuD·DNA complexes (data not shown). As suggested earlier, the appearance of the faint band in the crosslinking reaction in absence of RecA* which migrates to a position corresponding to about 72 kD is consistent with the formation of crosslinked dimers of dimers ($UmuD_4$ complexes).

Control experiments with CA24, the UmuD derivative that lacks cysteines are shown in Fig. 4, Panel A, lane 3 and lane 4. The CA24 protein was treated identically to the VC34 monocysteine derivative in that it was incubated with AIA, dialyzed, and mixed with RecA*. Photolysis of this mixture did not result in the appearance of any new crosslinked species. This is also evident from the Western analysis of this reaction (Fig. 4, Panel B, lanes 2 and 4). This indicates that protein-protein or protein-DNA crosslinking did not occur upon exposure of the reaction mixture to UV light in absence of a photocrosslinker, and that the formation of the new higher molecular weight complexes was not due to RecA·RecA crosslinking. Furthermore, in the experiments involving the CA24 UmuD derivative, photolysis resulted in negligible homodimer crosslinking as expected (see Fig. 4, Panel A, lane 4 and Fig. 4, Panel B, lane 2), thereby

demonstrating the specificity of the reagent. It is also interesting to note that in the case of the CA24 derivative, incubation with RecA* resulted in some cleavage of UmuD to UmuD' indicating that these reaction conditions for crosslinking were favorable for RecA*-UmuD interactions (Fig. 4, Panel A, lane 3).

We next examined the rest of the set of UmuD monocysteine derivatives using the same conditions. The results are shown in Fig. 5. The UmuD monocysteine derivatives in this set, modified with AIA, displayed different abilities to crosslink to RecA. Photolysis of the UmuD derivatives which were able to crosslink with RecA resulted in the appearance of higher molecular weight complexes. The most prominent species had molecular weights of approximately 64 kD and 72 kD, respectively. Apart from the differences in the overall efficiency of crosslinking to RecA, the number of such species and their exact mobilities varied quite strikingly between these derivatives. This observation suggests that the position of the crosslinker on the particular UmuD derivative affects its ability to react in particular ways with RecA and/or UmuD (i.e., it affects the position of the specific attachment of the crosslinker onto a neighboring protein) which in turn influences the exact mobility of the resulting complexes. Although we have shown that AIA is considerably more specific than *p*-azidophenacyl bromide, even this reagent is not perfectly cysteine-specific (36). The striking differences between various monocysteine derivatives also indicate that the major complexes we see are due to the presence of the reagent on the single cysteine rather than on a non-cysteine residue. As discussed above, these multiple bands could result from the following: (i) different conformational isomers of UmuD·RecA complexes containing the same molar ratio of UmuD to RecA; (ii) complexes containing UmuD, UmuD' and RecA; or (iii) complexes differing in the ratio of UmuD to RecA (i.e., RecA·UmuD, RecA·$UmuD_2$, RecA·$UmuD_4$, and RecA·$UmuD_6$). It is possible that such complexes would not have the mobility corresponding to their calculated molecular weight since such crosslinked species, when denatured, will not assume a totally linear conformation. For example, $UmuD_2$, which has a calculated molecular weight of 30 kD, has a mobility on an SDS acrylamide gel corresponding to approximately 40 kD.

With respect to the overall efficiency of crosslinking, VC34 and SC81 appear to most efficiently crosslink with RecA. To a lesser extent, others (SC57, SC67, SC112), also formed cross-linked species of approximately the same molecular weight. SC19, C24, LC44, and SC60 did not result in significant crosslinking. With the exception of SC19 and the wildtype protein, C24, all of the

monocysteine UmuD derivatives were modified by [14C]AIA to approximately the same degree, therefore, the amount of crosslinked UmuD-RecA can be qualitatively compared. SC19 and the wildtype UmuD incorporated roughly half the amount of [14C]AIA of the other mutants. However, this lower degree of AIA incorporation cannot fully account for the absence of the higher molecular weight species corresponding to UmuD·RecA, since even with this level of modification, a significant amount of crosslinked $UmuD_2$ is present in the same reaction mixture.

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

Discussion

We have extended our investigations of the interactions of UmuD using the monocysteine approach to study not only interactions of UmuD in the homodimer, but also interactions of UmuD with RecA. We have used the cysteine-specific photoactivatable crosslinker, *p*-azidoiodoacetanilide (AIA) (36), for these investigations. All the monocysteine derivatives in this set had previously been tested for their reactivity with [^{3}H]iodoacetate and had been found to be quite accessible to solvent (14). We found the reactivity of these derivatives to [^{14}C]AIA to be quite similar to their reactivities to iodoacetate with most of the derivatives reacting to an extent of 60% to 80% in one hour. The reactivities of SC19 and C24 (wildtype) were only slightly lower (approximately 50% modification after one hour). These results are consistent with our previous inferences that the unique cysteines at these positions were exposed to solvent (14). We observed small differences in relative reactivities for some of the derivatives, particularly SC19 and C24(wildtype), which were relatively less reactive with AIA than with iodoacetate. These small differences in reactivity could be due to the small influences of neighboring residues in interacting with the two different alkylating agents (AIA has a hydrophobic aromatic ring and iodoacetate has a negative charge).

In contrast to the rather similar reactivities of the monocysteine derivatives to iodoacetate and AIA, the various monocysteine derivatives modified with AIA displayed striking differences in their ability to crosslink to another UmuD in the $UmuD_2$ homodimer and in turn differed in their abilities to crosslink into a RecA containing complex if activated RecA is present. In interpreting these results we have taken into account the following factors: (i) crosslinking is highly dependent on the distance of the reactive radical of the activated crosslinker to the adjacent residue because the half life of the activated AIA-derived crosslinker is rather short (only 0.1 to 5 μsec) (2), and (ii) crosslinking is dependent on the chemical nature of the residue with which it is to react (i.e., this residue must be nucleophilic). AIA is only 9 Å long; therefore, those monocysteine derivatives which were able to be crosslinked by this crosslinker in the homodimer were probably within about 9 Å of the adjacent UmuD monomer. The results of the ability of this set of monocysteine derivatives to be crosslinked using AIA support previous inferences concerning the relative closeness of these positions to the $UmuD_2$ homodimer interface (14). We found the monocysteine derivatives, C24

(wildtype UmuD), VC34, and LC44 to crosslink most efficiently using this reagent. On the basis of iodine and *bis*-maleimidohexane crosslinking (BMH) we suggested that residues at positions 24, 34, and 44 are closer to the dimer interface than the other residues tested. Our results of homodimer crosslinking using AIA are consistent with this inference. $UmuD_2$ homodimer crosslinking results using AIA also suggest that the residue at position 57 is relatively closer to the dimer interface. Because crosslinking using AIA does not require that there be a cysteine residue in the other interacting protein, this strategy of probing protein interactions may be a better predictor of the relative closeness of adjacent residues than the use of cysteine-specific homobifunctional reagents. Thus position 57 in one UmuD monomer might be fairly close to the dimer interface but not necessarily as close to position 57 of the adjacent UmuD monomer. The monocysteine derivative with a cysteine substitution at position 19 also crosslinked with moderate efficiency. We found previously that SC19 was crosslinked rather efficiently with BMH but was crosslinked less efficiently upon oxidation using iodine (14). These observations led to the suggestion that the serines at position 19 in the homodimer are not as close to the dimer interface as residues 24, 34 and 44, but are within the 13.9 Å span of BMH. The observation that SC19 can be crosslinked in the homodimer with AIA (which is 9 Å in length) is consistent with this previous finding. Our present results also suggest that residues at position 60, 67, 81 and 112 are relatively farther from the dimer interface than the others tested and again this supports our previous inferences made on the basis of iodine and BMH crosslinking (14).

Peat et al. (23) have recently solved the structure of the cleaved form of UmuD, UmuD', to 2.5Å. The structure of UmuD' consists of a globular head (residues 50 to 135) and an extended amino terminal tail. Peat et al. have proposed that UmuD' forms a filament with two types of interactions: interactions between two UmuD' protomers (which they term the molecular dimer interactions) and interactions between two $UmuD'_2$ dimers (i.e., dimer of dimer interactions, which they term the filament dimer interactions). In the molecular dimer, residues Tyr52, Val54, Ile87, Phe94 and Phe128 are involved in hydrophobic interactions at the dimer interface and Glu93 and Lys55 form salt bridges with their dimer partners on both sides of the interface. The amino terminal tails which include residues 25 through 44, protrude in opposite directions in the $UmuD'_2$ molecular dimer, precluding interactions of the residues in this region within the molecular dimer. In the "filament dimer" associations,

however, the amino termini (one from each pair of dimers) interact with each other at residues Leu40, Asn41, Leu43, Leu44, and Ile45. This is reminiscent of our findings that the LC44 derivative of the intact $UmuD_2$ homodimer can be crosslinked. In fact, it is easier to rationalize all our observations using monocysteine UmuD derivatives by a model for the UmuD interface that is more closely related to the UmuD' "filament dimer" interface than to the UmuD' "molecular dimer" interface. In such a model for the quaternary structure of UmuD, the amino terminal region (including Val34 and Leu44) would be close to or would compose part of the dimer interface. In addition, pairs of Ser67 and Ser112 residues in the UmuD dimer would not be located on opposite sides of the dimer (as in the molecular dimer), but instead would be located on the "inner" surfaces of the dimer perhaps closer together than in the filament dimer in the UmuD' crystal. It would not be surprising to find the structure of intact UmuD to be slightly distorted from what is seen in the crystal structure for the UmuD' filament dimer. Our observations of poor disulfide crosslinking but efficient crosslinking using *bis*-maleimidohexane in the UmuD dimer at positions 67 and 112 (14) would be simple to explain by hypothesizing that the carboxy terminal globular heads are actually closer in the intact UmuD dimer than they are in the UmuD' filament dimer. In addition, a parallel arrangement of the amino terminal tails in the region including residues 34 to 44 might account for the efficient crosslinking of the VC34 and LC44 derivatives with various reagents (14) as well as for the efficient spontaneous oxidation of disulfide bonds in the dimer upon dialysis for the derivatives with single cysteine substitutions within this region (12).

If our hypothesis for the intact UmuD dimer interface is correct, it suggests another level of subtlety in the modulation of UmuD activity. Intact UmuD forms dimers which resemble the UmuD' filament dimers. Presumably, the residues that are involved in the $UmuD'_2$ "molecular dimer" interactions are not available in intact UmuD. Perhaps these residues are buried in intact UmuD or else obscured by the presence of the amino terminal 24 amino acids. Upon RecA-mediated cleavage of the first 24 amino acids, the residues at the "molecular dimer" interface become available for interaction. Consequently, the $UmuD'_2$ homodimer adopts a different quaternary conformation than the intact $UmuD_2$ homodimer using this alternative interface and forming the active species which is observed in the crystal structure as "molecular dimers".

In the crosslinking of the derivatives C24 (wildtype) and VC34 using AIA, we observed the appearance of higher order complexes consistent with the crosslinking of dimers of dimers. One explanation is that, like UmuD' dimers, UmuD dimers are able to undergo dimer dimer associations perhaps by an interaction that bears some relationship to the molecular dimer interactions in the UmuD' crystal (23). An alternative explanation is that the amino terminal region of one UmuD dimer interacts with the active site of another UmuD dimer. Kim and Little (13) have shown that LexA can serve as an enzyme in the cleavage of other LexA repressors by demonstrating the ability of LexA to cleave in *trans*. Thus it is possible for the amino terminal region of one UmuD molecule to interact with another UmuD at its active site. Whether these complexes of UmuD are physiologically significant requires further investigation. Only a small proportion of UmuD was crosslinked in these complexes suggesting that the UmuD dimer is the more prominent species in solution.

Amino acid positions in UmuD which are closer to the UmuD-RecA interface than the others tested. We then used the AIA-modified UmuD monocysteine derivatives to explore the nature of UmuD interactions with RecA. When AIA-modified UmuD derivatives were incubated with activated RecA and then irradiated with UV light to initiate the photolysis reaction, only a subset of the modified derivatives crosslinked to activated RecA. Of these derivatives, VC34 and SC81 seemed to crosslink the most efficiently. This suggests that positions 34 and 81 are closer to the UmuD-RecA interface than the other residues tested. The suggestion that position 34 is closer to the UmuD-RecA interface is consistent with our previous characterizations of this derivative. In experiments assessing ability to perform in mutagenesis and ability to undergo RecA-mediated cleavage, we found VC34 to be impaired in mutagenesis and most severely deficient of all the monocysteine derivatives in RecA-mediated cleavage. We interpreted these results to suggest that this residue is important for the direct interactions with RecA which lead to cleavage (14). As we have discussed previously (14), position 34 is also found within a region corresponding to the region in λ repressor suggested by Sauer and Gimble to be involved in RecA interactions (11).

In their study of UmuD', Peat et al. (23) suggested the importance of the extended amino terminal region of UmuD' in contacting RecA. They observed that while the wildtype UmuD' was able to be crosslinked to activated RecA using glutaraldehyde, a UmuD' mutant lacking residues 25 to 45 crosslinked to RecA

much more poorly using glutaraldehyde. Taken together with our findings, this suggests that this region is important for RecA interactions both in intact UmuD and cleaved UmuD'. RecA possibly interacts differently with UmuD than it does with UmuD'. In intact UmuD, the cleavage site must be brought to the active site proteolytic residues at one end of a cleft in the globular structure (23). After cleavage, the amino terminus extends out from the globular structure as observed in the crystal and this extended amino terminal tail is thought to interact with RecA.

We observed that SC81 was quite proficient in UV mutagenesis and RecA-mediated cleavage. Interestingly, position 81 is found within a small region of amino acids that is not conserved within the UmuD-LexA family of homologous proteins. Crosslinking with RecA indicates that it is close to the UmuD-RecA interface though this position may not be critical for mutagenesis or RecA-mediated cleavage. It is possible that the small region around position 81 contributes to the specific interactions with RecA which distinguishes it from other mutagenesis and repressor proteins.

The other residues which crosslinked moderately efficiently to RecA with respect to overall efficiency were the residues located in the central region of the UmuD amino acid sequence, SC57, SC67, and SC112. Since the incorporation of [14C]AIA is roughly equivalent for each of these derivatives, the amount of UmuD-RecA crosslinking can be qualitatively compared. SC67 seemed to crosslink more efficiently than SC57 and SC112. Our observation that the SC67 mutation affects the UV mutagenesis phenotype more dramatically than it affects RecA-mediated cleavage led us to suggest that this position is more important for the subsequent role of UmuD' in mutagenesis than it is for the cleavage reaction (14). Perhaps this role may involve interactions with RecA in its third role in mutagenesis. Alternatively, SC67 may interact with RecA during RecA-mediated cleavage, however, the substitution of serine for cysteine in this case does not greatly affect the interactions of RecA with UmuD which result in cleavage.

Amino acid changes that affect RecA-mediated cleavage in LexA (16), λ repressor (11), and UmuD (1, 22) have been found in the regions of these proteins that correspond to the Ser57 and Ser112 regions of UmuD. These positions correspond in UmuD to residues Val54, Lys55, Ala56, Gly58, Leu107 and Asn111. SC57 and SC112 crosslink to RecA, albeit less efficiently than the derivatives previously mentioned. These residues may be more important in

maintaining the whole architecture of the autocleavage domain than in the critical contacts between UmuD and RecA.

If a plane is drawn longitudinally through the active site cleft in the UmuD' crystal structure (23), Ser57, Ser67, and Ser112 would be located on one side of the cleft facing outward and Ser81 would be on the other side of the cleft, facing outward. If UmuD resembles UmuD' in this region, this would suggest that during RecA-mediated cleavage, RecA interacts with this region of UmuD.

Crosslinking of RecA to the UmuD derivatives VC34 and SC81 unexpectedly resulted in the appearance of more than just the complexes which would be consistent with RecA·UmuD and RecA·$UmuD_2$. This observation suggests that interaction with RecA causes a conformational change in UmuD that makes new interactions possible besides simply RecA's interaction with a monomer or dimer of UmuD. The new complexes arise only in the presence of activated RecA indicating a dependence of the crosslinking of such complexes on the presence of RecA*. Since the presence of each new crosslinker can only arise from the introduction of an additional UmuD monomer to the complex, the presence of RecA* may be causing a change in the ability of UmuD to interact, perhaps by allowing UmuD to interact in a new way with another UmuD within the RecA·UmuD complex, or alternatively, by allowing multiple UmuD proteins to interact with a single RecA monomer.

Cys24 and Ser60: cleavage site and active site residues. In the UmuD' crystal structure (23), the active site serine is located in a cleft. In light of the suggestion mentioned above that RecA interacts on the face of UmuD around the cleft region, it is interesting to note that SC60 did not crosslink to activated RecA although with low efficiency, it can crosslink in the dimer. If UmuD is similar in conformation to UmuD' in this region, the absence of crosslinking could be explained by the inaccessibility of the cysteine at position 60 to be crosslinked to RecA. One possibility is that as the cleavage site is brought near the active site in the RecA-mediated cleavage reaction, the amino terminal region covers the active site residues and hinders direct interactions with RecA. Interestingly, wildtype UmuD with a cysteine at position 24 (of the Cys24-Gly25 cleavage site) also did not crosslink to RecA but did crosslink very efficiently in the dimer. If, when UmuD interacts with RecA, the cleavage site becomes buried within the active site cleft region, this cysteine would also be inaccessible for direct interactions with RecA. This is consistent with the role of RecA being a coprotease in facilitating the otherwise latent capacity of UmuD to autodigest. Mutations have

been found at the cleavage site in LexA, λ repressor, and UmuD which severely affect the ability of these proteins to undergo RecA mediated cleavage (1, 11, 16, 22). Since our results suggest that Cys24 at the cleavage site does not directly contact RecA, these mutations may affect cleavage by altering interactions between the cleavage site and the active site (14, 17) rather than by altering direct interactions with RecA.

Ser-19 and Leu-44. The relatively conservative substitution of cysteine for serine at position 19 resulted in a significant (70 to 80%) reduction in RecA-mediated cleavage (14). Mutations in the corresponding residue of LexA also caused severe impairment of the ability of the mutant proteins to undergo RecA-mediated cleavage and autodigestion (16, 17). However, the monocysteine derivative with the cysteine substitution at position 19, SC19, did not crosslink significantly with RecA. Assuming that the lack of crosslinking observed is predominantly due to the distance of the crosslinker from a nucleophilic residue on RecA, this result indicates that this position is farther from the UmuD-RecA interface than the other derivatives tested. This suggests that the substitution of cysteine for serine may not affect RecA-mediated cleavage simply by affecting the direct UmuD-RecA interactions. Ser19 is located in the N-terminal domain of UmuD which is removed by RecA-mediated cleavage. Perhaps this N-terminal region is involved in maintaining the UmuD conformation which is distinct from the UmuD' conformation. The introduction of amino acid substitutions may alter the UmuD conformation that is favorable for interactions with RecA that lead to cleavage and not necessarily alter the specific contacts between UmuD and RecA.

