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Conformational dynamics of the *Escherichia coli* DNA polymerase manager proteins UmuD and UmuD'

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Abstract

The expression of *Escherichia coli* *umuD* gene products is upregulated as part of the SOS response to DNA damage. UmuD is initially produced as a 139-amino acid protein, which subsequently cleaves off its N-terminal 24-amino acids in a RecA/ssDNA-dependent reaction, giving UmuD'. The two forms of the *umuD* gene products play different roles in the cell. UmuD is implicated in a primitive DNA damage checkpoint and prevents DNA pol IV-dependent –1 frameshift mutagenesis, while the cleaved form facilitates UmuC-dependent mutagenesis via formation of DNA pol V (UmuD'2C). Thus, the cleavage of UmuD is a crucial switch that regulates replication and mutagenesis via numerous protein-protein interactions. A UmuD variant, UmuD3A, has been identified that is noncleavable but is a partial biological mimic of the cleaved form, UmuD'. We used hydrogen-deuterium exchange mass spectrometry (HXMS) to probe the conformations of UmuD, UmuD', and UmuD3A. In HXMS experiments, backbone amide hydrogens that are solvent-accessible or not involved in hydrogen bonding become labeled with deuterium over time. Our HXMS results reveal that the N-terminal arm of UmuD, which is truncated in the cleaved form UmuD', is dynamic. Residues that are likely to contact the N-terminal arm show more deuterium exchange in UmuD' and UmuD3A than in UmuD. These observations suggest that noncleavable UmuD3A mimics the cleaved form UmuD' because in both cases the arms are relatively unbound from the globular domain. Gas phase hydrogen exchange experiments, which specifically probe the exchange of side-chain hydrogens and are carried out on shorter time scales than solution experiments, show that UmuD' incorporates more deuterium than either UmuD or UmuD3A. This work indicates that these three forms of the UmuD gene products are highly flexible, which is likely of critical importance for their many protein interactions.

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Keywords

umuD gene products; thermofluor; hydrogen exchange; mass spectrometry; SOS response

Introduction

All cells are constantly exposed to a variety of natural and synthetic agents such as chemicals and UV radiation that can damage DNA and interfere with efficient and accurate DNA replication. If not repaired, such DNA damage can cause mutations that may harm cell fitness.^{1, 2} Furthermore, if these mutations are in genes that normally control cell proliferation or suppress tumor growth, they can lead to cancer. Cells have therefore developed mechanisms for tolerance and repair of DNA damage. Failure of these repair mechanisms can lead to disease and death.²

Escherichia coli (*E. coli*) responds to DNA damage with the SOS response, which involves the upregulation of at least 57 genes with roles in DNA repair, DNA damage tolerance, and cell division.^{3, 4} Among the SOS-regulated genes are those in the *umuDC* operon whose products are involved in DNA damage-induced mutagenesis.^{1, 5, 6} Products of the *E. coli umuD* gene, UmuD₂ and UmuD'₂, play key roles in coordinating the switch from accurate DNA repair to mutagenic translesion DNA synthesis (TLS) during the SOS response to DNA damage.^{1, 4, 7, 8, 9, 10}

UmuD₂ is a homodimeric protein that is the predominant species produced during the first approximately 20–40 min after the SOS response is activated.^{1, 7, 11, 12} In response to replication-blocking DNA damage, UmuD₂, together with UmuC, serves a non-catalytic role as part of a primitive DNA damage checkpoint control, inhibiting DNA replication and allowing time to repair damaged DNA.^{7, 9, 13} After the SOS response is induced, UmuD₂ binds the RecA/ssDNA nucleoprotein filament, which stimulates the cleavage of UmuD₂ to form UmuD'₂. The main conversion event is cleavage of the N-terminal 24 amino acids of UmuD₂ although additional structural rearrangements may also occur in the core of UmuD₂ (Fig. 1).^{14, 15}

