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NOTES

Inhibition of RecA-Mediated Cleavage in Covalent Dimers of UmuD

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Disulfide-cross-linked $UmuD_2$ derivatives were cleaved poorly upon incubation with activated RecA. Reducing the disulfide bonds prior to incubating the derivatives with RecA dramatically increased their extent of cleavage. These observations suggest that the UmuD monomer is a better substrate for the RecA-mediated cleavage reaction than the dimer.

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 products of three genes: *umuD*, *umuC*, and *recA*. These genes are 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 [22]) and a nucleotide cofactor, mediates the proteolytic cleavage of LexA by facilitating an otherwise latent capacity of LexA to autodigest (15, 16). Activated RecA, often referred to as RecA*, also mediates the posttranslational cleavage of UmuD at its Cys-24–Gly-25 bond by a similar mechanism (2, 24), removing the first 24 amino acids and thereby activating UmuD to the form designated UmuD′ for its role in SOS mutagenesis (17).

UmuD shares homology with the carboxyl-terminal regions of LexA, the repressors of bacteriophages λ, φ80, 434, and P22, and the UmuD anologs that play roles in mutagenesis, such as MucA and ImpA (1, 4, 19, 23). This homology has functional significance in that all these proteins undergo RecA-mediated cleavage and autodigestion at an alkaline pH. The cleavage reaction for this family of proteins is proposed to occur in 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 (25).

UmuD and UmuD′ form both homodimers and heterodimers (27), with the UmuD-UmuD′ heterodimer being more stable than 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 the 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, 20). Phizicky and Roberts (20) 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, *ind*^{s} −1, revealed that (i) at concentrations at which most of the wild-type λ repressor exists as dimers, a greater proportion of *ind*^{s} −1 remains monomeric; and (ii) *ind*^{s} −1 undergoes RecA-mediated cleavage much more efficiently than the wild-type λ repressor. These findings support 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 than the wild type and displayed a reduced ability to dimerize. From their results they suggested that the hypersensitivity of these mutants to RecA-mediated cleavage is due to the reduced ability of these mutants to dimerize. In contrast, LexA exists predominantly as a monomer in solution and dimerizes as it cooperatively binds to DNA (9).

Our strategy for determining the preferred UmuD substrate (the dimer or the monomer) for the RecA-mediated cleavage reaction involved disulfide cross-linking of certain UmuD monocysteine derivatives and incubation of the disulfide-cross-linked dimers with activated RecA. To maximize the probability of obtaining disulfide-cross-linked UmuD derivatives that were in their native conformation, we chose those derivatives that had cysteine substitutions at positions which we had inferred from previous studies to be closer to the dimer interface than the others tested, i.e., C24 (wild-type UmuD), VC34, IC38, and LC44 (8, 11). The relative ease of disulfide bond formation in the homodimer for each of the derivatives suggests that (i) the residues are relatively closer to the dimer interface (8, 11) or (ii) 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 interpreting our observations, we have assumed that the conformation of each of the cross-linked homodimers of UmuD and its monocysteine derivatives substantially resembles that of an untreated $UmuD_2$ homodimer. The results of the present study suggest that cross-linking of UmuD dimers substantially reduces the ability of UmuD to be cleaved by RecA.

Wild-type UmuD and selected UmuD monocysteine derivatives (VC34, IC38, and LC44) at a concentration of 38 μM in

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50 mM HEPES (pH 8.1)–100 mM NaCl were incubated with 0.57 mM Cu^{2+} and 0.74 mM phenanthroline at 22°C for 40 min to catalyze the air oxidation of the disulfide bonds in the $UmuD_2$ homodimer. Each of the chosen UmuD monocysteine derivatives was able to be disulfide cross-linked almost quantitatively (from 80 to 90%) in the $UmuD_2$ homodimer form. Control reactions with CA24, the UmuD derivative lacking cysteine, were performed under the same conditions; as expected, there was no cross-linking at all (data not shown). The cross-linked derivatives were then dialyzed against 40 mM TRIS–0.1 mM EDTA–100 mM NaCl (pH 8.0) to remove the Cu^{2+}–phenanthroline.

To test whether activated RecA was able to mediate the cleavage of these disulfide-cross-linked UmuD derivatives, each of the disulfide-cross-linked dimers at a 20 μM concentration was incubated with 5.2 μM activated RecA at 37°C for 1 h. RecA was activated in the presence of 0.11 mM ATPγS, 5.7 ng of $poly(dT)_{27}$ (Pharmacia) per μl, and 11.3 mM $MgCl_2$. In addition, a portion of each of the cross-linked $UmuD_2$ homodimers was preincubated with 10 mM dithiothreitol (DTT) to reduce the disulfide bonds prior to incubation with activated RecA. The protein mixtures were resolved by electrophoresis on a nonreducing (Fig. 1A) and a reducing (Fig. 1B) sodium dodecyl sulfate (SDS)–13% polyacrylamide gel and visualized by Coomassie blue staining. In a control experiment, 20 μM UmuD derivatives which were not initially treated with any oxidizing or reducing agents were also incubated with 5.2 μM activated RecA at 37°C for 1 h. As shown in Fig. 1C, untreated 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 cross-linked $UmuD_2$ homodimers which were reduced prior to their incubation with RecA were able to undergo cleavage to approximately the same extent in 1 h 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. 1B with Fig. 1C).