From the present and previous (14) crosslinking studies we proposed that Leu44, along with Cys24 (of the Cys24-Gly25 cleavage site) and residue Val34, is in the region which is closer to the $UmuD_2$ homodimer interface. LC44, however, did not crosslink to RecA in these experiments whereas VC34, which also crosslinked very efficiently in the $UmuD_2$ homodimer, crosslinked to RecA the most efficiently of the UmuD monocysteine mutants we tested. This is interesting considering that in the crystal structure of the cleaved from, UmuD' (23), Leu44 appears to be located on the same face as Ser57, Ser67 and Ser112 which we observed to crosslink with RecA. As suggested above, the amino terminal region is probably brought near the active site cleft so that the cleavage site can be properly positioned inside the cleft (23). This would necessarily entail a conformational change from the structure observed in the crystal of UmuD' (23) as well as the proposed structure of intact UmuD in the homodimer. Our results

suggest that Leu44 does not participate in RecA interactions as UmuD associates with RecA in this new conformation.

The repressor binding site on the RecA nucleofilament. From their three dimensional reconstruction of electron micrographs of the RecA-LexA complex, Yu and Egleman (35) determined that LexA bound within the deep groove of the activated RecA filament, with two strong contacts with the RecA filament surface spanning adjacent RecA protomers. If UmuD also bound within the deep groove of the activated RecA filament, our observation that residues from different sides of the UmuD molecule crosslinked to RecA is not surprising. The first site, site A, is a discreet contact on the inner surface of a pendulous lobe on each subunit. The identification of this site is in agreement with the repressor binding site proposed in the RecA crystal structure (32) including residues 229 and 243, positions at which mutations in RecA affect repressor cleavage. The second contact, site B, maps onto the region of the crystal structure containing residues 156 and 165 and an intervening disordered loop region, L1, which has been suggested to be the secondary DNA binding site within the RecA filament (32).

Nastri and Knight (21) have introduced many single and multiple amino acid substitutions using a combinatorial cassette mutagenesis procedure into the region in RecA defined by the residues 152-159 overlapping the disordered loop region, L1. They found that a few specific substitutions at Ile155 and Glu156, and a number of different substitutions at Gly157 and Glu158, gave constitutive coprotease mutants. From their results they suggested that many of the mutations they found affect coprotease activity by altering part of the repressor binding site.

It is possible that residues in site A or site B of RecA interact directly with UmuD and are crosslinked to UmuD by the photoactivatable crosslinker, AIA. We have used AIA with the UmuD monocysteine derivatives to make inferences regarding the regions within UmuD which might be closer to the UmuD-RecA interface. Further experiments which include digesting the various RecA/UmuD complexes, isolating crosslinked tryptic fragment and identifying the crosslinked peptide using mass spectrometric techniques and sequencing may yield biochemical results which would help to more specifically elucidate these UmuD-RecA interactions.

Acknowledgments

We are grateful to H. Gobind Khorana and Peter Kim and to the members of the laboratory for many helpful discussions, suggestions and comments on the manuscript.

This work was supported by Public Health service grant CA21615 awarded by the National Cancer Institute. M. H. L. was supported by Predoctoral Training Grant 5T32GM07287 from the National Institute of Health.

References

1. **Battista, J. R., T. Ohta, T. Nohmi, W. Sun and G. C. Walker**. 1990. Dominant negative *umuD* mutations decreasing RecA-mediated cleavage suggest roles for intact UmuD in modulation of SOS mutagenesis. Proc. Natl. Acad. Sci. USA **87:**7190-7194.

2. **Bayley, H.** 1983. Photogenerated reagents in biochemistry and molecular biology. Elsevier, New York.

3. **Bennet, S. E., O. N. Jensen, D. F. Barofsky and D. W. Mosbaugh**. 1994. UV-catalysed crosslinking of E. Coli uracil-DNA glycosylase to DNA. The Journal of Biological Chemistry **269:**21870-21879.

4. **Burckhardt, S. E., R. Woodgate, R. H. Scheuermann and H. Echols**. 1988. UmuD mutagenesis protein of *Escherichia coli*: Overproduction, purification, and cleavage by RecA. Proc. Natl. Acad. Sci. U.S.A. **85:**1811-1815.

5. **Clauser, K. R., S. C. Hall, D. M. Smith, J. W. Webb, L. E. Andrews, H. M. Tran, L. B. Epstein and A. L. Burlingame**. 1995. Rapid mass spectrometric peptide sequencing and mass matching for characterization of human melanoma proteins isolated by two-dimensional PAGE. Proc. Natl. Acad. Sci. USA **92:**5072-5076.

6. **Cohen-Fix, O. and Z. Livneh**. 1992. Biochemical analysis of UV mutagenesis in *Escherichia coli* by using a cell-free reaction coupled to a bioassay: identification of a DNA repair-dependent pathway. Proc. Natl. Acad. Sci. U.S.A. **89:**3300-3304.

7. **Dutreix, M., P. L. Moreau, A. Bailone, F. Galibert, J. R. Battista, G. C. Walker and R. Devoret**. 1989. New *recA* mutations that dissociate the various RecA protein activities in *Escherichia coli* provide evidence for an additional role for RecA protein in UV mutagenesis. J. Bacteriol. **171:**2415-2423.

8. **Eguchi, Y., O. T. and O. H.** 1988. Cleavage of bacteriophage $\phi 80$ *c*I repressor by RecA protein. J. Mol. Biol. **202:**565-573.

9. **Frank, E. G., J. Hauser, A. S. Levine and R. Woodgate**. 1993. Targeting of the UmuD, UmuD' and MucA' mutagenesis proteins to DNA by RecA protein. Proc. Natl. Acad. Sci. U.S.A. **90:**8169-8173.

10. **Friedberg, E. C., G. C. Walker and W. Siede.** 1995. DNA repair and mutagenesis. The American Society for Microbiology, Washington, DC.

11. **Gimble, F. S. and R. T. Sauer**. 1986. λ repressor inactivation: properties of purified ind$^-$ proteins in the autodigestion and RecA-mediated cleavage reactions. J. Mol. Biol. **192:**39-47.

12. **Guzzo, A., M. H. Lee, K. Oda and G. C. Walker**. 1995. Analysis of the region between amino acids 30 to 42 of intact UmuD by a monocysteine approach. in preparation.

13. **Kim, B. and J. W. Little**. 1993. LexA and λ cI repressors as enzymes: specific cleavage in an intermolecular reaction. Cell **73:** 1165-1173.

14. **Lee, M. H., T. Ohta and G. C. Walker**. 1994. A monocysteine approach for probing the structure and interactions of the UmuD Protein. J. Bacteriol. **176:** 4825-4837.

15. **Lee, T. D. and J. E. Shively**. 1990. Enzymatic and chemical digestion of proteins for mass spectrometry. Methods in Enzymology **193:** 361-374.

16. **Lin, L. L. and J. W. Little**. 1988. Isolation and characterization of noncleavable (Ind^-) mutants of the LexA repressor of *Escherichia coli* K-12. J. Bacteriol. **170:** 2163-2173.

17. **Lin, L. L. and J. W. Little**. 1989. Autodigestion and RecA-dependent cleavage of Ind^- mutant LexA proteins. J. Mol. Biol. **210:** 439-452.

18. **Little, J. W.** 1984. Autodigestion of LexA and phage λ repressors. Proc. Natl. Acad. Sci. U.S.A. **81:** 1375-1379.

19. **Little, J. W., S. H. Edmiston, Z. Pacelli and D. W. Mount**. 1980. Cleavage of the *Escherichia coli* LexA protein by the RecA protease. Proc. Natl. Acad. Sci. U.S.A. **77:** 3225-3229.

20. **Moreau, P. L. and M. F. Carlier**. 1989. RecA protein-promoted cleavage of LexA repressor in the presence of ADP and structural anologues of inorganic phosphate, the fluoride complexes of aluminum and beryllium. J. Biol. Chem. **264:** 2302-2306.

21. **Nastri, H. G. and K. L. Knight**. 1994. Identification of residues in the L1 region of the RecA protein which are important to recombination or coprotease activities. J. Biol. Chem. **269:** 26311-26322.

22. **Nohmi, T., J. R. Battista, L. A. Dodson and G. C. Walker**. 1988. RecA-mediated cleavage activates UmuD for mutagenesis: Mechanistic relationship between transcriptional derepression and posttranslational activation. Proc. Natl. Acad. Sci. U.S.A. **85:** 1816-1820.

23. **Peat, T. S., E. G. Frank, J. P. McDonald, A. S. Levine, R. Woodgate and W. A. Hendrickson**. 1995. Structure of the UmuD' protein as related to its role in the response to DNA damage. Nature submitted.

24. **Perry, K. L., S. J. Elledge, B. Mitchell, L. Marsh and G. C. Walker**. 1985. *umuDC* and *mucAB* operons whose products are required for UV light- and chemical-induced mutagenesis: UmuD, MucA, and LexA proteins share homology. Proc. Natl. Acad. Sci. USA **82:** 4331-4335.

25. **Rajagopalan, M., C. Lu, R. Woodgate, M. O'Donnell, M. F. Goodman and H. Echols**. 1992. Activity of the purified mutagenesis proteins UmuC, UmuD', and RecA in replicative bypass of an abasic DNA lesion by DNA polymerase III. Proc. Natl. Acad. Sci. U.S.A. **89:** 10777-10781.

26. **Roberts, J. W. and C. W. Roberts**. 1981. Two mutations that alter the regulatory activity of *E. coli recA* protein. Nature (London) **290:** 422-424.

27. **Sassanfar, M. and J. W. Roberts**. 1990. Nature of the SOS-inducing signal in *Escherichia coli*: the involvement of DNA replication. J. Mol. Biol. **212:** 79-96.

28. **Sauer, R. T., R. R. Yocum, R. F. Doolittle, M. Lewis and C. O. Pabo**. 1982. Homology among DNA-binding proteins suggests use of a conserved super-secondary structure. Nature (London) **298:** 447-451.

29. **Shinagawa, H., H. Iwasaki, T. Kato and A. Nakata**. 1988. RecA protein-dependent cleavage of UmuD protein and SOS mutagenesis. Proc. Natl. Acad. Sci. U.S.A. **85:** 1806-1810.

30. **Slilaty, S. N. and J. W. Little**. 1987. Lysine-156 and serine-119 are required for LexA repressor cleavage: a possible mechanism. Proc. Natl. Acad. Sci. U.S.A. **84:** 3987-3991.

31. **Sommer, S., A. Bailone and R. Devoret**. 1993. The appearance of the UmuD'C protein complex in *Escherichia coli* switches repair from homologous recombination to SOS mutagenesis. Molec. Microbiology **10:** 963-971.

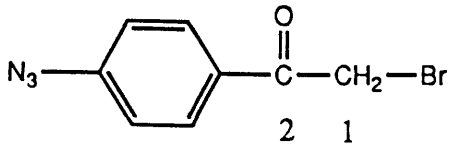
32. **Story, R. M., I. T. Weber and T. A. Steitz**. 1992. The structure of the *E. coli recA* protein monomer and polymer. Nature **355:** 318-325.

33. **Sweasy, J. B., E. M. Witkin, N. Sinha and V. Roegner-Maniscalco**. 1990. RecA protein of *Escherichia coli* has a third essential role in SOS mutator activity. J. Bacteriol. **172:** 3030-3036.

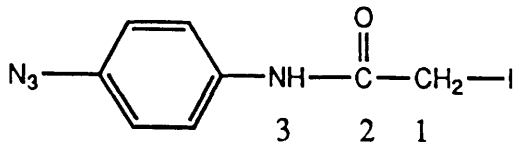
34. **Woodgate, R., M. Rajagopalan, C. Lu and H. Echols**. 1989. UmuC mutagenesis protein of *Escherichia coli:* Purification and interaction with UmuD and UmuD'. Proc. Natl. Acad. Sci. USA **86:** 7301-7305.

35. **Yu, X. and E. H. Egelman**. 1993. The LexA repressor binds within a deep helical groove of the activated RecA filament. J. Mol. Biol. **231:** 29-40.

36. **Zhang, J., M. H. Lee and G. C. Walker**. 1995. *p*-Azidoiodoacetanilide, a new short photocrosslinker that has a greater cysteine specificity than *p*-azidophenacyl bromide and *p*-azidobromoacetanilide. Biochemical and Biophysical Research Communications **217:** 1177-1184.



p-Azidophenacyl bromide (APB)



p-Azidoiodoacetanilide (AIA)

Figure 1. Chemical structures of *p*-azidophenacyl bromide (APB) and *p*-azidoiodo-acetanilide (AIA).

Figure 2. Reactivity of UmuD monocysteine mutant proteins with [^{14}C]AIA. The percent of total protein modified by AIA in 60 min. was measured. UmuD mutant derivatives at a 20 μM concentration in 50 mM HEPES (pH 8.0) containing 500 mM NaCl was incubated with a 10 fold molar excess of [2-^{14}C]*p*-azidoiodoacetanilide in the dark at 37 °C for 1 h.

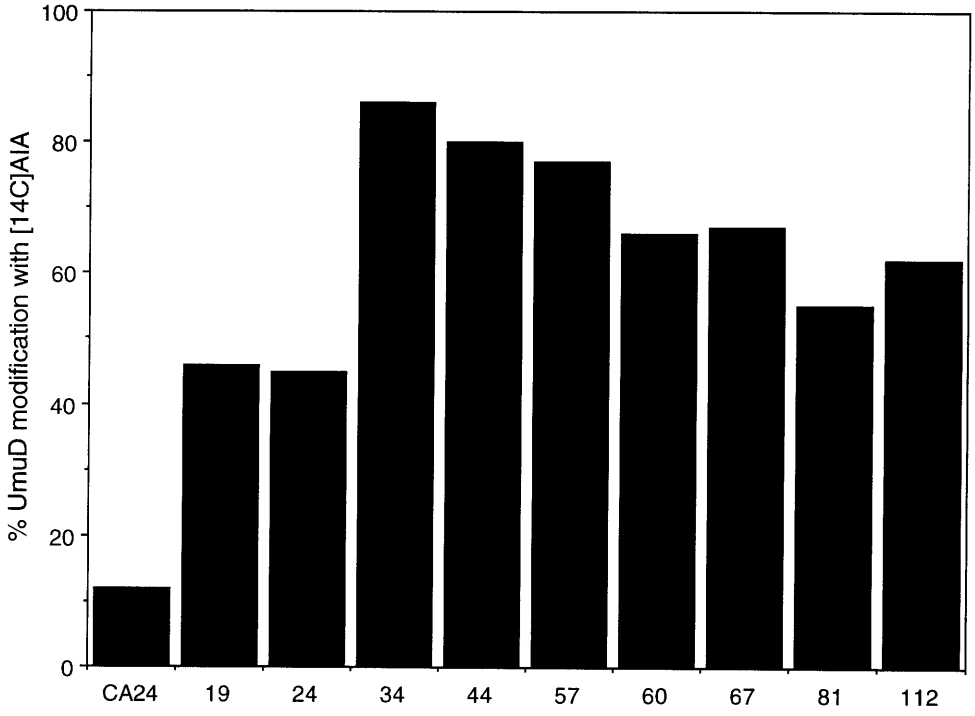
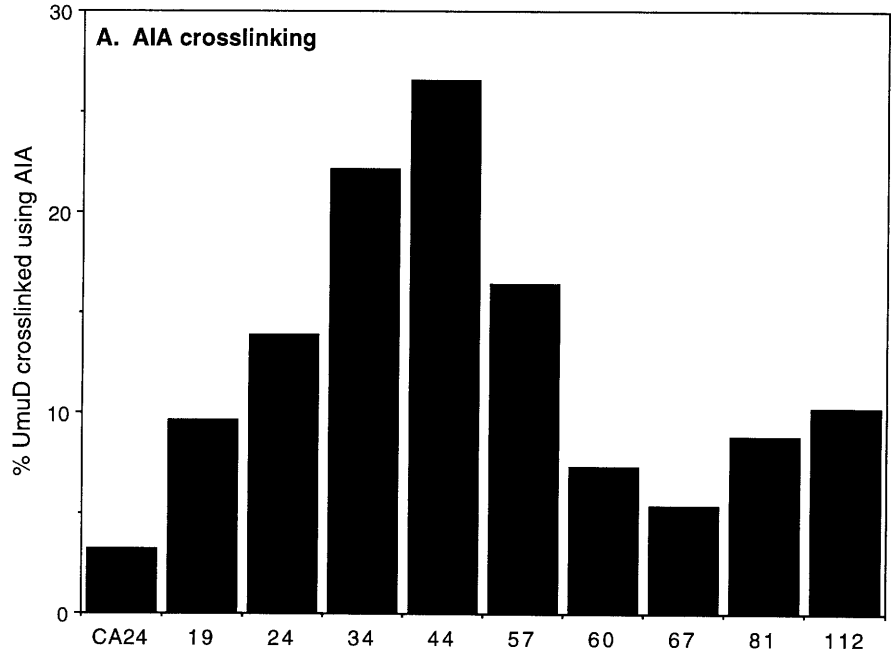

% UmuD modification with [14C]AIA
100
80
60
40
20
0
CA24
19
24
34
44
57
60
67
81
112

Figure 3. Percent of UmuD crosslinked by using AIA. Panel A. Quantitation of UmuD crosslinking from densitometric scans of Coomassie stained gels. UmuD derivatives at a 80 μM concentration in 50 mM HEPES (pH 8.0) containing 500 mM NaCl was incubated with a 10 fold molar excess of AIA in the dark at 37 °C for 1 h. The reaction mixture was dialyzed in a microdialyzer to remove excess reagent. UmuD derivatives at a final concentration of 60 μM were then exposed to UV light at 320 nm on ice for 15 min to initiate photolysis reaction. Panel B. Normalization of crosslinking data. UmuD crosslinking data was normalized to account for the differences in UmuD modification by AIA. The maximum value for the percent of UmuD crosslinking for a given mutant derivative was obtained by dividing the actual percentage of the crosslinked UmuD by the percent of UmuD modified as described in the Methods section.