UmuD₂ and UmuD'₂ display differential interactions with components of the DNA polymerase III replisome that are believed to be caused by their conformational differences.^{16, 17} In particular, UmuD₂ binds more strongly to the β processivity clamp than UmuD'₂ does, whereas UmuD'₂ binds more strongly to the α polymerase subunit than UmuD₂ does.¹⁶ UmuD₂ and UmuD'₂ both interact with the ε proofreading subunit.¹⁶ UmuD₂ has also been found to bind Y family DNA polymerase IV (DinB) and modulate its mutagenic activity.¹⁸ Approximately 40 min after induction of the SOS response, UmuD'₂ becomes the predominant species and plays two principal roles. First, it may release the DNA damage checkpoint control by its weaker interaction with the β clamp protein compared to UmuD₂.^{11, 19} Second, UmuD'₂ together with UmuC constitute the Y family DNA polymerase V (UmuD'₂C), a low fidelity DNA polymerase that can replicate damaged DNA by TLS.^{8, 10, 20, 21} Thus, by interacting differentially with components of DNA polymerases, UmuD₂ and UmuD'₂ manage the actions of Y family DNA polymerases and coordinate them with those of the replication machinery.^{16, 22} Once the SOS response is no longer required, the potentially mutagenic activity of DNA polymerase V must be controlled to prevent spurious mutations. Three mechanisms accomplish this: UmuDD' heterodimers form and prevent UmuC-dependent mutagenesis,^{23, 24} UmuD and UmuC are degraded by the Lon protease,^{25, 26} and UmuD₂ directs its bound partner, whether UmuD₂ or UmuD'₂, to the ClpXP protease for degradation.^{25, 26, 27, 28}

Since the *umuD* gene products play crucial roles in managing the biological responses to DNA damage, differences in the conformation and dynamics between UmuD₂ and UmuD'₂ are of great interest. However, to date the structure of full-length UmuD₂ remains to be determined. Suitable crystals of UmuD₂ were not obtained for X-ray crystallography²⁹ and Ferentz *et al.* found that the peaks in the NMR spectrum of UmuD₂ were too broad to allow structure determination.³⁰ The NMR^{30, 31} (Fig. 1b) and crystal²⁹ structures of UmuD'₂ have been solved and reveal overall similar secondary structures, but the difference between the two structures is significant.³⁰ Specifically, the RMSD between the backbone atoms of UmuD'₂ residues 40–139 in the two structures is 4.59 Å.³⁰ Two possible dimer interfaces of UmuD'₂ were suggested from the X-ray crystallographic data: the “filament” dimer interface, which is the interface observed in NMR experiments³¹, and the “molecular” dimer interface.^{29, 32} Our observations are consistent with UmuD'₂ having the dimer interface observed by NMR.

As UmuD₂ has been resistant to structural investigations by NMR and X-ray crystallography, we turned to other biophysical methods for conformational analysis including fluorescence and mass spectrometry. We determined that UmuD₂ undergoes two melting transitions while UmuD' undergoes only one. Hydrogen exchange mass spectrometry (HXMS) was used to probe the conformation and dynamics of UmuD₂ and compare it with UmuD'₂ and a previously identified UmuD₂ variant (T14A, L17A, F18A, “UmuD3A₂” indicated in Fig. 1a). UmuD3A₂ acts as a mimic of UmuD'₂, even though the N-terminal arms of UmuD3A₂ are non-cleavable.³³ Our observations suggest that the N-terminal arm is a key component affecting the dynamics and conformation of UmuD₂ and UmuD'₂. We observed that many regions of UmuD₂ were flexible in solution, especially its N-terminal arm, consistent with previous suggestions.^{30, 34} In the absence of the N-terminal 24 residues in UmuD'₂, regions of the globular domain likely to contact the arm underwent more exchange than in UmuD₂. In order to better understand the conformational and functional changes in UmuD₂ caused by the presence or absence of the N-termini, we investigated UmuD3A₂. We observed a high degree of local flexibility in the N-terminal arms of both wild-type UmuD₂ and UmuD3A₂. We also observed that the regions of the globular domain that would contact the arm of UmuD3A₂ underwent more exchange than in UmuD₂. This suggests that the mutations in UmuD3A₂ disrupt the binding of the N-terminal arms over the C-terminal globular domain. Conformational analysis by gas-phase hydrogen exchange (HX)³⁵ showed that side-chain residues of UmuD3A₂ and UmuD₂ were labeled to the same extent during a 0.3 ms exposure to ND₃ gas, whereas side-chain sites on UmuD'₂ were labeled more than either UmuD3A₂ and UmuD₂ even though it contains fewer residues. Taken together, these observations demonstrate a significant role for the N-terminal arms of UmuD₂ in the conformational transition this protein undergoes upon cleavage.