As another control, CA24 (the UmuD derivative lacking cysteine) was subjected to the same treatment as the other derivatives. We found that CA24 which had been treated with Cu^{2+} and phenanthroline and then dialyzed was able to undergo RecA-mediated cleavage in the absence or presence of DTT; however, the amount of cleavage in the absence of DTT was about 35% of the amount in the presence of DTT. 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 substantial RecA-mediated cleavage of UmuD and that these conditions did not significantly perturb the native structure of UmuD (Fig. 1A and B).

Without prior reduction of the disulfide bonds in the $UmuD_2$ homodimers, the disulfide-cross-linked dimers exhibited a significant reduction in their ability to undergo RecA-mediated cleavage (Fig. 1A and B). As shown in Fig. 1A, lanes 2 and 3, and Fig. 1B, lanes 2 and 3, incubation of cross-linked dimers of C24 (wild type) and VC34, respectively, with activated RecA did not result in the appearance of any detectable UmuD′. A reasonable explanation for the observation that cross-linked C24 (wild type) was not able to be cleaved is that the cross-linking of the cysteines at the cleavage site sterically hinders the interactions of the cleavage site (the position 24-25 peptide bond) with the active site, which is presumed to involve Ser-60 and Lys-97 (17, 18, 25). Mutations have been found at the cleavage sites of the LexA repressor (13), λ repressor (6),

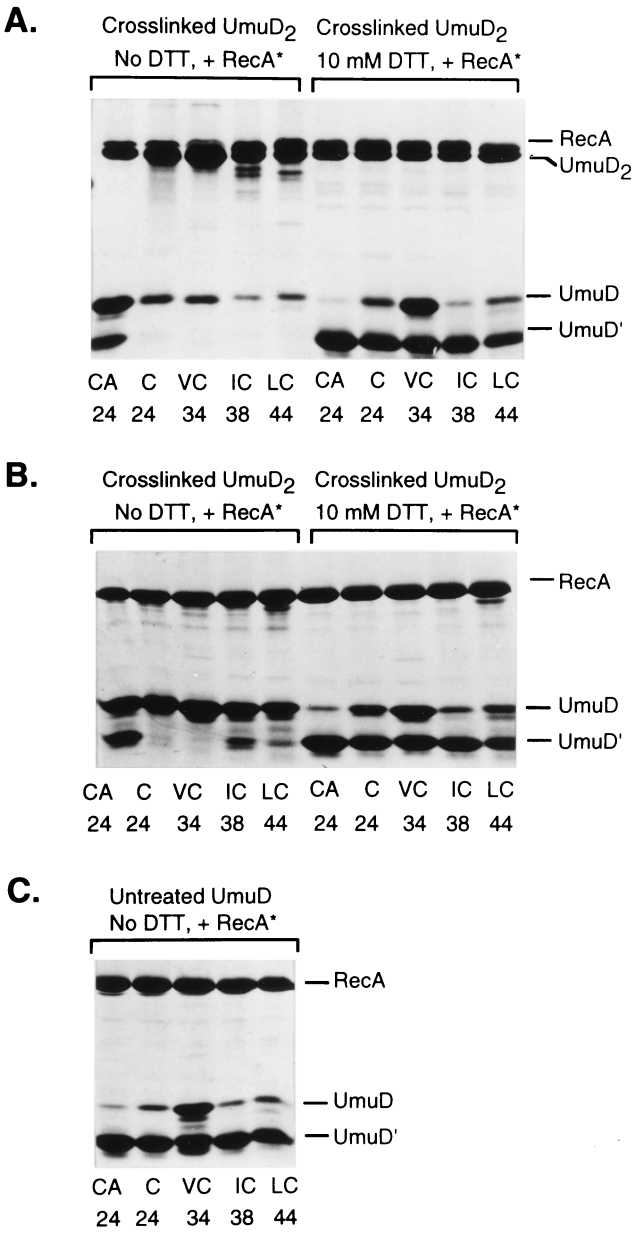

FIG. 1. RecA-mediated cleavage of cross-linked UmuD dimers. Disulfide-cross-linked dimers of UmuD monocysteine derivatives at a 20 μM concentration were incubated with 5.2 μM activated RecA at 37°C for 1 h or were reduced in the presence of 10 mM DTT prior to incubation with activated RecA. Reactions were quenched, and proteins from the reaction mixture were resolved by electrophoresis on a nonreducing (A) or reducing (B) SDS-polyacrylamide gel and visualized by Coomassie blue 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 (C).

and UmuD (1, 17) 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 sites of the different proteins (14). In the crystal structure of the cleaved form of UmuD (UmuD′), reported by Peat et al. (18), the putative active-site residues (Ser-60 and Lys-97) 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. On the basis of 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. Cross-linking at the cleavage site might preclude these interactions by preventing a necessary conformational change or sterically hindering the residues from occupying the correct positions within the cleft.