A. AIA crosslinking
% UmuD crosslinked using AIA
30
20
10
0
CA24
19
24
34
44
57
60
67
81
112

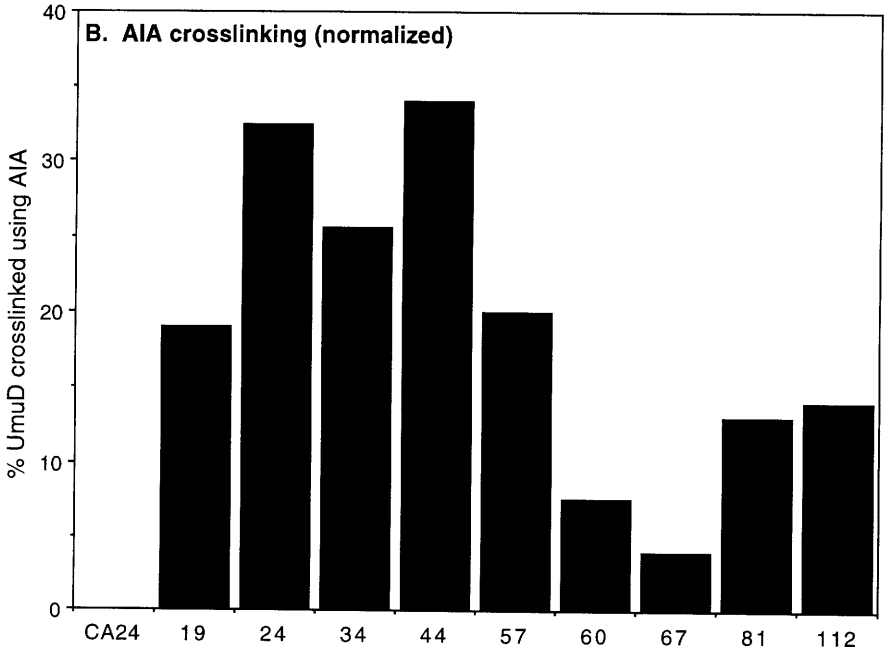

B. AIA crosslinking (normalized)
% UmuD crosslinked using AIA
40
30
20
10
0
CA24
19
24
34
44
57
60
67
81
112

Figure 4. Crosslinking of the UmuD derivatives, VC34 and CA24, to RecA* using AIA. UmuD derivatives at a concentration of 80 μM were modified with AIA then dialyzed to remove excess reagent. Modified UmuD derivatives at a final concentration of 45 μM were incubated with 8 mM RecA activated with in the presence of ATP γS and ssDNA for 5 min at 37 °C in the dark, then exposed to UV light for 15 min to initiate photolysis reaction. Panel A. Coomassie stained gel of crosslinked species resolved by electrophoresis on an SDS-polyacrylamide gel. Lanes: 1, VC34 in the presence of activated RecA; 2, VC34 only; 3, CA24 in the presence of activated RecA; 4, CA24 only. Panel B. Western analysis of crosslinked complexes by blotting with αUmuD or αRecA antibodies and visualizing with chemiluminescence. Lanes 1 and 2: samples were visualized using αUmuD antibodies; Lanes 3 and 4: samples were visualized using αRecA antibodies. Lanes 1 and 3: VC34 in the presence of activated RecA; lanes 2 and 4: CA24 in the presence of RecA.

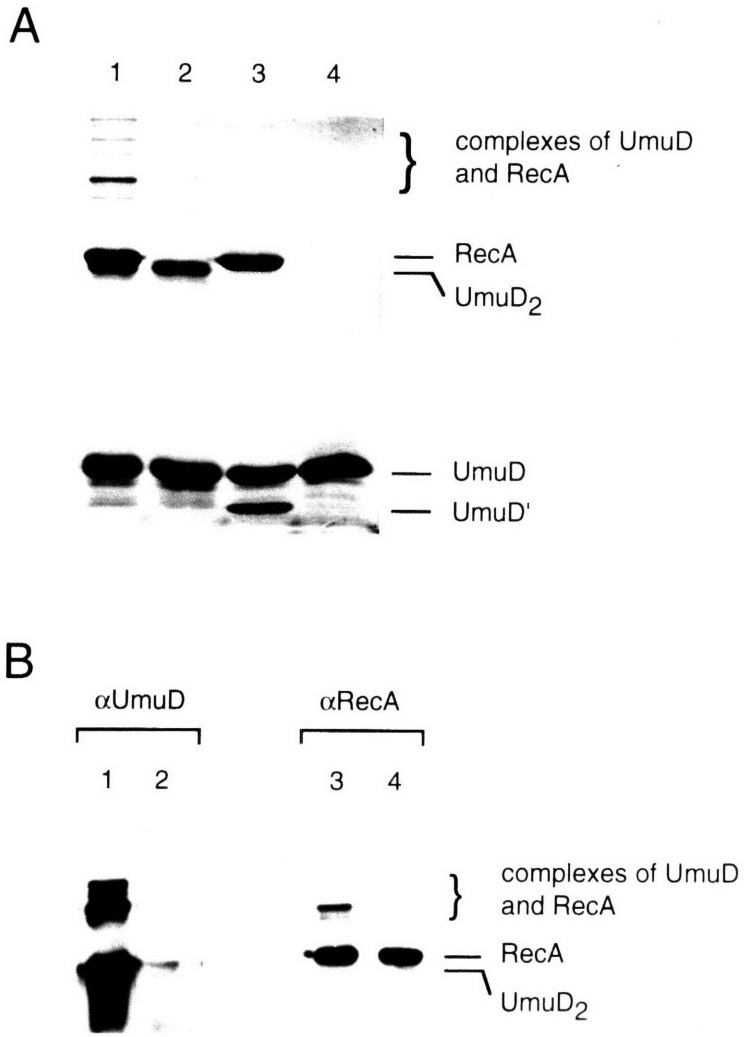
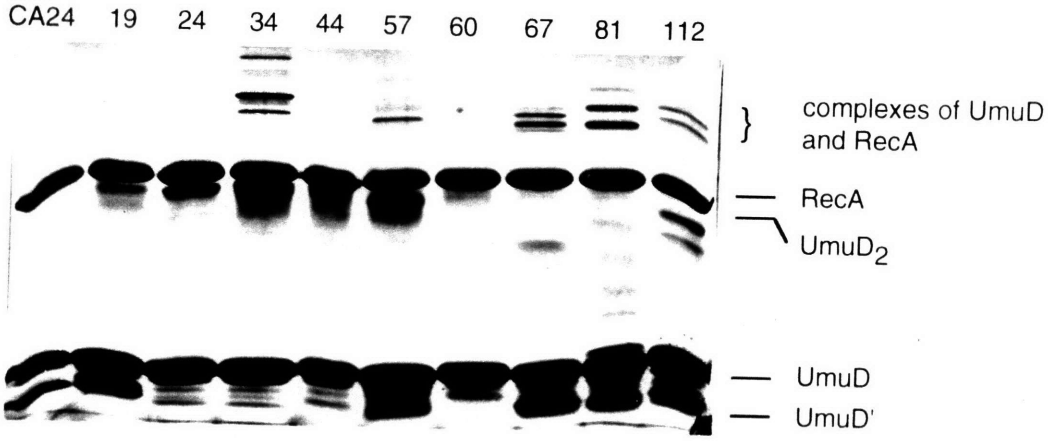

A
1
2
3
4
complexes of UmuD
and RecA
RecA
UmuD2
UmuD
UmuD'
B
αUmuD
αRecA
1
2
3
4
complexes of UmuD
and RecA
RecA
UmuD2

Figure 5. Crosslinking of UmuD monocysteine derivatives to activated RecA using AIA. UmuD derivatives modified with AIA were dialyzed to remove excess reagent then incubated with activated RecA for 5 min at 37 °C in the dark. The photocrosslinking reaction was then initiated by exposing the reaction mixtures to UV light for 15 min on ice as described in the Methods section. All lanes contain both a UmuD derivative and activated RecA. Lanes are identified by the position of the cysteine substitution of each particular monocysteine mutant in the crosslinking reaction. CA24 is the UmuD derivative without cysteines.


CA24
19
24
34
44
57
60
67
81
112
complexes of UmuD and RecA
RecA
UmuD2
UmuD
UmuD'

Chapter 5

Analysis of the region between amino acids 30 to 42 of intact UmuD by a monocysteine approach

This work was done in collaboration with Angelina Guzzo and Karen Oda.

Abstract. Based on previous characterizations of our UmuD monocysteine derivatives we suggested that positions 24, 34 and 44 were closer to the intact UmuD homodimer interface than other positions tested. Because this region of UmuD also appeared to be important for interactions with RecA, we followed up our previous study by constructing a second set of monocysteine UmuD derivatives with single cysteine substitutions at positions 30 to 42. The observation that purified proteins of the UmuD derivatives RC37 and IC38 could be disulfide crosslinked quantitatively upon addition of iodine and yet be poorly modified with iodoacetate led us to suggest that the pairs of residues at 37 and 38 are at the UmuD homodimer interface. We assessed the ability of these UmuD derivatives to perform in UV mutagenesis and in vivo and in vitro RecA mediated cleavage and found monocysteine mutants with substitutions at positions 32, 33, 34, and 35 to be most severely affected by a cysteine substitution indicating that the residues in this region are important for UV mutagenesis and RecA-mediated cleavage. In addition we found LC40 to be deficient in UV mutagenesis and RecA-mediated cleavage suggesting that a cysteine substitution for a leucine at this position, conserved among the mutagenesis proteins, is also important for the RecA-mediated cleavage and the cleaved UmuD' function in UV mutagenesis.

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 (36). Genetically, this process was shown to require the expression of the products of three genes: *umuC*, *umuD* and *recA* (20, 34), which are regulated as part of the SOS regulon (17). The 15 kDa UmuD protein is subsequently cleaved in a RecA-mediated fashion to yield the 12 kDa carboxy-terminal derivative, designated UmuD' (9, 24, 32). Genetic evidence suggests that DNA polymerase I and II are not required for mutagenesis (3, 29), and indicates that DNA polymerase III is required (6, 7, 8). 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 (27). UmuD was found to inhibit the process. UmuD and UmuD' form homodimers, as well as a heterodimer. The heterodimer was shown to be more stable *in vitro* (5), and has been postulated to play a posttranslational role in negative regulation of UV mutagenesis which is perhaps particularly important during the shut down of the SOS response.

RecA plays at least three roles in UV mutagenesis. After exposure to a mutagen, RecA is activated to RecA* by binding to single-stranded DNA generated during a cell's attempt to replicate its damaged DNA (30). RecA* serves as a coprotease that facilitates the latent ability of LexA (23) and UmuD (9) to autodigest. Cleavage of LexA is required for expression of several genes under the control of the SOS regulon, including the *umuDC* operon (17). Cleavage of UmuD to UmuD' (24, 32) activates the protein for its role in UV and chemical mutagenesis (1, 24). The third role of RecA in SOS mutagenesis is not yet known, but several *recA* mutants defective in this role have been isolated (2, 10, 24) .

Several hypotheses have been proposed for the mechanism of SOS mutagenesis including: i) UmuD' and UmuC affect the processivity of DNA polymerase III (4, 35) ii) UmuD' and UmuC bind to the RecA-single-stranded DNA causing it to switch from a recombinational to a mutagenic bypass mode (33), and iii) UmuD' and UmuC inhibit the ε (proofreading) subunit of DNA polymerase III (14). Several experiments have indicated an interaction between UmuD' (15) or UmuC (16) and a RecA-single-stranded DNA complex. Furthermore, interactions between UmuC and UmuD or UmuC and UmuD' have also been noted (38). The different interactions between UmuD/UmuD', UmuC and a RecA-single-stranded DNA complex are consistent with the suggestion that

these proteins are targeted to the lesion. The crystal structure of UmuD', solved by Peat *et al.* (25), revealed that it forms filaments and they suggested that UmuD' may form a scaffold on the RecA-DNA filament that positions UmuC appropriately for interactions with the DNA polymerase III holoenzyme.

Other homologs of UmuD and UmuC have been identified. The best characterized of these is the *mucAB* operon of the plasmid pKM101 that can suppress the nonmutability of *umuD* and *umuC* mutant strains (37). Cloning and sequencing of the *mucAB* operon from pKM101 and the *E. coli umuDC* operon revealed that the UmuD and MucA proteins share homology with the carboxy-terminal domains of the LexA repressor and the repressors of phage λ, 434 and P22 (26, 31).

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 which contains an alanine substituted for its single cysteine (designated CA24) is identical to wild-type UmuD in all the 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, 126) and several of their genetic and biochemical properties were characterized (21). Oxidation with iodine of the purified monocysteine UmuD proteins revealed that derivatives having a single cysteine at positions 24, 34 and 44 crosslinked into the homodimer to a higher extent than the derivatives having cysteines at the other positions tested. Interestingly, mutations in the corresponding homologous region in λ cI (111 to 132) were found to abolish RecA-mediated cleavage but not self-cleavage ((18)), 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 at amino acids 30 to 42. In this paper we report our analyses of the effects of the single cysteine changes on biological activity by assaying UV mutagenesis and the effect 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 crosslinked after oxidation with iodine and copper phenanthroline. Moreover, the ability of the cysteines to spontaneously crosslink in a dimer after dialysis into a buffer lacking DTT was assessed, and the results obtained from the different methods of crosslinking 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 are listed in Table 1. Antibiotics were used at the following concentrations: ampicillin (100 μg/ml); chloramphenicol (30 μg/ml); kanamycin (50 μg/ml); 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 (21). All of the UmuD mutants are under the control of the T7 promoter. *Escherichia coli* strain SG1611 was used for the overproduction of the UmuD derivatives EC35, QC36, DC39, LC40 and 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 (21) except that the buffer of the UmuD-containing fractions eluted from the Mono Q column was not exchanged.

UV mutability and RecA cleavage assays. UV mutagenesis was carried out according to Elledge and Walker (11) using strain GW3200. *In vivo* RecA-mediated cleavage was performed by the following procedure using strain GW8017. 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/ml thiamine and antibiotics was diluted 1:10 into LB containing the appropriate antibiotics. At an A_{600} of 0.4 to 0.6, IPTG was added to a 0.5 mM final concentration. After 1 hour 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 4000 rpm for 10 min, resuspended in an equal volume of LB containing antibiotics and incubated for 45 minutes at 37°C. UmuD cleavage was assessed by centrifuging the cells and resolving the protein from 0.05 A_{600} units of cells by electrophoresis on a 15% SDS-polyacrylamide gel, transferring the protein to polyvinylidene difluoride transfer membrane (Immobilon-P, Bedford, MA) and blotting with a 1:10,000 dilution of affinity-purified antibodies raised against UmuD'. Cross-reacting material was visualized by chemiluminescence (Tropix, Bedford, MA) which was quantitated using the LKB Bromma 2202 Ultroscan Laser densitometer.

In vitro RecA-mediated cleavage was carried out according to Lee *et al.* (21) 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 (dithiothreitol) 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 hour.

Reactivity of mutant UmuD proteins to [^{3}H]iodoacetate and cross-linking of UmuD mutant derivatives with iodine and copper phenanthroline. Reactivity to [^{3}H]iodoacetate was conducted as previously described (21) except that 0.6 mM DTT was present in the reaction mixture. Reactions with iodine and copper phenanthroline were performed as previously described (21) 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 minutes 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 Na-phosphate (pH 6.8), 100 mM NaCl, 0.4 mM DTT was dialyzed against 10 mM Na-phosphate (pH 6.8), 100 mM NaCl, 5 mM EDTA using the System 100 Microdialyzer (Pierce, Rockford, IL) at 4°C for 2 hours. SDS sample buffer was added after dialysis and the proteins were resolved by electrophoresis on a 13% SDS polyacrylamide gel. The Coomassie-stained bands corresponding to the monomeric and dimeric forms of UmuD were quantitated using the LKB Bromma 2202 Ultroscan Laser densitometer.

Results

Activity of the UmuD mutant proteins in UV mutagenesis and RecA-mediated cleavage. In this study, our strategy for choosing the sites for the single substitutions in this set of derivatives differed from that described previously (21). In that case we chose sites which would maximize the probability of obtaining biologically active molecules spanning the entire length of the UmuD protein (21), 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 effect 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 regarding 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* mutation to Arg^+. Most of the UmuD monocysteine derivatives tested retained a substantial ability to participate in mutagenesis (40 to 90% of wildtype activity). The UmuD monocysteine derivatives which were most severely impaired by the cysteine substitution were DC32, YC33, EC35 and LC40 which retained less than 30% of wildtype activity (Fig. 2), suggesting that the residues in these positions are important for some function of UmuD in mutagenesis.

To test the ability of these monocysteine UmuD derivatives to productively interact with RecA in a manner which leads to UmuD cleavage, both *in vivo* and *in vitro* RecA-mediated cleavage of each of the monocysteine derivatives was assessed. To determine the extent of RecA-mediated cleavage *in vivo*, a *ΔumuDC* 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 minute incubation at 37°C, the extent of cleavage was determined by Western blotting using affinity-purified UmuD antisera (5) and was found to be around 75% for wild-type UmuD under these conditions. In addition, the UmuD monocysteine derivatives were purified to homogeneity and their ability to be cleaved by RecA *in vitro* was assessed. We found that wild-type UmuD was cleaved to an extent of around

60% in one hour under the conditions described in the Materials and Methods section.

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 (5). One exception was the AC31 derivative, which displayed a low 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 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*. It is possible that some factor in the *in vivo* reaction that is not present in the *in vitro* reaction allows these derivatives to be cleaved more efficiently *in vivo*. Furthermore, these mutants may be like AC31 in that they are impaired in their ability to perform in some role of 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 hour, where 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 (12). The mutants could be divided into three classes of solvent accessibility. The UmuD derivatives that displayed a high level of reactivity were AC30, AC31, YC33, EC35, DC39, LC40 and QC42; those derivatives which had a moderate level of reactivity were C24 (wildtype UmuD), DC32, VC34, QC36, NC41 and LC44; and the derivatives that reacted poorly with [^{3}H]iodoacetate were RC37 and IC38. Moderate or high reactivity suggests that the thiol group of the UmuD derivative is accessible to solvent, whereas a low reactivity suggests that the thiol group is buried within the interior of the protein or possibly within the dimer interface.

Crosslinking of the UmuD monocysteine derivatives with iodine and 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 become crosslinked by disulfide bond formation. This crosslinking was carried out by the addition of iodine (Figure 3B) or copper phenanthroline (Figure 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 crosslinked 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 concentration of DTT in the buffer of the stocks of the mutant proteins to a 1 mM final concentration for all the UmuD mutant proteins 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) (21). 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 using iodine, both RC37 and IC38 resulted in nearly quantitative crosslinking. 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 sulfhydryls were reasonably exposed to solvent. Even within this small region, the position of the cysteine substitution greatly affects the ability of the UmuD derivatives to be crosslinked 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 hour at 37°C without any oxidizing agent, and no detectable disulfide bonds were formed (data not shown), ruling out the possibility that the observed crosslinking was due to spontaneous disulfide bond formation.

Interestingly, the oxidation reaction using iodine occurs so rapidly that we were unable to follow the kinetics of the crosslinking reaction. Although under our standard reaction conditions, the proteins are exposed to iodine at 22°C for 20 minutes, we found no detectable difference in the amount of disulfide bond formation even when the reaction was carried out at 4°C for 1 minute (data not shown). Furthermore, the amount of disulfide crosslinked dimers cannot be

further increased upon a second addition of iodine. A possible explanation is the following: iodine oxidation of the thiol group of the UmuD derivative, resulting in a sulfenyl iodide intermediate (36), occurs very rapidly. The sulfenyl iodide intermediate is very labile and susceptible to nucleophilic attack. A reaction with another thiol group of the associating UmuD monomer results in the formation of a disulfide bond and the crosslinking of the dimer. This reaction requires a close proximal relationship between the two thiol groups (36). However, hydrolysis of this compound results in the formation of a protein sulfenic acid which is no longer available for crosslinking. Because this reaction proceeds very rapidly (less than one minute), the extent of $UmuD_2$ disulfide crosslinking promoted by iodine probably closely reflects the proportion of cysteines that are in close proximity in the dimer within a small window of time .