Results

UmuD exhibits two melting transitions

A thermal-shift assay, often used in drug discovery screening³⁶ and in optimization of conditions for protein crystallography³⁷, was used to compare the stability of UmuD₂, UmuD'₂, and the UmuD3A₂ variant. This assay involves the use of a hydrophobic fluorescent dye whose fluorescence intensity is proportional to the hydrophobicity of its environment. When a folded protein is present, the dye is in a mostly aqueous environment and so the fluorescence intensity is low. As the temperature reaches the melting point of the protein, the dye will bind to the exposed hydrophobic interior of the unfolding protein, increasing the fluorescence intensity. By observing the fluorescence intensity as a function of temperature, the folded and unfolded states of the protein can be distinguished. The temperature at the midpoint of the transition between these two states is the T_m .

The melting curves in Fig. 2 show that UmuD₂ and UmuD'₂ each has a T_m of 60 °C, and UmuD3A₂ has a T_m of 59 °C. Additionally, the UmuD₂ melting curve shows another melting transition at a T_m of approximately 30 °C, which is not present in the case of UmuD'₂. The absence of a second transition in the UmuD'₂ melting curve indicates that the melting transition near 30 °C is due to dissociation of the N-terminal arms of UmuD₂ from its globular domain. Moreover, the melting curve of UmuD3A₂ suggests the presence of an additional very broad transition, which is likely due to the arms being exposed to the dye even at the lowest temperature of the experiment, 25 °C. Molecular modeling experiments suggested that the arms of UmuD₂ can exist in an “elbows up” and an “elbows down” configuration,³³ indicating a high degree of conformational flexibility in this region of the protein.^{30, 34}

Hydrogen exchange mass spectrometry

The mechanisms of HXMS have been reviewed extensively.^{38, 39} In summary, HXMS probes the exchange of backbone amide hydrogens in proteins with hydrogens in solution; if the all-H₂O solution is replaced with D₂O, the amide hydrogens in the target protein will become deuterated. The rate and level of deuterium incorporation is an indication of the combined effects of hydrogen bonding and solvent accessibility, making the method sensitive to changes in protein conformation. Mass spectrometry can be used to determine the deuterium incorporation as a function of time, both for whole proteins and for proteins that are digested into fragments after the deuterium exchange reaction has been quenched. Proteolytic digestion of the protein of interest allows one to localize deuterium exchange in a protein and thereby draw conclusions about the conformation and conformational changes in specific regions.