VC34 (which has a cysteine substitution within the region corresponding to the region in λ repressor suggested to be involved in RecA-mediated cleavage [1, 6]) (i) is severely defective for RecA-mediated cleavage compared with other monocysteine derivatives (11) and (ii) can be cross-linked to activated RecA by the photoactivatable cross-linker *p*-azidoiodoacetanilide (12). These observations have led us to suggest that there might be elements close to the $UmuD_2$ homodimer interface which are also involved in interactions with RecA (8, 11, 12) and, in particular, that Val-34 appears to be important in both UmuD dimer interactions and UmuD interactions with RecA. Thus, cross-linking at this position might affect cleavage by sterically blocking sites of UmuD interaction with RecA, thereby preventing interactions which lead to UmuD cleavage. The view that amino acid 34 must engage in interactions with activated RecA rather than with a second UmuD molecule for efficient RecA-mediated cleavage to occur is consistent with the conclusion that UmuD monomers are cleaved preferentially to $UmuD_2$ homodimers in the RecA-mediated cleavage reaction.

Interestingly, in contrast, incubation of the cross-linked IC38 and LC44 derivatives with activated RecA resulted in a small amount of cleavage. Since CA24 was cleaved to about 35% under these conditions, we expected that if cross-linking the dimers had no effect on its ability to be cleaved, these cross-linked dimers would also be cleaved to 35%. Normalizing the observed cleavage data by this factor, cross-linked IC38 derivative was cleaved to 28% the extent of the CA24 derivative and the LC44 derivative was cleaved to 10% the extent of the CA24 derivative under these conditions. Since a small proportion of these derivatives were not disulfide cross-linked, one possibility is that free UmuD molecules are cleaved in a RecA-mediated fashion and are subsequently cross-linked in a disulfide exchange reaction with a cross-linked UmuD homodimer. An alternative explanation is that the UmuD dimer can be cleaved in a RecA-mediated fashion, although with a greatly reduced efficiency. If this is true, it suggests that although the UmuD derivatives IC38 and LC44 are locked in the $UmuD_2$ homodimer form by the disulfide cross-link, all the essential interactions that lead to RecA-mediated cleavage can still occur.

The ability of the disulfide-cross-linked derivatives which were incubated with 10 mM DTT prior to incubation with RecA to undergo RecA-mediated cleavage increased dramatically, 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. 1C). These results together suggest that UmuD monomers are more efficiently cleaved than $UmuD_2$ homodimers.

Residues 34, 38, and 44 lie within a region that is seen as an extended N-terminal tail in the crystal structure of the $UmuD'_2$ homodimer (18). Thus, it seems possible that the region may have some degree of flexibility in the intact UmuD molecule as well. It has been clearly established that the autodigestion of LexA under alkaline conditions is unimolecular (15, 26), and thus this must involve the protein adopting a conformation that brings the cleavage site to the active serine of the same protein molecule. In contrast, the exact mechanism of the RecA-mediated cleavage reaction is not yet equivalently understood. It is possible that the autodigestion of UmuD that is facilitated by interactions with the RecA nucleoprotein filament is unimolecular with respect to UmuD, as is often tacitly assumed. If so, it is possible that an individual UmuD monomer, despite being linked to another monomer by a C-38–C-38 or a C-44–C-44 disulfide bond, might be able to undergo the interactions with activated RecA (perhaps involving position 34 of UmuD) that facilitate the unimolecular cleavage of its own position 24-25 peptide bond.

Alternatively, it is possible that the RecA-mediated autodigestion of UmuD is bimolecular with respect to UmuD. In such a model for UmuD cleavage, a function of the RecA nucleoprotein filament would be to allow an arrangement of UmuD monomers that would permit the UmuD position 24-25 bond to be cleaved by the Ser-60 of an adjacent UmuD; the cleavage event would still be a facilitated autodigestion but would be intermolecular rather than intramolecular. The apparent cooperativity of the RecA-UmuD interaction observed in our cross-linking experiments discussed in one of the accompanying papers (12) is consistent with this possibility, as is our observation in our *p*-azidoiodoacetanilide cross-linking experiments (12) that some cross-linked species most likely consist of one RecA molecule and more than one UmuD molecule. Perhaps one of the reasons that the UmuD monomers are a better substrate for RecA-mediated cleavage than $UmuD_2$ homodimer is that they can rearrange along the RecA nucleoprotein filament in a way that will permit such an intermolecular autodigestion of UmuD. With respect to the applicability of such a model to RecA-mediated cleavage of LexA, modest cooperativity has been reported for the RecA-LexA interaction (28), and Kim and Little (10) 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*. Both models are consistent with the suggestion of Roland et al. (21) that cleavage of LexA and related molecules involves a conformational change that creates a local environment around the cleavage site and active site that is favorable for cleavage.

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