As observed previously (21), the copper phenanthroline catalyzed oxidation of the UmuD monocysteine mutant proteins in the homodimer resulted in crosslinking data that is consistent with that obtained from iodine oxidation although the results are less striking. RC37 and IC38 are still the most efficiently crosslinked; however, all of the other mutants were also able to crosslink with moderate efficiency using this reagent. A simple explanation for these observations is that local flexibility in this region allows two sulfhydryls to come close enough for crosslinking during the more prolonged copper phenanthroline catalyzed air oxidation of the thiol groups. No crosslinking was observed when the UmuD derivative lacking a cysteine, CA24, was reacted with iodine or copper phenanthroline (data not shown).

Spontaneous crosslinking of UmuD dimers after removal of DTT by dialysis. Based on the observation that a significant proportion of a subset of UmuD monocysteine derivatives spontaneously disulfide crosslinked in the presence of low concentrations of DTT, we thought it would be interesting to survey the ability of each of the UmuD monocysteine derivatives in this set, in addition to those created previously, to spontaneously crosslink upon removal of DTT by dialysis. The monocysteine derivatives in a buffer containing 0.4 mM DTT was dialyzed for 2 hours at 4°C, and the resulting percent of disulfide crosslinked dimers is plotted in Figure 4. Dialysis of the derivatives with cysteine substitutions within the region between positions 37 and 41 resulted in a high degree of crosslinking. Other derivatives which resulted in efficient dimer crosslinking are C24 (wild type UmuD), AC30, AC31, and LC44. SC19, SC60, SC112 and DC126, and derivatives with cysteine substitutions within the region

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

Discussion

Based on the crosslinking results of several monocysteine derivatives of UmuD, we previously suggested that the region including the Cys24-Gly25 cleavage site, Val34 and Leu44 is closer to the UmuD homodimer interface than the other residues tested (21). Other evidence also suggested this region to be important for UmuD interactions with RecA(5, 18, 22). 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 to 42 inclusively. We then genetically and biochemically characterized all the mutant derivatives. Our results suggest that Arg37 and Ile38 are very close to the dimer interface of UmuD. This inference is based on the ease of disulfide crosslinking of these derivatives upon treatment with iodine and copper phenanthroline and upon removal of reducing agent by dialysis. Nearly quantitative disulfide crosslinking of the RC37 and IC38 derivatives occurred upon oxidation with iodine, in contrast to the crosslinking efficiency of the other derivatives tested which ranged from approximately 10% to 50%. Interestingly, when the ability of each of the mutants 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 to iodoacetate (Figure 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.

Peat *et al.* have solved the crystal structure of the cleaved form of UmuD, UmuD' to 2.5 Å (25). In the crystal structure of UmuD', the amino terminal tail (including amino acids 30 to 42) extends outward in a random coil from a globular head. Peat et al. propose that UmuD' participates in two types of dimer interactions. The first type involves the association of two UmuD' monomers (termed "molecular dimer" interactions). Residues Tyr52, Val54, Ile87, Phe94, and Phe128 from each protomer participate in the hydrophobic interactions of this dimer interface. In addition, a salt bridge is formed between Glu93 and Lys55 of the associating dimer. The amino terminal tails in this dimer protrude out in opposite directions and do not participate in dimer interactions. To support the hypothesis that this is the structure of the dimer found in solution, Peat et al. constructed a UmuD' mutant lacking the amino terminal residues 25 through 45. The resulting mutant retained the ability to dimerize in solution as suggested by gel filtration, native gel electrophoresis, and glutaraldehyde crosslinking (25). In

this model of dimer interactions, residues 30 through 42, in particular, residues 37 and 38, could not be involved in the dimer interactions observed in the UmuD'$_2$ crystal structure.

However, the crystal structure of UmuD' (25) revealed a second type of UmuD' dimer interactions, i.e., dimer of dimer interactions, which are referred to by Peat et al. as "filament" dimer interactions. In these filament dimer interactions, the amino termini (one from each pair of dimers) interact with each other at residues Leu40, Asn41, Leu43, Leu44, and Ile45. Furthermore, amino acids at the carboxy terminal end, particularly, residues 134 through 36, form hydrogen bonds across the interface with residues 134 through 136 of the associating UmuD' (25). The present and previous (21, 22) crosslinking results of the UmuD monocysteine derivatives are more easily rationalized using a model for the intact UmuD homodimer that more closely resembles the "filament dimer" seen in the crystal structure. Based on these results we have proposed that the structure of intact UmuD is actually slightly distorted from the structure of UmuD' observed in the crystal (25). We have suggested that in the homodimer of intact UmuD, the carboxy terminal globular domains are closer together than in UmuD' and that a small region of the amino termini are arranged in parallel to each other (22). Our suggestion that, in intact UmuD, Arg37 and Ile38 are involved in dimer interactions is simple to explain using such a model for the quaternary arrangement of UmuD.

Since Peat et al. observed that the extrapolated cleavage site from the UmuD' crystal structure (Cys24-Gly25) is still remote from the active site cleft in UmuD', they suggest that the UmuD conformation of this region is significantly different from that of the UmuD' filament conformation seen in the crystal structure (25). Their interesting observation that a mutant UmuD' lacking residues 25-45 can crosslink with another mutant UmuD' or with wildtype UmuD' but not with UmuD (25) is consistent with our inference that UmuD and UmuD' have different structural characteristics in this region. These findings also suggest to us that the homodimer interfaces of UmuD$_2$ and UmuD'$_2$ may be 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 dimer interactions but also for interactions with RecA (5, 18, 22)), our strategy involved choosing this particularly interesting region of UmuD to make successive single cysteine substitutions. Consequently, some derivatives have cysteine substitutions at sites which are conserved within the set of analogous

mutagenesis proteins and other derivatives in this set have cysteine substitutions which do not necessarily represent conservative substitutions (Fig. 1). When the ability of the mutants to perform in UV mutagenesis and their ability to be cleaved by RecA both *in vivo* and *in vitro* was assessed (Figure 2), we observed that although many of the monocysteine derivatives retained a significant ability to perform in these capacities, some derivatives were severely impaired (DC32, YC33, EC35, and LC40) suggesting that the residues in this region are important for UmuD and possibly UmuD' interactions with RecA. We found that the VC34 UmuD derivative was also impaired in its ability to perform in these functions (21), consistent with the present results. In addition, we were able to crosslink the VC34 derivative to RecA using the cysteine-specific photoactive crosslinker, *p*-azidoiodoacetanilide (22). In a screen for λ cI mutants with a reduced capacity for RecA-mediated cleavage, Gimble and Sauer (18) found that more than 50% of the mutants they obtained were still able to autodigest. These mutations include EK117, TI122, GD124, DV125, DY125, DN125 and EK127 which correspond to Ala30, Glu35, Arg37, Ile38, and Leu40 of UmuD.

In their analysis of the crystal structure of UmuD', Peat et al. (25) suggested that the extended amino terminal tail (residues 25 to 45) in the UmuD' protein is important in its interactions with RecA. Supporting this interpretation is their observation that a UmuD' mutant lacking residues 25-45 crosslinked to a RecA filament using glutaraldehyde much less efficiently than the wildtype UmuD' protein. If residues 32-35, and 40 are important for the interactions of UmuD' with RecA, this suggests that the cysteine substitutions for residues in this region affect not only the interactions with RecA which lead to the initial cleavage reaction but also may affect interactions with RecA which are important for the subsequent role of UmuD' in mutagenesis. In addition, in the crystal structure of UmuD', Leu40 is involved is various hydrophobic contacts in the filament dimer of UmuD' (25). We infer from our observations that a substitution of a cysteine for leucine at this position may disrupt some of these important interactions.

Although Ala30 and Gln36 are conserved throughout the set of mutagenesis proteins (Fig. 1), we observed that a cysteine substitution for alanine at position 30 or for glutamine at position 36 did not significantly affect the ability of the resulting derivative to participate in UV mutagenesis or RecA-mediated cleavage. A previous screen for UmuD mutants which were defective in UV mutagenesis yielded the mutant AT30 which was subsequently shown to also be defective in RecA-mediated cleavage (5). It is interesting that the subtle differences in the

side chain substitution at this position can significantly affect the activity of the resulting derivative. In contrast, the mutant derivative AC31 has a cysteine substitution at a position which is not conserved within the family of analogous mutagenesis proteins. This derivative, however was more defective in its ability to perform in UV mutagenesis than in its ability to participate in RecA-mediated cleavage. This observation suggests that the mutation more severely affects the function of UmuD' in mutagenesis, perhaps by affecting interactions in the filament dimers of UmuD' (25). DC39, NC41, and QC42 all retain a reasonable ability to be cleaved in the RecA-mediated cleavage reaction. NC41 is the most defective of the three derivatives in participating in UV mutagenesis. In UmuD', the asparagine residue at position 41 contributes to the stability of the UmuD' filament dimer by making a hydrogen bond with the main chain nitrogen of an associating UmuD' protomer (25). Our results suggest that a mutation at this position may affect these interactions.

The ability of the UmuD monocysteine derivatives to crosslink in the dimer was determined by three different means: oxidation with iodine, oxidation with oxygen catalyzed by copper phenanthroline, and spontaneous oxidation with oxygen upon removal of reducing agent by dialysis. Of the three methods for discerning the relative proximity of cysteine residues by disulfide crosslinking, iodine oxidation seemed the most discriminatory. From the iodine crosslinking data, RC37 and IC38 clearly crosslinked the most efficiently of the derivatives that we tested. Oxidation with copper phenanthroline also yielded the same results, however, a cysteine substituted in other positions also formed disulfide bonds in the dimer to a moderate degree. It is possible that this region (residues 30 to 42) is quite flexible - in the cleaved form, UmuD', this amino terminal tail (residues 25 to 36) is thought to be disordered (25). If this is the case, then the oxidation of the derivatives catalyzed by copper phenanthroline could be capturing these transient intermediates when the residues of associating dimers are brought close enough to be disulfide crosslinked (13, 21). 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 for Arg37 and Ile38.

Surprisingly, we found that the spontaneous oxidation of thiols to form disulfide bonds during dialysis was not as discriminatory as the oxidation of thiols using iodine. We expected that since spontaneous air oxidation of thiols appears to be a very mild method of oxidation, this procedure would be highly selective

for only those pairs of cysteines which are optimally positioned in the native structure. Oxidation of monocysteine derivatives by dialysis has also been used in structure/function investigations of the γδ resolvase protein (19) in which the crystal structure of the catalytic subunit is known (29). Inspection of the crystal structure and computer modeling was employed to identify positions for cysteine substitutions which would minimize distortion of the dimer interface in the disulfide crosslinked dimer. The engineered mutants were tested for their ability to crosslink via disulfide bonds during a 12 h dialysis against buffer with air bubbling through it. Two of the mutants crosslinked completely in the dimer and one mutant crosslinked to a level of 50%. These results are consistent with what was expected from computer modeling and suggest that oxidation of disulfide bonds using the mild procedure of dialysis 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 crosslinked quite efficiently during dialysis (Fig. 4). In our experiments, we only allowed dialysis to proceed for 2 h although an additional 2 h of dialysis resulted in a higher yield of crosslinked dimers (data not shown). Moreover, we found that in addition to those derivatives in the first set of monocysteine proteins which were expected to crosslink efficiently (SC19, C24, VC34, and LC44), the UmuD derivatives SC60, SC112, and DC126 also appeared to crosslink relatively more efficiently during dialysis than upon treatment with iodine (determined previously) (21). These results suggest that inferences about the solution structure made from the spontaneous disulfide crosslinking of a single pair of cysteines should be made with caution. Efficient crosslinking in the dimer by this method does not necessarily reflect a close spatial relationship as might be suggested in a crystal structure. Although the dialysis experiments on resolvase are consistent with what is observed in its crystal structure (19), a survey of the crosslinking of derivatives with cysteines in other positions might have yielded misleading results regarding the proximity of certain residues to the dimer interface. We suggest from these results that for the purposes of discriminating the relative proximity of cysteine residues, iodine oxidation is the superior method.

Structural information of UmuD' suggests that the interactions of the UmuD' protomers in the UmuD' filament are more complex than might have been expected. Whether intact UmuD also interacts in a complex like UmuD' is still

unclear. Our results are more easily rationalized using a model for intact UmuD which more closely resembles the "filament dimer" observed in the UmuD' crystal structure (25). Thus an alternative hypothesis is the following: intact UmuD forms a dimer which resemble the "filament dimer" of UmuD' with residues 37 through 41, particularly 37 and 38, participating in the dimer interactions at the amino terminal region. This region has some flexibility, characteristic of a hinge region in a protein structure. During RecA-mediated cleavage, amino acids 32 through 35 interact with RecA as a conformational change in UmuD brings the cleavage site close to the active site cleft of the protein. Upon cleavage, UmuD' forms a dimer using an alternative dimer interface, the "molecular dimer" interface (25). Presumably, this interface is obscured in the intact UmuD form and becomes available once the amino terminal 24 amino acids are cleaved. The amino terminal tails of the active UmuD'$_2$ molecular dimer (which protrude in opposite directions from the globular structure) then interact with the activated RecA filament for the subsequent role of UmuD' in mutagenesis (25).

It will be interesting to evaluate the structure of UmuD with results obtained from this monocysteine approach. Furthermore, analysis of the UmuD' crystal structure using a monocysteine approach will also provide insights regarding the interactions of UmuD', not only in the "molecular dimer" and "filament dimer" but also in interactions 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 in a comparison of the structure and interactions of UmuD and UmuD'.

Acknowledgments

We thank Per Malcus for helping us to generate this set of mutants. We also thank the members of the laboratory for many helpful suggestions. This work was supported by Public Health service grant CA21615 awarded by the National Cancer Institute. A. G. was supported by a fellowship from the Medical Research Council of Canada. M. H. L. was supported by Predoctoral Training Grant 5T32GM07287 from the National Institute of Health.

References

1. **Bailone, A., S. Sommer, J. Knezevic and R. Devoret**. 1991. Substitution of UmuD' for UmuD does not affect SOS mutagenesis. Biochimie **73:**471-478.

2. **Bailone, A., S. Sommer, J. Knezevic, M. Dutreix and R. Devoret**. 1991. A RecA protein deficient in its interaction with the UmuDC complex. Biochimie **73:**479-484.

3. **Bates, H., S. K. Randall, C. Rayssiguier, B. A. Bridges, M. F. Goodman and M. Radman**. 1989. Spontaneous and UV-induced mutations in *Escherichia coli* K-12 strains with altered or absent DNA polymerase I. J. Bacteriol. **171:**2480-2484.

4. **Battista, J. R., T. Nohmi, C. E. Donnelly and G. C. Walker.** 1988. Role of UmuD and UmuC in UV and chemical mutagenesis., 455-459. *In* E. C. Friedberg and P. C. Hanawalt (ed.), Mechanisms and Consequences of DNA Damage Processing. Alan R. Liss, Inc., New York.

5. **Battista, J. R., T. Ohta, T. Nohmi, W. Sun and G. C. Walker**. 1990. Dominant negative *umuD* mutations decreasing RecA-mediated cleavage suggest roles for intact UmuD in modulation of SOS mutagenesis. Proc. Natl. Acad. Sci. USA **87:**7190-7194.

6. **Bridges, B. A. and H. Bates**. 1990. Mutagenic repair in *Escherichia coli.* XVIII. Involvement of DNA polymerase III α-subunit (DnaE) in mutagenesis after exposure to UV light. Mutagenesis **5:**35-38.

7. **Bridges, B. A. and R. P. Mottershead**. 1976. Mutagenic DNA repair in *Escherichia coli.* Molec. Gen. Genet. **144:**53-58.

8. **Bryan, S. K., M. Hagensee and R. E. Moses**. 1990. Holoenzyme DNA polymerase III fixes mutations. Mutat. Res. **243:**313-318.

9. **Burckhardt, S. E., R. Woodgate, R. H. Scheuermann and H. Echols**. 1988. UmuD mutagenesis protein of *Escherichia coli*: Overproduction, purification, and cleavage by RecA. Proc. Natl. Acad. Sci. U.S.A. **85:**1811-1815.

10. **Dutreix, M., P. L. Moreau, A. Bailone, F. Galibert, J. R. Battista, G. C. Walker and R. Devoret**. 1989. New *recA* mutations that dissociate the various RecA protein activities in *Escherichia coli* provide evidence for an additional role for RecA protein in UV mutagenesis. J. Bacteriol. **171:**2415-2423.

11. **Elledge, S. J. and G. C. Walker**. 1983. Proteins required for ultraviolet light and chemical mutagenesis: identification of the products of the *umuC* locus of *Escherichia coli.* J. Mol. Biol. **164:**175-192.

12. **Falke, J. J., A. F. Dernburg, D. A. Sternberg, N. Zalkin, D. L. Milligan and D. E. Koshland, Jr.** 1988. Structure of a bacterial sensory receptor. A site-directed sulfhydryl study. J. Biol. Chem. **263:**14850-14858.

13. **Falke, J. J. and D. E. Koshland, Jr.** 1987. Global flexibility in a sensory receptor: a site-directed cross-linking approach. Science **237:** 1596-1600.

14. **Foster, P. L. and A. D. Sullivan**. 1988. Interactions between epsilon, the proofreading subunit of DNA polymerase III, and proteins involved in the SOS response of *Escherichia coli*. Mol. Gen. Genet. **214:** 467-473.

15. **Frank, E. G., J. Hauser, A. S. Levine and R. Woodgate**. 1993. Targeting of the UmuD, UmuD' and MucA' mutagenesis proteins to DNA by RecA protein. Proc. Natl. Acad. Sci. U.S.A. **90:** 8169-8173.

16. **Freitag, N. and K. McEntee**. 1989. "Activated"-RecA protein affinity chromatography of LexA repressor and other SOS-regulated proteins. Proc. Natl. Acad. Sci. U.S.A. **86:** 8363-8367.

17. **Friedberg, E. C., G. C. Walker and W. Siede.** 1995. DNA repair and mutagenesis. The American Society for Microbiology, Washington, DC.

18. **Gimble, F. S. and R. T. Sauer**. 1986. λ repressor inactivation: properties of purified ind$^-$ proteins in the autodigestion and RecA-mediated cleavage reactions. J. Mol. Biol. **192:** 39-47.

19. **Hughes, R. E., P. A. Rice, T. A. Steitz and N. D. F. Grindley**. 1993. Protein-protein interactions directing resolvase site-specific recombination: a structure-function analysis. EMBO **12:** 1447-1458.

20. **Kato, T. and Y. Shinoura**. 1977. Isolation and characterization of mutants of *Escherichia coli* deficient in induction of mutations by ultraviolet light. Mol. Gen. Genet. **156:** 121-131.

21. **Lee, M. H., T. Ohta and G. C. Walker**. 1994. A monocysteine approach for probing the structure and interactions of the UmuD Protein. J. Bacteriol. **176:** 4825-4837.

22. **Lee, M. H. and G. C. Walker**. 1995. Crosslinking of UmuD to activated RecA using *p*-azidoiodoacetanilide. in preparation.

23. **Little, J. W.** 1984. Autodigestion of LexA and phage λ repressors. Proc. Natl. Acad. Sci. U.S.A. **81:** 1375-1379.