Analysis of intact proteins by HXMS: UmuD is dynamic

Deuterium incorporation was assessed for intact UmuD₂, UmuD'₂ and UmuD3A₂ by exposing the proteins to D₂O for various periods of time ranging from 30 seconds to 8 h and measuring the resulting mass increase. Prior to HXMS, the purity and correct synthesis of each recombinant protein was verified by electrospray mass spectrometry (Supplementary Fig. 1). The HXMS analysis for all three proteins was done under identical experimental conditions, which allowed for the direct comparison of the deuterium incorporation without back-exchange corrections.³⁹ The mass increase reported here is only due to the deuteration on backbone amides because side-chain deuterium is washed away during the chromatography step.³⁹ Note that although UmuD is expected to be a dimer at the concentrations used in these experiments,¹⁷ for simplicity we will discuss it as a monomer in terms of interpreting deuterium exchange. The average mass increase of each protein was determined and plotted. As shown in Fig. 3, in UmuD approximately 58 residues of one monomer exchanged in 30 s, or 45% of the available backbone amide hydrogens. In UmuD, there are 128 backbone amide hydrogens of 139 residues available to exchange, calculated subtracting the number of prolines and one for the N-terminus³⁹ from the total number of residues. Rapid exchange of amide hydrogens, within the first 30 seconds of labeling, can generally be attributed to highly exposed and non-hydrogen bonded positions.^{40, 41} These results suggest that UmuD has a relatively open and/or dynamic structure capable of rapidly exchanging with D₂O. The mass of UmuD continued to increase upon longer incubation times, up to approximately 100 deuterons (more than 75% of the available NHs) after 8 hours of labeling. Overall, the exchange data for intact UmuD indicate that the protein is highly dynamic in solution and therefore able to incorporate a large amount of deuterium. By comparison, other much more stable proteins may only be deuterated less than 50% after 8 hours in deuterium.⁴² If no conformational motions occurred, the deuterium level at 30 s would be equal to the deuterium level at 8 h, for example.

For the cleaved form, UmuD', there are 108 residues that could become deuterated while the UmuD variant UmuD3A has the same maximum number of backbone amide hydrogens as UmuD, 128. Comparing exchange into intact UmuD with exchange into the UmuD3A variant,

it appears that the mutations (T14A L17A F18A) cause a minor change in the conformation of UmuD (Fig. 3). UmuD3A underwent slightly more deuterium exchange during this time than UmuD, suggesting that the mutations cause some regions of UmuD3A to undergo more exchange than wild-type UmuD. The differences seen in this intact protein experiment are close to the error one would expect in an HXMS experiment of a protein of this mass. However, digestion experiments (see below) suggest that the changes seen at the intact protein level are significant. Finally, comparing UmuD' with UmuD3A, we found the relative slope of UmuD3A and UmuD' deuterium up-take curves to be similar. UmuD' is 24 residues shorter than the other two constructs, hence its lower overall incorporation of deuterium. The observation that the slopes of the deuterium uptake curves are similar implies that the overall dynamics of UmuD3A are similar to UmuD' and UmuD.

Localizing deuterium exchange to different regions of UmuD by pepsin digestion analysis

In order to localize HX to specific regions of UmuD, the deuterium labeled proteins were digested with pepsin. Note that deuterium exchange captures all the conformational information at physiological pH (in this case pH 7.5) and digestion occurs after the exchange reaction has been quenched. All peptic peptides produced during digestion of the three proteins were identified by MS^E and accurate mass measurements. The map of the peptides studied in this work is shown in Supplementary Fig. 2. The sequence coverage was more than 90% for UmuD, UmuD', and UmuD3A. Examples of the chromatographic separation of the peptic peptides of UmuD (4.0–11.5 min) and representative mass spectra from peptides of different charge states and m/z are shown in Supplementary Fig. 3. The data were of very high quality. The exchange-in measurements were performed for the proteins under identical conditions so differences could be reliably identified for parts of the sequence in common between the two proteins. The deuterium incorporation as a function of time for each peptic peptide was determined in duplicate experiments and provided good reproducibility. The uptake curves for 15 peptides (UmuD and UmuD3A) that cover 92% of the sequence of UmuD and 7 peptides of UmuD' that cover 90% of the sequence of UmuD' are provided in Supplementary Fig. 4 (redundant and overlapping peptides not shown). To ease data interpretation, UmuD sequence numbering was used for all proteins; UmuD' sequence numbering starts from 24 (there is an extra methionine at the N-terminus as a result of protein expression that is not found in UmuD, see also Supplementary Fig. 2).