24. **Nohmi, T., J. R. Battista, L. A. Dodson and G. C. Walker**. 1988. RecA-mediated cleavage activates UmuD for mutagenesis: Mechanistic relationship between transcriptional derepression and posttranslational activation. Proc. Natl. Acad. Sci. U.S.A. **85:** 1816-1820.

25. **Peat, T. S., E. G. Frank, J. P. McDonald, A. S. Levine, R. Woodgate and W. A. Hendrickson**. 1995. Structure of the UmuD' protein as related to its role in the response to DNA damage. Nature submitted.

26. **Perry, K. L., S. J. Elledge, B. Mitchell, L. Marsh and G. C. Walker**. 1985. *umuDC* and *mucAB* operons whose products are required for UV light- and chemical-induced mutagenesis: UmuD, MucA, and LexA proteins share homology. Proc. Natl. Acad. Sci. USA **82:** 4331-4335.

27. **Rajagopalan, M., C. Lu, R. Woodgate, M. O'Donnell, M. F. Goodman and H. Echols**. 1992. Activity of the purified mutagenesis proteins UmuC, UmuD', and RecA in replicative bypass of an abasic DNA lesion by DNA polymerase III. Proc. Natl. Acad. Sci. U.S.A. **89:** 10777-10781.

28. **Sambrook, J., E. F. Fritsch and T. Maniatis.** 1989. Molecular cloning-a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

29. **Sanderson, M. R., P. S. Freemont, P. A. Rice, A. Goldman, G. F. Hatfull, N. D. F. Grindley and T. A. Steitz**. 1990. The crystal structure of the catalytic domain of the site-specific recombination enzyme γδ resolvase at 2.7 Å. Cell **63:** 1323-1329.

30. **Sassanfar, M. and J. W. Roberts**. 1990. Nature of the SOS-inducing signal in *Escherichia coli*: the involvement of DNA replication. J. Mol. Biol. **212:** 79-96.

31. **Sauer, R. T., R. R. Yocum, R. F. Doolittle, M. Lewis and C. O. Pabo**. 1982. Homology among DNA-binding proteins suggests use of a conserved super-secondary structure. Nature (London) **298:** 447-451.

32. **Shinagawa, H., H. Iwasaki, T. Kato and A. Nakata**. 1988. RecA protein-dependent cleavage of UmuD protein and SOS mutagenesis. Proc. Natl. Acad. Sci. U.S.A. **85:** 1806-1810.

33. **Sommer, S., A. Bailone and R. Devoret**. 1993. The appearance of the UmuD'C protein complex in *Escherichia coli* switches repair from homologous recombination to SOS mutagenesis. Molec. Microbiology **10:** 963-971.

34. **Steinborn, G.** 1978. *Uvm* mutants of *Escherichia coli* K12 deficient in UV mutagenesis. I. Isolation of *uvm* mutants and their phenotypical characterization in DNA repair and mutagenesis. Mol. Gen. Genet. **165:** 87-93.

35. **Tadmor, Y., R. Ascarelli-Goell, R. Skaliter and Z. Livneh**. 1992. Overproduction of the β subunit of DNA polymerase III Holoenzyme reduces UV mutagenesis in *Escherichia coli.* J. Bacteriol. **174:** 2517-2524.

36. **Trundle, D. and L. W. Cunningham**. 1969. Iodine oxidation of the sulfhydryl groups of creatine kinase. Biochemistry **8:** 1919-1925.

37. **Walker, G. C. and P. P. Dobson**. 1979. Mutagenesis and repair deficiencies of *Escherichia coli umuC* mutants are suppressed by the plasmid pKM101. Mol. Gen. Genet. **172:** 17-24.

38. **Woodgate, R., M. Rajagopalan, C. Lu and H. Echols**. 1989. UmuC mutagenesis protein of *Escherichia coli:* Purification and interaction with UmuD and UmuD'. Proc. Natl. Acad. Sci. USA **86:** 7301-7305.

Table 1: Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or description	Reference or source
Strains		
AB1157	*argE3*	
GW3200	As AB1157, but *umuD44*	(28)
SG1161	JM101 derivative; Δ(*lac-pro*) Δ*gal* Δ*lon510 supE thi*/(F' traD36 *proAB*$^+$ *lacI*^{q} *lacZΔM15*)	(20)
GW8017	As AB1157, but Δ(umuDC)::cam^R	Sumati Murli
GW8400	As SG1161, but *recA*::cam^R	Melissa Lee
Plasmids		
pGW6070	UmuD expressed from T7 promoter	(23)
pGW6100	70TGT to GCC; Cys-24 to Ala; pGW6070 derivative	(23)
pGW7041	103GAA to TGT; Glu-35 to Cys; pGW6070 derivative	this work
pGW7051	106CAG to TGT; Gln-36 to Cys; pGW6070 derivative	this work
pGW7061	109CGC to TGC; Arg-37 to Cys; pGW6070 derivative	this work
pGW7071	112ATC to TGC; Ile-38 to Cys; pGW6070 derivative	this work
pGW7081	115GAT to TGT; Asp-39 to Cys; pGW6070 derivative	this work
pGW7091	118CTG to TGC; Leu-40 to Cys; pGW6070 derivative	this work
pGW7101	121AAT to TGT; Asn-41 to Cys; pGW6070 derivative	this work
pGW7111	124CAA to TGT; Gln-42 to Cys; pGW6070 derivative	this work
pGW7131	88GCA to TGC; Ala-30 to Cys; pGW6070 derivative	this work
pGW7141	91GCA to TGC; Ala-31 to Cys; pGW6070 derivative	this work
pGW7151	94GAT to TGT; Asp-32 to Cys; pGW6070 derivative	this work
pGW7161	97TAC to TGT; Tyr-33 to Cys; pGW6070 derivative	this work

Figure 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 Battista et al. (5). Positions of λ cI (18), LexA (25, 26), UmuD (5, 28) where amino acid substitutions have been shown to yield stable proteins that are defective in RecA-mediated cleavage are indicated by squares. Positions of λ cI where an amino acid substitution has been shown to interfere with dimer formation are indicated by circles (19) . Amino acids that are identical in the three mutagenesis proteins but are not shared with LexA or the three bacteriophage repressors are indicated by bold lettering.

Figure 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 $UmuD^+$ was 74.5 $\pm$ 5.5%. Extent of *in vitro* RecA-mediated cleavage for $UmuD^+$ was 60.5 $\pm$ 3.0%.

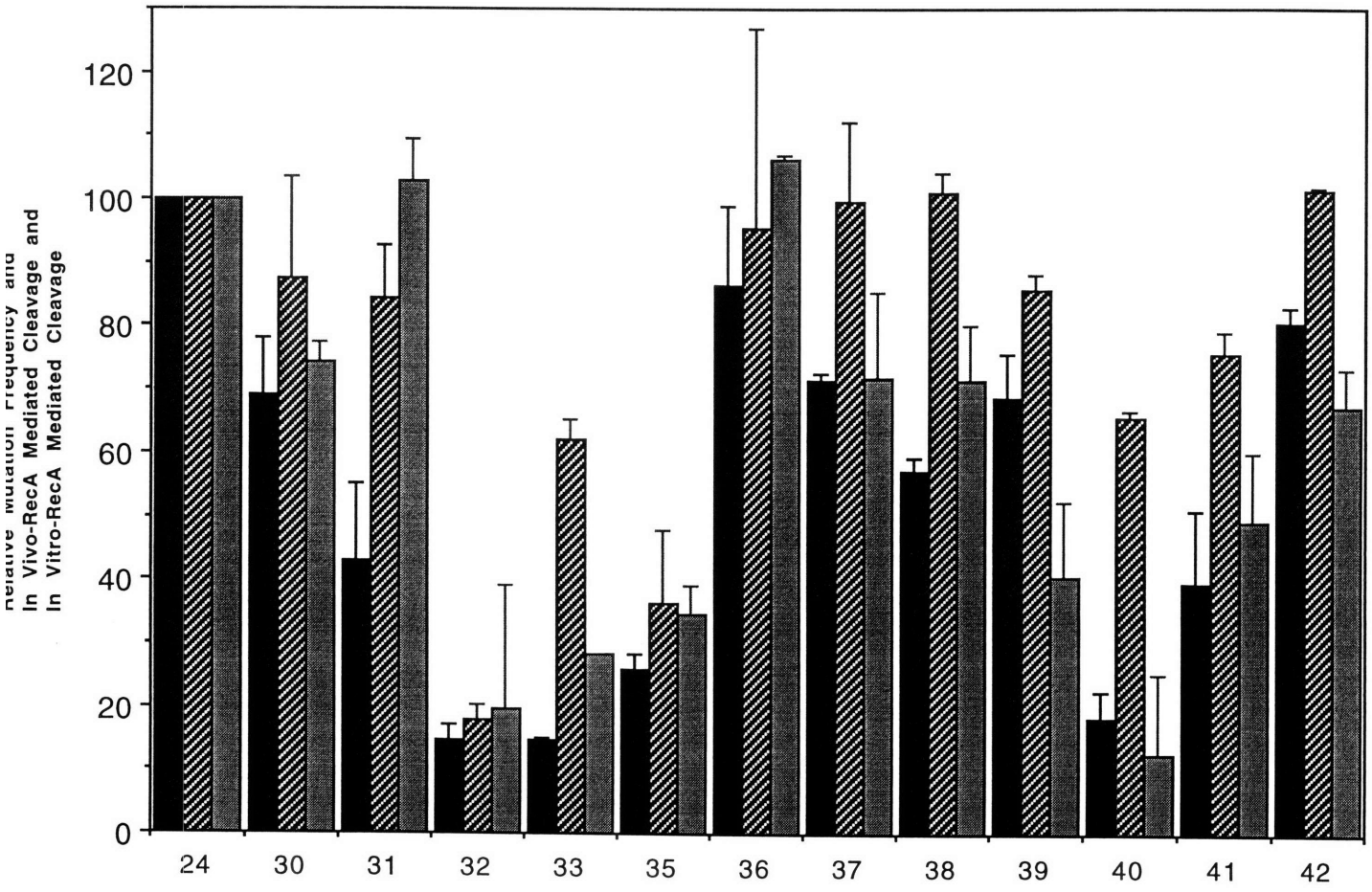
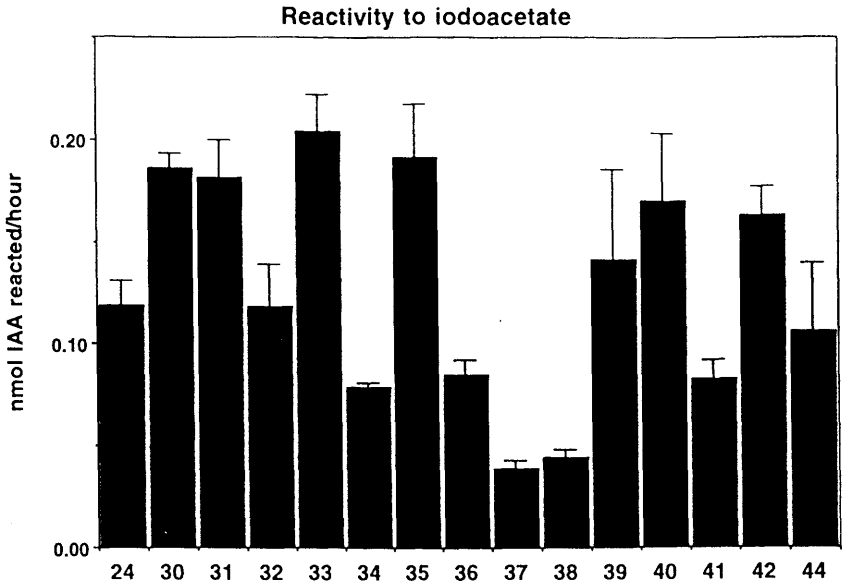
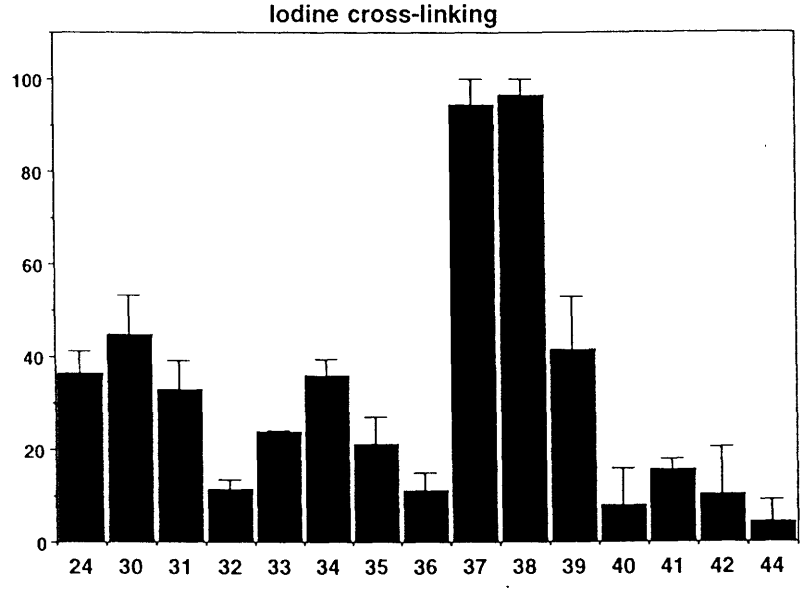

Relative Mutation Frequency and
In Vivo-RecA Mediated Cleavage and
In Vitro-RecA Mediated Cleavage
120
100
80
60
40
20
0
24
30
31
32
33
35
36
37
38
39
40
41
42

Figure 3: Iodoacetate reactivities and crosslinking 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 minutes 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 minutes 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) Percent UmuD crosslinked by using iodine (I_2). UmuD (10 μM) was incubated with 1 mM iodine for 20 minutes at 22°C as described in Materials and Methods. C) Percent UmuD crosslinked 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 minutes at 0°C in 50 mM HEPES (pH 8.1), 100 mM NaCl as described in Materials and Methods.


Reactivity to iodoacetate
nmol IAA reacted/hour
0.20
0.10
0.00
24 30 31 32 33 34 35 36 37 38 39 40 41 42 44


Iodine cross-linking
100
80
60
40
20
0
24 30 31 32 33 34 35 36 37 38 39 40 41 42 44

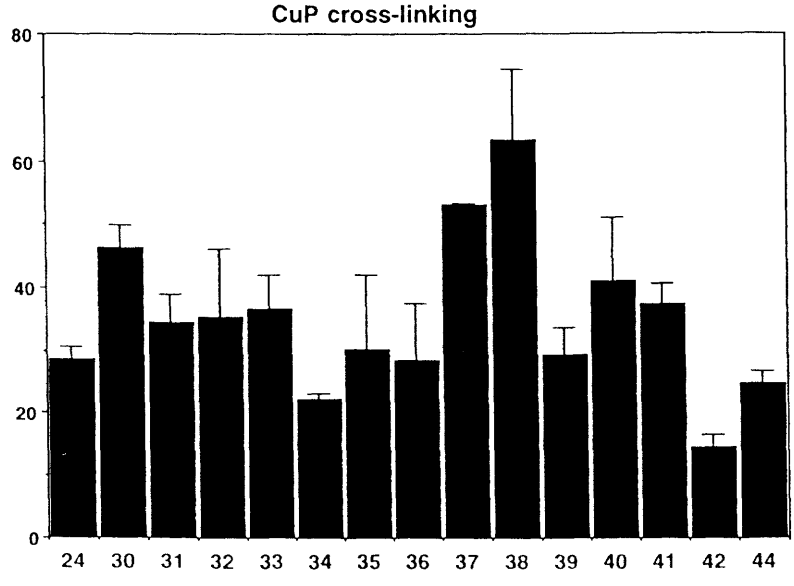
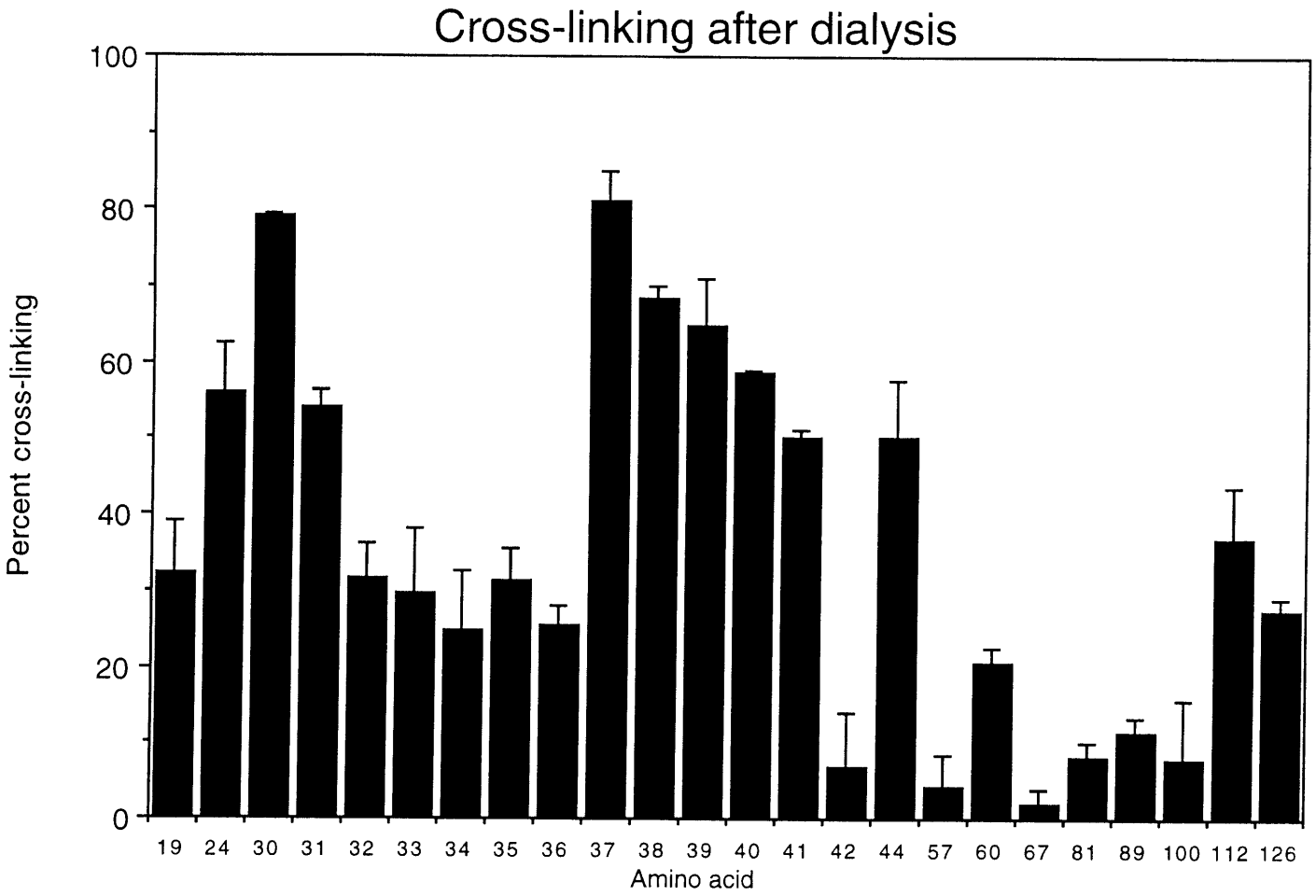

CuP cross-linking
80
60
40
20
0
24 30 31 32 33 34 35 36 37 38 39 40 41 42 44

Figure 4: Crosslinking 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 hours at 4°C against 10 mM Na phosphate (pH 6.8), 100 mM NaCl, 5 mM EDTA as described in Materials and Methods.


Cross-linking after dialysis
Percent cross-linking
100
80
60
40
20
0
19 24 30 31 32 33 34 35 36 37 38 39 40 41 42 44 57 60 67 81 89 100 112 126
Amino acid

Chapter 6

Preferential RecA-mediated cleavage of UmuD monomers

This work was done in collaboration with Angelina Guzzo

Abstract. We have applied the monocysteine approach for probing UmuD protein interactions to a strategy which addresses the question of whether UmuD is more efficiently cleaved as a monomer or a dimer. Selected UmuD derivatives were disulfide crosslinked in dimers by exposure to oxidizing conditions and then incubated with activated RecA to determine whether such crosslinked dimers could undergo RecA-mediated cleavage. To maximize the probability of obtaining disulfide crosslinked UmuD derivatives that were in their native conformations, we chose those derivatives that had cysteine substitutions at positions which were found previously to most efficiently crosslink by disulfide bonds (8, 10) [C24 (wildtype UmuD), VC34, IC38 and LC44]. Generally, we found that the crosslinked $UmuD_2$ derivatives were cleaved very poorly upon incubation with activated RecA compared to a UmuD derivative lacking cysteines that had been treated identically. However, if these disulfide crosslinked derivatives were incubated with DTT prior to incubation with RecA, reducing the disulfide bonds and thereby allowing free conversion between the dimeric and monomeric states, then the resulting extent of cleavage dramatically increased for each derivatives. The level cleavage for each of the derivatives after reduction of the disulfide bonds was comparable to the extent of RecA-mediated cleavage for the respective UmuD derivatives which were not pretreated with either an oxidizing or reducing agent. This result suggests that the monomeric form of UmuD is a better substrate for the RecA-mediated cleavage reaction than the dimeric form.

SOS mutagenesis in *Escherichia coli* occurring after exposure to UV light and various chemicals appears to result from a specialized process of translesion synthesis which requires the participation of the product of three genes: *umuD*, *umuC*, and *recA*. These genes regulated as part of the *recA*$^+$*lexA*$^+$-dependent SOS response (5). and are induced when RecA, activated in the presence of single-stranded DNA generated by the cell's attempt to replicate damaged DNA (21) and a nucleotide cofactor, mediates the proteolytic cleavage of LexA by facilitating an otherwise latent capacity of LexA to autodigest (14, 15). Activated RecA, RecA*, also mediates the post-translational cleavage of UmuD at its Cys24-Gly25 bond by a similar mechanism (2, 23), removing the first 24 amino acids and activating UmuD (designated UmuD') for its role in mutagenesis (16). New mutations in the cell arise when the replicative machinery involving UmuD', UmuC, RecA and DNA polymerase III encounters a non-coding or miscoding lesion, inserts an incorrect nucleotide across from the lesion, and then continues elongation (5).

UmuD shares homology with the carboxyl-terminal regions of LexA, the repressors of the bacteriophages λ, φ80, 434, and P22, and with the UmuD anologs that play roles in mutagenesis such as MucA and ImpA (1, 4, 18, 22). 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 manner similar to that of serine proteases in which a nucleophile, apparently a serine residue conserved in all members of the family, is activated by a lysine residue, also conserved in all members of the family (24).

Both UmuD and UmuD' form homodimers and heterodimers (26), and the interactions of the UmuD·UmuD' heterodimer are more stable than that of either of the homodimers (1). Thus, it is unclear whether the substrate for the RecA-mediated cleavage reaction (which converts UmuD to UmuD') is the monomeric form or dimeric form of UmuD. In the case of λ repressor which shares homology with UmuD, evidence has been presented which suggests that the repressor monomer is the preferred substrate (3, 7, 19). Phizicky and Roberts (19) observed that the rate of RecA-mediated cleavage of λ repressor decreased as the concentration of λ repressor was increased and suggested that λ repressor becomes a less efficient substrate for the cleavage reaction at higher concentrations when it exists mostly as a dimer in solution. In other experiments, characterizations of a hyperinducible λ repressor, λ*ind*^{S} -1, revealed that (i) at

concentrations at which most of wild type λ repressor exists as dimers, a greater proportion of *ind*^{S} -1 remains monomeric; and (ii) unlike wild type λ repressor, *ind*^{S} -1 undergoes RecA-mediated cleavage much more efficiently. These findings are supportive of the suggestion that the repressor monomer is the preferred substrate for the RecA-mediated cleavage reaction (3). In addition, Gimble and Sauer (7) isolated three mutants of λ repressor which were better substrates for RecA-mediated cleavage and displayed a reduced ability to dimerize. They suggested from their results that the hypersensitivity of these mutants to RecA-mediated cleavage is due to the reduced ability of these mutants to dimerize.

We have devised a strategy to address the issue of whether UmuD is more efficiently cleaved as a monomer using the monocysteine approach for investigating protein-protein interactions (10). UmuD has one cysteine in its amino acid sequence located at the Cys24-Gly25 cleavage site. The substitution of this cysteine with an alanine results in a derivative whose function is indistinguishable from wild type (10). This observation has allowed us to construct a family of UmuD proteins differing only in the position of the unique cysteine residue. From our initial characterizations of the UmuD monocysteine derivatives we had made serveral inferences concerning the relative topological arrangement of certain residues of UmuD in relation to the dimer interface (10). The assignments were primarily based on solvent accessibility of the cysteines at these positions as determined by iodoacetate reactivities, and the relative ease of homodimer crosslinking of the monocysteine derivatives by formation of disulfide bonds upon mild oxidation with iodine or by reaction with the cysteine-specific crosslinker, *bis*-maleimidohexane. From these results we suggested that Cys24 (of the Cys24-Gly25 cleavage site), Val34, and Leu44 are relatively closer to the dimer interface than the others tested. In a follow up study characterizing UmuD derivatives with single cysteine substitutions within the region of residues 30 and 42 of UmuD, we found that the UmuD derivatives RC37 and IC38 crosslinked almost quantitatively upon iodine oxidation. Based on these findings we suggested that Arg37 and Ile38 are particularly close to the UmuD interface (8).

Our strategy for determining the preferred UmuD substrate (the dimer or the monomer) for the RecA-mediated cleavage reaction involved disulfide crosslinking of certain UmuD monocysteine derivatives, removal of excess reagents by dialysis, incubation of the disulfide crosslinked dimers with activated RecA, and quantifying the resulting cleavage products. To maximize the

probability of obtaining disulfide crosslinked UmuD derivatives that were in their native conformations, we chose those derivatives that had cysteine substitutions at positions which we had inferred to be closer to the dimer interface than the others tested, i.e., C24 (wild type UmuD), VC34, IC38, and LC44. The results of the present study suggest that UmuD is more efficiently cleaved as a monomer.

Materials and Methods

UmuD monocysteine mutant derivatives were produced and purified as described (8, 10).

Disulfide crosslinking of UmuD derivatives using Cu^{2+}/phenanthroline. The oxidation of disulfide bonds in UmuD with oxygen catalyzed by Cu^{2+}/phenanthroline was conducted by reacting UmuD monocysteine mutant derivatives at a concentration of 38 μM in 50 mM HEPES [pH 8.1]-100 mM NaCl with 0.57 mM Cu^{2+} and 0.74 mM phenanthroline at 22 °C for 40 min (10). The reaction mixtures were then dialyzed against 40 mM TRIS -0.1 mM EDTA and 100 mM NaCl, pH 8.0- to remove excess reagent.

RecA-mediated cleavage of disulfide crosslinked UmuD dimers or reduced disulfide crosslinked dimers. To test whether UmuD is more efficiently cleaved as a monomer or a dimer, disulfide crosslinked dimers of UmuD monocysteine derivatives (prepared as described above) at a 20 μM concentration were incubated with activated RecA at 37 °C for 1 h or were reduced in the presence of 10 mM dithiothreitol (DTT) prior to incubation with activated RecA. RecA, at a concentration of 5.2 μM was activated in the presence of 0.11 mM ATPγS, 5.7 ng/μL $p(dT)_{27}$ (Pharmacia), and 11.3 mM $MgCl_2$. Reactions were quenched and proteins from the reaction mixture were resolved by electrophoresis on a reducing or non-reducing 13% SDS polyacrylamide gel and visualized by Coomassie staining. Control reactions of RecA-mediated cleavage of untreated UmuD were performed as follows: 20 μM UmuD was incubated with 5.2 μM RecA (activated as above) at 37 °C for 1 h. Cleavage products were resolved by electrophoresis on reducing or non-reducing polyacrylamide gels as described above. UmuD cleavage products were quantitated from the Coomassie blue-stained reducing gels using the LKB Bromma 2202 Ultroscan Laser densitometer.

Results

The UmuD monocysteine derivatives at a concentration of 38 μM were incubated at 22 °C for 40 min with Cu^{2+}/phenanthroline to catalyze the air oxidation of the disulfide bonds in the $UmuD_2$ dimer. Of the reagents we had tested (air oxidation catalyzed by copper/phenanthroline, iodine oxidation, and spontaneous air oxidation) (8, 10), Cu^{2+}/phenanthroline appeared to be the best reagent for quantitatively oxidizing the thiols into disulfide bonds. Because oxidation of thiols using iodine leads not only to the formation of disulfide bonds but also to the formation of sulfenic acid (25), this reagent, although useful for discriminating the relative proximity of cysteine residues, is not as suitable as Cu^{2+}/phenanthroline for the purposes of these experiments (8). As shown in Fig. 1, each of the UmuD monocysteine derivatives was able to be disulfide crosslinked almost quantitatively (from 80% to 90%). Control reactions using CA24, the UmuD derivative with no cysteine, were also performed under the same conditions and, as expected, did not crosslink at all. The crosslinked derivatives were then dialyzed to remove the Cu^{2+}/phenanthroline.

To test whether RecA* was able to mediate the cleavage of these disulfide crosslinked UmuD derivatives, an aliquot of each of the disulfide crosslinked dimers at a concentration of 20 μM was incubated with 5.2 μM activated RecA at 37 °C for 1 h. An equivalent aliquot of crosslinked dimers was preincubated with 10 mM DTT to reduce the disulfide bonds prior to incubation with RecA. After the reaction, the protein mixtures were resolved by electrophoresis on a non-reducing (Fig. 2, Panel A) and a reducing (Fig. 2, Panel B) gel and visualized by Coomassie staining. In a control experiment, UmuD derivatives which were not initially treated with any oxidizing or reducing agents were also incubated with activated RecA for 1 hr. As shown in Fig. 2, Panel C, CA24, C24 (wild type UmuD), IC38, and LC44 were cleaved to similar extents (about 90% of the UmuD present in the reaction) and VC34 was cleaved to a lesser extent (around 50%). The crosslinked dimers which were reduced prior to their incubation with RecA were able to undergo cleavage to approximately the same extent in 1 hr as the UmuD derivatives which were not initially treated with any oxidizing or reducing agents indicating that successive oxidation and reduction of these derivatives did not significantly affect their ability to be cleaved in a RecA-mediated fashion (compare Fig. 2, Panel B with Fig. 2, Panel C).

As another control, CA24 (the derivative with no cysteine) was treated identically to the other derivatives. We found that CA24 treated with Cu^{2+}/phenanthroline and then dialyzed, was able to undergo RecA-mediated cleavage in the absence and presence of DTT, however, the amount of cleavage in the absence of DTT was about 35% of the amount in the presence of DTT. Perhaps this is not surprising given that RecA has three cysteines in its amino acid sequence. Nevertheless, this result indicates that the reaction conditions could accommodate RecA-mediated cleavage of UmuD and that the reaction conditions did not significantly perturb the native structure of UmuD (Fig. 2, Panel A and Panel B).

Without prior reduction of the disulfide bonds in the UmuD dimers, the disulfide crosslinked dimers exhibited a significant reduction in their ability to undergo RecA-mediated cleavage (see Fig. 2, Panel A and Panel B). As shown in Fig. 2, Panel A, lanes 2 and 3 and Fig. 2, Panel B, lanes 2 and 3, incubation of crosslinked dimers of C24 (wild type) and VC34, respectively, with activated RecA did not result in the appearance of any detectable UmuD'. However, the appearance of a small amount of UmuD' can be detected for the crosslinked derivatives, IC38 and LC44. Since CA24 was cleaved to about 35% under these conditions, we expected that if crosslinking the dimers had no effect on its ability to be cleaved, these crosslinked dimers would also cleave to 35%. Normalizing the cleavage data to this amount, crosslinked IC38 was cleaved to 28% and LC44 was cleaved to 10% under these conditions. This cleavage may be due to a small amount of cleavage of crosslinked IC38 or LC44. Alternatively, these derivatives could be cleaved as monomers which, as a result of disulfide exchange, become covalently linked in the dimer. About 10% of the crosslinked IC38 preparation was not crosslinked in the dimer and about 20% of the crosslinked LC44 preparation was not crosslinked in the dimer. Nevertheless, the disulfide crosslinked derivatives which were incubated with 10 mM DTT prior to incubation with RecA increased dramatically in their ability to undergo RecA-mediated cleavage, their efficiency of cleavage approaching that of the UmuD monocysteine derivatives in the control reactions which were not treated with either an oxidizing or a reducing agent (Fig. 2, Panel C). The amount of UmuD cleavage products for each of the reactions was quantitated and the results are summarized in Fig. 3. These results suggest that prior reduction of the disulfide bonds is necessary for RecA to efficiently mediate the cleavage of UmuD and that the UmuD derivatives are less efficiently cleaved as a dimer.

Discussion

We have applied the monocysteine approach for probing UmuD protein interactions to a strategy which addresses the question of whether UmuD is more efficiently cleaved as a monomer or a dimer. Selected UmuD derivatives were disulfide crosslinked in dimers by treatment with an oxidizing agent and then incubated with activated RecA to determine whether such dimers could undergo RecA-mediated cleavage. We had hoped that the crosslinking of these selected UmuD monocysteine derivatives would not significantly perturb their native dimeric conformation. Therefore, we chose those derivatives which were found previously to crosslink by disulfide bonds most efficiently (8, 10) [C24 (wild type UmuD), VC34, IC38 and LC44]. IC38, for instance, spontaneously crosslinks in dimers almost quantitatively upon removal of the reducing agent by dialysis, and crosslinks completely in the dimer upon oxidation with iodine (8). The relative ease of disulfide bond formation in the dimer for each of the derivatives suggests (i) that the residues are relatively closer to the dimer interface (8, 10); or (ii) that the positions of the cysteine substitutions are in regions of local flexibility which allow frequent encounters of the two cysteine residues within the dimer. Either possibility suggests that the formation of the disulfide bonds in the dimer does not impose significant strain on the conformation of UmuD so that the basic structure of UmuD which interacts with other proteins, particularly RecA, is still intact. Thus, in simplifying the interpretation of these results, we have assumed that the crosslinked UmuD dimer resembles an untreated UmuD dimer in its conformation.

Generally, we found that the crosslinked $UmuD_2$ derivatives were cleaved very poorly upon incubation with activated RecA. Incubation of crosslinked C24 (wild type UmuD) with activated RecA resulted in no detectable cleavage. A reasonable explanation for this observation is that the crosslinking of the cysteines at the cleavage site stearically hinders the interactions of the cleavage site with the active site. Mutations have been found at the cleavage site of the LexA repressor (12), λ repressor (6), and UmuD(1, 16) which abolish the ability of these proteins to be cleaved in a RecA-mediated fashion. These mutations have been hypothesized to interfere with the interaction of the cleavage site with the active site of the different proteins (13). Peat et al.(17) have solved the crystal structure of the cleaved form of UmuD, UmuD' to 2.5 Å. In the structure, the putative active site residues are located at one end of a cleft region in the globular domain

of the molecule. An amino terminal tail (including residues 25 to 45) extends outward from the globular domain. Based on their observations of structural similarities between this structure and other serine proteases they suggest that the cleavage of the UmuD/LexA/λ cI family of proteins occurs by a similar mechanism. The cleft region is proposed to be the binding site of the amino terminus where the cleavage site is located. Presumably, the cleavage site must be properly located within the active site cleft in order for cleavage to occur. Crosslinking at the cleavage site might preclude these interactions by preventing a necessary conformational change or stearically hindering the residues from occupying the correct positions within the cleft.

The crosslinked VC34 derivative also was not able to be cleaved in a RecA-mediated fashion. VC34 (which has a cysteine substitution within the region corresponding to the region suggested in λ repressor to be involved in RecA-mediated cleavage (1, 6)) (i) is severely defective for RecA-mediated cleavage compared to other monocysteine derivatives (10); and (ii) can be crosslinked to activated RecA using the photoactivatable crosslinker, *p*-azidoiodoacetanilide (11). These observations led to the suggestion that there might be elements close to the $UmuD_2$ homodimer interface which might also be involved in interactions with RecA (10, 11). Val34 appears to be important in both UmuD dimer interactions as well as UmuD interactions with RecA. Thus, crosslinking at this position might affect cleavage by stearically blocking sites of UmuD interactions with RecA, thereby preventing interactions which lead to UmuD cleavage.

Interestingly, incubation of the crosslinked IC38 and LC44 derivatives with activated RecA resulted in a small amount of cleavage (28% and 10% of CA24 treated under identical conditions, respectively). Since a small proportion of these derivatives were not disulfide crosslinked, one possibility is that free UmuD molecules are cleaved in a RecA-mediated fashion and are subsequently crosslinked in a disulfide exchange reaction with a crosslinked UmuD homodimer. An alternative explanation is that the UmuD dimer can be cleaved in a RecA-mediated fashion, although with greatly reduced efficiency. If this is true, it suggests that although the UmuD derivatives IC38 or LC44 are locked in the dimer by the disulfide crosslink, all the essential interactions that lead to RecA-mediated cleavage can still occur.

Peat et al. have proposed that UmuD', the cleaved form of UmuD, forms a filament with two types of interactions: interactions between two UmuD' protomers (which they term the "molecular dimer" interactions) and interactions

between two UmuD'$_2$ dimers (i.e., dimer of dimer interactions, which they term "filament dimer" interactions). We have found it easier to rationalize our crosslinking studies of the intact UmuD dimer by using a model for the intact UmuD dimer that is more closely related to the UmuD' "filament dimer" (8, 11). In the "filament dimer" associations of UmuD', the amino termini (one from each pair of dimers) interact with each other at residues Leu40, Asn41, Leu43, Leu44, and Ile45. Moreover, the active site cleft, which is covered by the dimer interface in the "molecular dimer", is exposed in the "filament dimer". Since, in this quaternary structure of UmuD, much of the active site is still exposed, it is possible for the amino terminal region of UmuD to find either its own active site cleft, or the active site of a different UmuD molecule (reminiscent of the cleavage of LexA *in trans* (9)) and be cleaved in a RecA-mediated fashion. Such a reaction is not expected to be very efficient but might account for the small amount of cleavage observed. The RecA-mediated cleavage of crosslinked LC44 derivative is probably mechanistically similar to that of the IC38 derivative. The difference in efficiency of cleavage most likely reflects the positional effect of the cysteine substitution and of the disulfide crosslink.

Incubation of the disulfide crosslinked derivatives with DTT prior to incubation with RecA, reducing the disulfide bonds and thereby allowing free conversion between the dimeric and monomeric states, resulted in a dramatic increase in the extent of cleavage for each of the derivatives. The level of cleavage for each of the derivatives after reduction of the disulfide bonds was comparable to the extent of RecA-mediated cleavage for the respective UmuD derivatives which were not pretreated with either an oxidizing or reducing agent. This result suggests that the monomeric form of UmuD is a better substrate for the RecA-mediated cleavage reaction than the dimeric form. From their analysis of mutant LexA proteins with an increased rate of cleavage, Roland, et al. (20) proposed a model for the cleavage of LexA which involves a conformational change that creates a local environment around the cleavage site and active site that is favorable for cleavage. They proposed that RecA preferentially binds to LexA proteins that are in the conformation that is favorable for cleavage. It is likely that the cleavage of LexA and UmuD are mechanistically similar. If the cleavage of these proteins does require a conformational change, such changes could be hindered by the dimerization of UmuD. Our present results suggest that the monomeric form of UmuD can more freely interconvert between the different

conformational states and therefore has a higher probability of interacting favorably with RecA than the dimeric form.

Acknowledgments

We thank the members of the laboratory for many helpful suggestions. This work was supported by Public Health service grant CA21615 awarded by the National Cancer Institute. M. H. L. was supported by Predoctoral Training Grant 5T32GM07287 from the National Institute of Health. A. G. was supported by a fellowship from the Medical Council of Canada.

References

1. **Battista, J. R., T. Ohta, T. Nohmi, W. Sun and G. C. Walker**. 1990. Dominant negative *umuD* mutations decreasing RecA-mediated cleavage suggest roles for intact UmuD in modulation of SOS mutagenesis. Proc. Natl. Acad. Sci. USA **87:**7190-7194.

2. **Burckhardt, S. E., R. Woodgate, R. H. Scheuermann and H. Echols**. 1988. UmuD mutagenesis protein of *Escherichia coli*: Overproduction, purification, and cleavage by RecA. Proc. Natl. Acad. Sci. U.S.A. **85:**1811-1815.

3. **Cohen, S., B. J. Knoll, J. W. Little and D. W. Mount**. 1981. Preferential cleavage of phage λ repressor monomers by *recA* protease. Nature (London) **294:**182-184.

4. **Eguchi, Y., O. T. and O. H.** 1988. Cleavage of bacteriophage ϕ80 *c*I repressor by RecA protein. J. Mol. Biol. **202:**565-573.

5. **Friedberg, E. C., G. C. Walker and W. Siede.** 1995. DNA repair and mutagenesis. The American Society for Microbiology, Washington, DC.

6. **Gimble, F. S. and R. T. Sauer**. 1986. λ repressor inactivation: properties of purified ind$^-$ proteins in the autodigestion and RecA-mediated cleavage reactions. J. Mol. Biol. **192:**39-47.

7. **Gimble, F. S. and R. T. Sauer**. 1989. Lambda repressor mutants that are better substrates for RecA-mediated cleavage. J. Mol. Biol. **206:**29-39.

8. **Guzzo, A., M. H. Lee, K. Oda and G. C. Walker**. 1995. Analysis of the region between amino acids 30 to 42 of intact UmuD by a monocysteine approach. in preparation.

9. **Kim, B. and J. W. Little**. 1993. LexA and λ cI repressors as enzymes: specific cleavage in an intermolecular reaction. Cell **73:**1165-1173.

10. **Lee, M. H., T. Ohta and G. C. Walker**. 1994. A monocysteine approach for probing the structure and interactions of the UmuD Protein. J. Bacteriol. **176:**4825-4837.

11. **Lee, M. H. and G. C. Walker**. 1995. Crosslinking of UmuD to activated RecA using *p*-azidoiodoacetanilide. in preparation.

12. **Lin, L. L. and J. W. Little**. 1988. Isolation and characterization of noncleavable (Ind$^-$) mutants of the LexA repressor of *Escherichia coli* K-12. J. Bacteriol. **170:**2163-2173.

13. **Lin, L. L. and J. W. Little**. 1989. Autodigestion and RecA-dependent cleavage of Ind$^-$ mutant LexA proteins. J. Mol. Biol. **210:**439-452.

14. **Little, J. W.** 1984. Autodigestion of LexA and phage λ repressors. Proc. Natl. Acad. Sci. U.S.A. **81:** 1375-1379.

15. **Little, J. W., S. H. Edmiston, Z. Pacelli and D. W. Mount**. 1980. Cleavage of the *Escherichia coli* LexA protein by the RecA protease. Proc. Natl. Acad. Sci. U.S.A. **77:** 3225-3229.

16. **Nohmi, T., J. R. Battista, L. A. Dodson and G. C. Walker**. 1988. RecA-mediated cleavage activates UmuD for mutagenesis: Mechanistic relationship between transcriptional derepression and posttranslational activation. Proc. Natl. Acad. Sci. U.S.A. **85:** 1816-1820.

17. **Peat, T. S., E. G. Frank, J. P. McDonald, A. S. Levine, R. Woodgate and W. A. Hendrickson**. 1995. Structure of the UmuD' protein as related to its role in the response to DNA damage. Nature submitted.

18. **Perry, K. L., S. J. Elledge, B. Mitchell, L. Marsh and G. C. Walker**. 1985. *umuDC* and *mucAB* operons whose products are required for UV light- and chemical-induced mutagenesis: UmuD, MucA, and LexA proteins share homology. Proc. Natl. Acad. Sci. USA **82:** 4331-4335.

19. **Phizicky, E. M. and J. W. Roberts**. 1980. Kinetics of RecA protein-directed inactivation of repressors of phage lambda and phage P22. J. Mol. Biol. **139:** 319-328.

20. **Roland, K. L., M. H. Smith, J. A. Rupley and J. W. Little**. 1992. *In vitro* analysis of mutant LexA proteins with an increased rate of specific cleavage. J. Mol. Biol. **228:** 395-408.

21. **Sassanfar, M. and J. W. Roberts**. 1990. Nature of the SOS-inducing signal in *Escherichia coli*: the involvement of DNA replication. J. Mol. Biol. **212:** 79-96.

22. **Sauer, R. T., R. R. Yocum, R. F. Doolittle, M. Lewis and C. O. Pabo**. 1982. Homology among DNA-binding proteins suggests use of a conserved super-secondary structure. Nature (London) **298:** 447-451.

23. **Shinagawa, H., H. Iwasaki, T. Kato and A. Nakata**. 1988. RecA protein-dependent cleavage of UmuD protein and SOS mutagenesis. Proc. Natl. Acad. Sci. U.S.A. **85:** 1806-1810.

24. **Slilaty, S. N. and J. W. Little**. 1987. Lysine-156 and serine-119 are required for LexA repressor cleavage: a possible mechanism. Proc. Natl. Acad. Sci. U.S.A. **84:** 3987-3991.

25. **Trundle, D. and L. W. Cunningham**. 1969. Iodine oxidation of the sulfhydryl groups of creatine kinase. Biochemistry **8:** 1919-1925.

26. **Woodgate, R., M. Rajagopalan, C. Lu and H. Echols**. 1989. UmuC mutagenesis protein of *Escherichia coli:* Purification and interaction with UmuD and UmuD'. Proc. Natl. Acad. Sci. USA **86:** 7301-7305.

Fig. 1. Disulfide crosslinking of UmuD derivatives. UmuD monocysteine mutant derivatives at a concentration of 38 μM in 50 mM HEPES [pH 8.1]-100 mM NaCl was incubated with 0.57 mM Cu^{2+} and 0.74 mM phenanthroline at 22 °C for 40 min (10). The reaction mixtures were then dialyzed against 40 mM TRIS -0.1 mM EDTA and 100 mM NaCl, pH 8.0- to remove excess reagent.

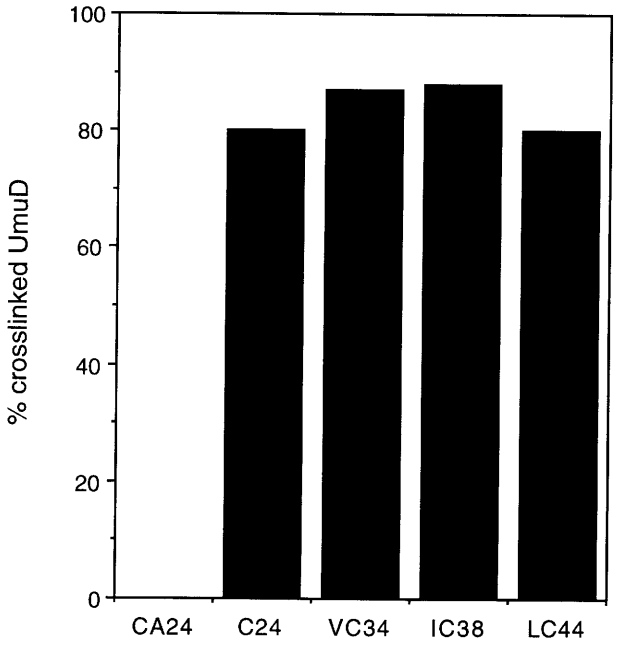

% crosslinked UmuD
100
80
60
40
20
0
CA24
C24
VC34
IC38
LC44

Fig. 2. RecA-mediated cleavage of crosslinked UmuD dimers. Disulfide crosslinked dimers of UmuD monocysteine derivatives at a 20 μM concentration were incubated with 5.2 μM activated RecA at 37 °C for 1 h or were reduced in the presence of 10 mM dithiothreitol (DTT) prior to incubation with activated RecA. Reactions were quenched and proteins from the reaction mixture were resolved by electrophoresis on a non-reducing (Panel A) or reducing (Panel B) SDS polyacrylamide gel and visualized by Coomassie staining. Control reactions of RecA-mediated cleavage of untreated UmuD were performed by incubating 20 μM UmuD with 5.2 μM activated RecA at 37 °C for 1 h. Cleavage products were resolved by electrophoresis on a reducing polyacrylamide gel (Panel C).

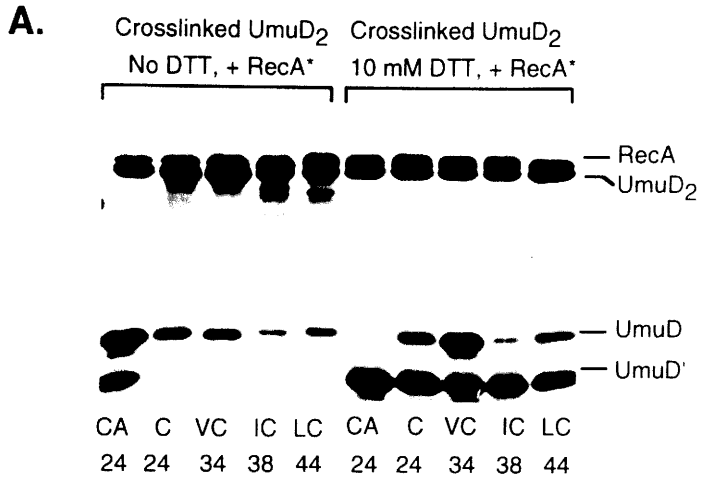
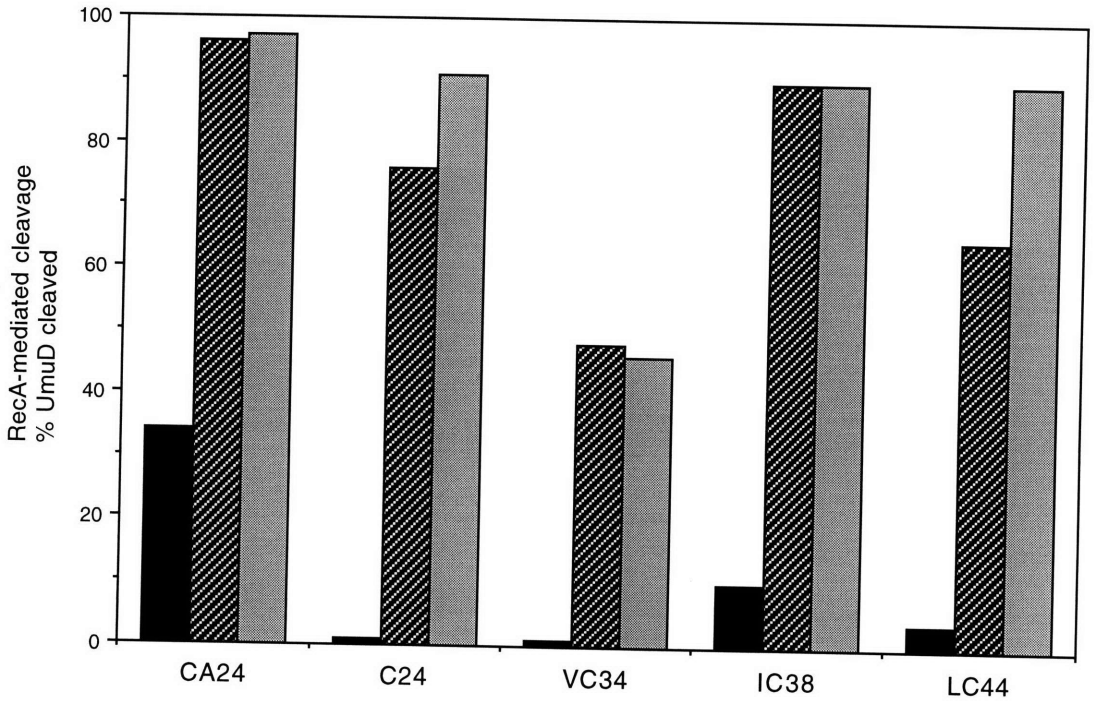

A.
Crosslinked UmuD2
No DTT, + RecA*
Crosslinked UmuD2
10 mM DTT, + RecA*
RecA
UmuD2
UmuD
UmuD'
CA C VC IC LC CA C VC IC LC
24 24 34 38 44 24 24 34 38 44
B.
Crosslinked UmuD2
No DTT, + RecA*
Crosslinked UmuD2
10 mM DTT, + RecA*
RecA
UmuD
UmuD'
CA C VC IC LC CA C VC IC LC
24 24 34 38 44 24 24 34 38 44
C.
Untreated UmuD
No DTT, + RecA*
RecA
UmuD
UmuD'
CA C VC IC LC
24 24 34 38 44

Fig. 3. Quantitation of UmuD RecA-mediated cleavage products. Cleavage products from each of the reactions were quantitated from the reducing polyacrylamide gel using a densitometer. Solid bars: RecA-mediated cleavage of crosslinked UmuD derivatives; hatched bars: RecA-mediated cleavage of crosslinked UmuD derivatives which were reduced with DTT prior to incubation with activated RecA; Dotted bars: RecA-mediated cleavage of UmuD derivatives which were not treated with either an oxidizing agent or reducing agent prior to incubation with activated RecA.


RecA-mediated cleavage
% UmuD cleaved
100
80
60
40
20
0
CA24
C24
VC34
IC38
LC44

Conclusions

In this thesis, we have described the utilization of the monocysteine approach to investigate different interactions of the UmuD protein. 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) represented conservative substitutions. Although this strategy was largely successful, certain of the monocysteine derivatives did have biological or biochemical characteristics that shed additional light on the functional elements of UmuD. We have also 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 cysteine. We have used simple interpretations of the results to make inferences regarding the three-dimensional structure of the UmuD protein and its interactions within the homodimer and with activated RecA. The assumptions we have made in interpreting our data are (i) that the UmuD derivatives are in conformations similar to the wild type UmuD; (ii) that the reactivity of the sulfhydryl group with iodoacetate and the cysteine-specific photoactive crosslinker, *p*-azidoiodoacetanilide (AIA), is predominantly influenced by its accessibility (except for the case of SC60 , the putative nucleophile, which might be more reactive because of its local environment); (iii) that the crosslinking results primarily reflect inter-residue distances in proteins with the same conformation. In interpreting the results using the cysteine-specific photoactive crosslinker, *p*-azidoiodoacetanilide (AIA), we also have taken into account the following factors: (i) because the half-life of the activated AIA-derived crosslinker is rather short (only 0.1 to 5 μsec, crosslinking is highly dependent on the distance of the reactive radical of the activated crosslinker to the adjacent residue, and (ii) crosslinking is dependent on the chemical nature of the residue with which it is to react (i.e., this residue must be nucleophilic).

Interactions of UmuD in the homodimer. All of the monocysteine derivatives were tested for solvent accessibility using [^{3}H]iodoacetate and [^{14}C]AIA. We found most derivatives reacted with either reagent to an extent of 40% to 80% indicating that the sulfhydryls in these positions were quite exposed to solvent. The exceptions were AC89, QC100, and DC126 which had relatively

low reactivities suggesting that the residues in these positions are most likely buried within the interior of the protein or at least minimally exposed to the exterior environment. We observed small differences in relative reactivities for some derivatives between these two reagents, particularly SC19 and C24 (wildtype UmuD), which were less reactive with AIA than with iodoacetate. We suggested that these small differences are due to the small influences of neighboring residues in interacting with the two different alkylating agents.

The relative closeness of certain residues to the dimer interface was investigated using various cysteine-specific reagents: the zero-length crosslinkers (which promote disulfide bond formation), iodine and oxygen in a reaction catalyzed by copper/phenanthroline or in a spontaneous oxidation reaction in the absence of reducing agent; a 13 Å homobifunctional crosslinker, *bis*-maleimidohexane; and a 9 Å heterobifunctional photoactive crosslinker, *p*-azidoiodoacetanilide. The observation that the ability of the monocysteine UmuD derivatives to be crosslinked differed from their ability to be modified by the two different alkylating agents suggests that the susceptibility of the derivatives to be crosslinked depends on the differences in the positions of the sulfhydryl groups rather than on the accessibility of the derivatives to reagents in solution.

On the basis of these crosslinking results we have made several inferences concerning the relative topological arrangement of certain residues of UmuD in relation to the dimer interface. We suggested that C24 (of the Cys24-Gly25 cleavage site), Val34, and Leu44 are closer to the homodimer interface than the other residues tested based on the results of efficient crosslinking using each of the different crosslinkers. In contrast, the observation of poor crosslinking using the various reagents led us to suggest that Ser60, the site of the putative nucleophile in the cleavage reaction, is not as close to the dimer interface or is located in a cleft region. Poor disulfide crosslinking using iodine or upon removal of the reducing agent, but efficient crosslinking with BMH suggests that Ser19 (located in the N-terminal fragment of UmuD that is removed by RecA-mediated cleavage), Ser67, and Ser112 are further from the dimer interface, but that the pairs of serines in the $UmuD_2$ homodimer at positions 19, 67 and 112 are less than 13 Å apart. Interestingly, of these three derivatives, SC19 crosslinked the most efficiently with the photoactive agent AIA and SC67 crosslinked the least efficiently suggesting that Ser19, but not Ser67, is within 9 Å of the UmuD dimer interface. The serine to cysteine substitution at position 67 appeared to affect the UV mutagenesis phenotype much more dramatically than it appeared to affect

RecA-mediated cleavage suggesting that this position is important for the subsequent role of UmuD' in mutagenesis. The UmuD derivative, SC57 did not readily form disulfide bonds within the homodimer upon oxidation with iodine or copper phenanthroline or upon removal of the reducing agent. However, the observation that SC57 also crosslinked efficiently with AIA suggests that Ser57 is relatively close to the dimer interface. Because crosslinking using AIA does not require that there be a cysteine residue in the other interacting protein, this strategy of analyzing interactions might be a better predictor of the relative closeness of adjacent residues than the use of homobifunctional reagents. Thus Ser57 might be fairly close to the dimer interface but not necessarily close to position 57 of the adjacent UmuD monomer. The observation that SC57 does not crosslink well with BMH is consistent with this inference. SC81 did not crosslink efficiently upon oxidation with iodine or upon removal of the reducing agent by dialysis and also did not crosslink efficiently using BMH or the photoactive crosslinker, AIA. These observations suggest that SC81 is farther from the $UmuD_2$ homodimer interface than the other residues tested. Finally, the UmuD derivatives, AC89, QC100 and DC126, did not react well with any of the crosslinking reagents, consistent with the suggestion that the cysteines at these positions are not as accessible to solvent.

Because the region of UmuD including residues at position 24 to position 42 appeared to be important for both UmuD homodimer interactions and interactions with RecA, we felt that a closer study of this region using the monocysteine approach would yield interesting insights regarding these interactions. A set of monocysteine derivatives was constructed in which each derivative had a single cysteine substitution for a residue in this region. As with the first set of monocysteine derivatives, we assessed the ability of these UmuD derivatives to perform in UV mutagenesis and in vivo and in vitro RecA mediated cleavage. We found monocysteine mutants with substitutions at positions 32, 33, 34, and 35 to be most severely affected by the cysteine substitution indicating that the residues in this region are important for UV mutagenesis and RecA-mediated cleavage. In addition we found LC40 to be deficient in UV mutagenesis and RecA-mediated cleavage suggesting that a cysteine substitution for a leucine at this position, conserved among the mutagenesis proteins, is also important for UmuD function in UV mutagenesis and RecA-mediated cleavage. The observation that purified proteins of the UmuD derivatives RC37 and IC38 could be disulfide crosslinked quantitatively upon addition of iodine and yet be poorly

modified with iodoacetate led us to suggest that the pairs of residues at 37 and 38 are very close in the UmuD homodimer and in fact may be buried within the homodimer interface.

Crystal Structure of UmuD'

For the most part, the inferences drawn from the experiments described in this thesis were made in the absence of a UmuD crystal structure. During the writing of this thesis, the crystal structure of the cleaved form of UmuD, UmuD', was solved to 2.5 Å (1). As discussed in previous chapters, the amino terminal tail in the UmuD' crystal structure (including amino acids 30 to 42) extends outward in a random coil from a globular head. Peat et al. propose that UmuD' participates in two types of dimer interactions. The first type involves the association of two UmuD' monomers (termed "molecular dimer" interactions) (Please see Fig. 1). Residues Tyr52, Val54, Ile87, Phe94, and Phe128 from each protomer participate in the hydrophobic interactions of this dimer interface. In addition, a salt bridge is formed between Glu93 and Lys55 of the associating dimer. The amino terminal tails in this dimer protrude in opposite directions and do not participate in dimer interactions. To support the hypothesis that this is the structure of the dimer found in solution, Peat et al. constructed a UmuD' mutant lacking the amino terminal residues 25 through 45. The resulting mutant retained the ability to dimerize in solution as suggested by gel filtration, native gel electrophoresis, and glutaraldehyde crosslinking (1).

The crystal structure of UmuD' (1) revealed a second type of UmuD' dimer interactions, i.e., dimer of dimer interactions, which are referred to by Peat et al. as "filament" dimer interactions (Please see Fig. 2). In these filament dimer interactions, the amino termini (one from each pair of dimers) interact with each other at residues Leu40, Asn41, Leu43, Leu44, and Ile45. Furthermore, amino acids at the carboxy terminal end, particularly, residues 134 through 36, form hydrogen bonds across the interface with residues 134 through 136 of the associating UmuD' (1).

The observation that the amino termini of UmuD' interact with each other is reminiscent of our findings that the LC44 derivative of the intact $UmuD_2$ homodimer can be crosslinked. In fact, it is easier to rationalize all our observations using monocysteine UmuD derivatives by a model for the UmuD interface that is more closely related to the UmuD' "filament dimer" interface than to the UmuD' "molecular dimer" interface. In such a model for the quaternary

structure of UmuD, the amino terminal region (including Val34 and Leu44) would be close to or would compose part of the dimer interface. In addition, pairs of Ser67 and Ser112 residues in the UmuD dimer would not be located on opposite sides of the dimer (as in the molecular dimer), but instead would be located on the "inner" surfaces of the dimer perhaps closer together than in the filament dimer in the UmuD' crystal. It would not be surprising to find the structure of intact UmuD to be slightly distorted from what is seen in the crystal structure for the UmuD' filament dimer. Our observations of poor disulfide crosslinking but efficient crosslinking using *bis*-maleimidohexane in the UmuD dimer at positions 67 and 112 would be simple to explain by hypothesizing that the carboxy terminal globular heads are actually closer in the intact UmuD dimer than they are in the UmuD' filament dimer. In addition, a parallel arrangement of the amino terminal tails in the region including residues 34 to 44 might account for the efficient crosslinking of the VC34, RC37, IC38 and LC44 derivatives with various reagents as well as for the efficient spontaneous oxidation of disulfide bonds in the dimer upon dialysis for the derivatives with single cysteine substitutions within this region.

If our hypothesis for the intact UmuD dimer interface is correct, it suggests another level of subtlety in the modulation of UmuD activity. Intact UmuD forms dimers which resemble the UmuD' filament dimers. Presumably, the residues that are involved in the UmuD'$_2$ "molecular dimer" interactions are not available in intact UmuD. Perhaps these residues are buried in intact UmuD or else obscured by the presence of the amino terminal 24 amino acids. Upon RecA-mediated cleavage of the first 24 amino acids, the residues at the "molecular dimer" interface become available for interaction. Consequently, the UmuD'$_2$ homodimer adopts a different quaternary conformation than the intact UmuD$_2$ homodimer using this alternative interface and forming the active species which is observed in the crystal structure as "molecular dimers". This hypothesis has been discussed at greater length in the previous chapters.

Interactions of UmuD with RecA. We have also extended our study of UmuD monocysteine derivatives to explore interactions with RecA. Using the photoactivatable crosslinker, *p*-azidoiodoacetanilide (AIA), we identified regions of UmuD which probably interact with RecA. With respect to overall efficiency of crosslinking, VC34 and SC81 seemed to crosslink the most efficiently suggesting that positions 34 and 81 are closer to the UmuD-RecA interface than

the other residues tested. The other derivatives which crosslinked to RecA with moderate efficiency are SC57, SC67, and SC112. Interestingly, neither the wildtype protein, with a cysteine located at the cleavage site, nor SC60, the derivative with a cysteine substitution at the position of the putative nucleophile crosslinked significantly to RecA using AIA. This suggests that RecA needs not directly interact with the residues involved in the RecA-mediated cleavage reaction, consistent with the role of RecA being a coprotease in facilitating the autodigestion reactions of these repressors and mutagenesis proteins. SC19 and LC44 also did not significantly crosslink with RecA. From these results we are beginning to make inferences regarding the residues which are in the regions of UmuD homodimer interactions and UmuD-RecA interactions. The relationship of these interactions to the crystal structure of UmuD' have been discussed in detail in the previous chapters.

To further investigate RecA interactions with UmuD, we utilized the monocysteine approach in a strategy which addresses the question of whether UmuD is more efficiently cleaved as a monomer or a dimer. Selected UmuD derivatives were disulfide crosslinked in dimers by treatment with an oxidizing agent and then incubated with activated RecA to determine whether such crosslinked dimers could undergo RecA-mediated cleavage. To maximize the probability of obtaining disulfide crosslinked UmuD derivatives that were in their native conformations, we chose those derivatives that had cysteine substitutions at positions which were found previously to most efficiently crosslink by disulfide bonds [C24 (wildtype UmuD), VC34, IC38 and LC44]. For the most part, the crosslinked $UmuD_2$ derivatives were cleaved very poorly upon incubation with activated RecA. However, if these disulfide crosslinked derivatives were incubated with DTT prior to incubation with RecA, reducing the disulfide bonds and thereby allowing free conversion between the dimeric and monomeric states, then the resulting extent of cleavage dramatically increased for each derivatives. The level cleavage for each of the derivatives after reduction of the disulfide bonds was comparable to the extent of RecA-mediated cleavage for the respective UmuD derivatives which were not pretreated with either an oxidizing or reducing agent. This result suggests that the monomeric form of UmuD is a better substrate for the RecA-mediated cleavage reaction than the dimeric form.

Future directions. UmuD undergoes 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). These interactions may be similar to or different from the interactions of the cleaved and active form of UmuD, UmuD'. Use of the monocysteine approach for the investigations of the structure and interactions of UmuD has yielded insights into a subset of these interactions. Further elucidation of the properties of UmuD and UmuD' using a monocysteine approach to probe the interactions of UmuD and UmuD' with other proteins involved in SOS mutagenesis should shed additional light on the possible mechanistic roles of UmuD and UmuD' in mutagenesis.

Reference

1. **Peat, T. S., E. G. Frank, J. P. McDonald, A. S. Levine, R. Woodgate and W. A. Hendrickson**. 1995. Structure of the UmuD' protein as related to its role in the response to DNA damage. Nature Submitted.

Figure 1. Crystal structure of the cleaved form of UmuD, UmuD' at 2.5 Å, view of the molecular dimer interactions of UmuD' as a worm representation. The blue region of the structure includes residues 32 through 45. A mutant with a deletion of this region retains the ability to dimerize in solution as suggested by gel filtration, native gel electrophoresis, and glutaraldehyde crosslinking (1). About 1100 $Å^2$ of accessible surface area is buried when two protomers form a molecular dimer. Hydrophobic residues are in green, acidic residues are in red, basic residues are in blue, and all other residues are in yellow. UmuD' monomer A is in orange and UmuD' monomer B is in green. This figure was reproduced from ref. (1). Used by permission.

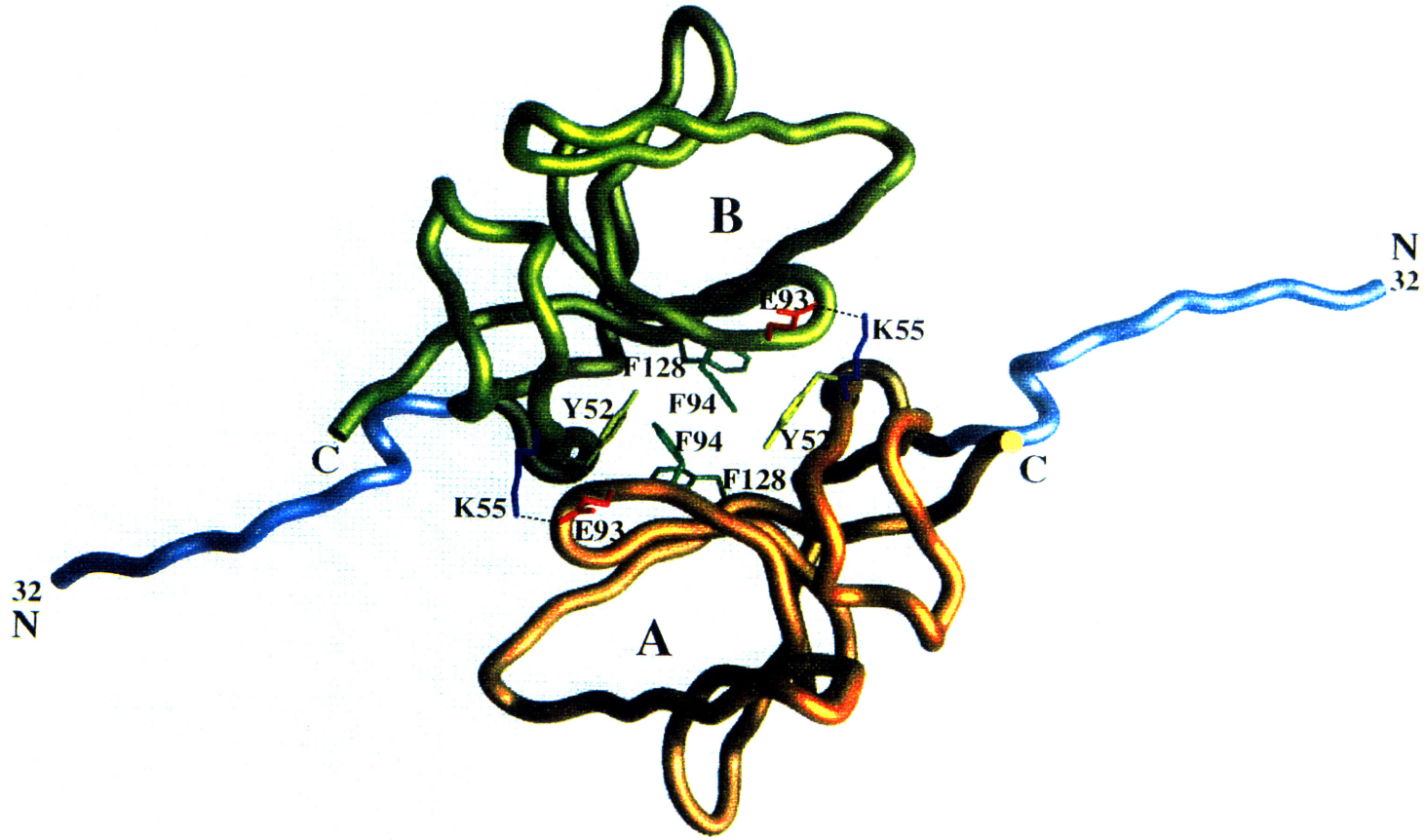
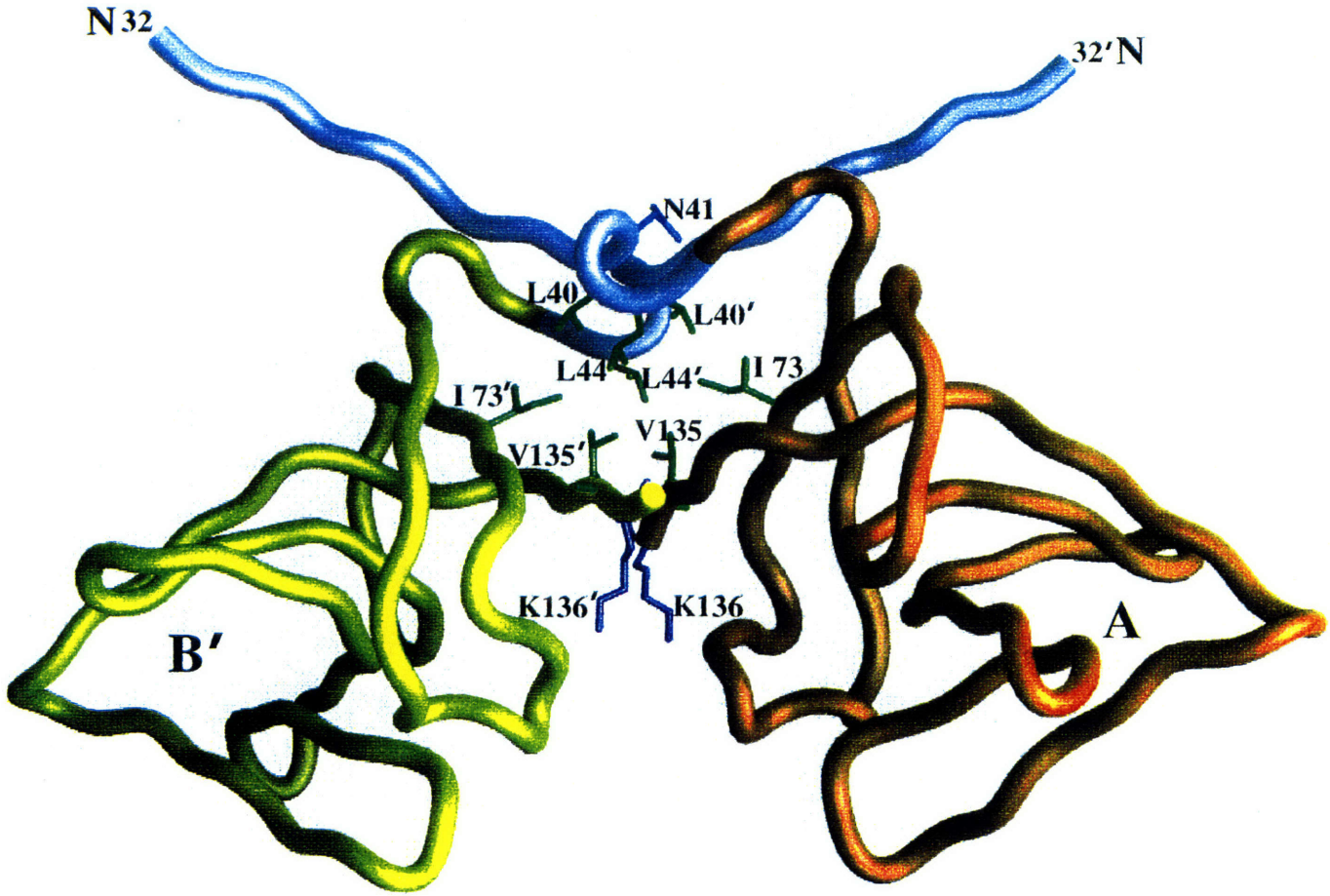

B
A
N
32
C
C
N
32
E93
K55
F128
Y52
F94
F94
Y52
F128
K55
E93

Figure 2. Crystal structure of the cleaved form of UmuD, UmuD' at 2.5 Å, view of the filament dimer interactions of UmuD' as a worm representation. The blue region of the structure includes residues 32 through 45. UmuD' monomer A is in orange and UmuD' monomer B is in green. Hydrophobic residues are in green, basic residues are in blue, and all other residues are in cyan. The amino termini (one from each pair of dimers) interact with each other at residues Leu40, Asn41, Leu43, Leu44, and Ile45. Furthermore, amino acids at the carboxy terminal end, particularly, residues 134 through 136, form hydrogen bonds across the interface with residues 134 through 136 of the associating UmuD'. About 1700 $Å^2$ of solvent accessible surface is buried when two protomers form a filament dimer. This figure was reproduced from ref. (1). Used by permission.


N 32
32′N
N41
L40
L40′
L44
L44′
I 73
I 73′
V135
V135′
K136′
K136
B′
A