The hydrogen exchange data for UmuD are summarized in graphical form in Fig. 4 where the relative percent deuteration is plotted onto a current model for the conformation of the UmuD protein³³ and onto a known structure for UmuD'.²⁹ An animation of the exchange data for UmuD, which incorporates all of the frames of Fig. 4a, can be found in the Supplementary materials (Movie 1). From this data summary, it is noteworthy to mention several regions of UmuD. Within the first 30 seconds of deuterium labeling, the N-terminal arm (residues 1 to 32, shown in red in Fig. 4a-1) exchanged >60% of the possible backbone amide hydrogen sites while other regions, including the C-terminal globular domain, remained more protected during this time. Such rapid deuteration of the N-terminal arm is indicative of high solvent exposure and minimal hydrogen bonding. In other regions of UmuD there was much more protection from deuteration; however, even some protected regions became >60% deuterated by 120 minutes of labeling (Fig. 4a-4). Other regions, such as several β -strands buried in the presumed dimeric interface, were more resistant to deuterium incorporation even after 8 hours (green in Fig. 4a-5). These observations indicate that this part of UmuD was solvent protected and/or protected from exchange by hydrogen bonds. Overall, the HXMS data of UmuD are consistent with the UmuD homology model³³ upon which they are plotted, validating the use of this model for further UmuD studies.

UmuD' incorporates more deuterium than UmuD

The deuterium incorporation into UmuD' was determined as described above and is shown in summary form in Fig. 4b, mapped onto the crystal structure of UmuD' ^{29, 32} ("filament" interface, Fig. 4b; "molecular" dimer interface, Supplementary Fig. 5) and onto the NMR structure of UmuD' ³⁰ (Supplementary Fig. 6). In comparing the conformation-function relationships between UmuD and UmuD', the N-terminal arms are intriguing because the cleaved form UmuD' lacks the N-terminal 24 amino acids. The shortened N-terminal arms, residues 33–40 (peptide 33–43) exchanged 50–60% of the possible backbone amide hydrogen sites within the first 30 seconds of labeling (Fig. 4b-1), and exchanged >60% by 10 minutes of labeling (Fig. 4b-2). The "top" of UmuD', residues 41–52, exchanged >60% of possible backbone hydrogens by 10 min of labeling (Fig. 4b-2). Some regions of UmuD', notably those located at the dimer interface shown in Fig. 4b, are resistant to deuteration. Our data are consistent with the UmuD'₂ dimer interface being the "filament" dimer interface from the X-ray crystal structure,²⁹ which is the one observed in the solution structure³¹ (Fig. 4b and Supplementary Fig. 6), and are not consistent with the "molecular" dimer interface that was also observed in the X-ray crystal structure^{29, 32} (Supplementary Fig. 5). An animation of the exchange data for UmuD', which incorporates all of the frames of Fig. 4b, can be found in the Supplementary materials (Movie 2).

We then determined the differences in exchange between UmuD and UmuD' and mapped them onto the UmuD homology model (Fig. 5a; the complete dataset is shown in Supplementary Fig. 4). The cumulative error of measuring deuterium uptake in these assays is approximately ± 0.2 Da. Any differences in deuterium uptake larger than that were considered significant for the purpose of comparing the two datasets. The changes were grouped according to obvious changes (>1.0 Da separating the deuterium incorporation curves at any one exchange point, shown in red) and subtle changes (0.4–1.0 Da difference at any one exchange point, shown in yellow). There were differences in exchange in residues 33–43, 41–52 and 73–88. The most obvious changes were in peptides covering residues 41–52 (Fig. 5a-2) and 73–88 (Fig. 5a-3), which are near the N-terminal arm in UmuD. The differences in exchange at residues 33–43 (Fig. 5a-1) are subtle. In each of these three peptides, UmuD' incorporated approximately 1–2 more deuterium atoms than UmuD, indicating a loosening of the structure or subtle exposure to more solvent in some part(s) of these regions of UmuD'. In the absence of the N-terminal arm, perhaps one more backbone amide in each of these peptides is now more solvent exposed. The lack of the N-terminal arm in UmuD' caused more residues of these regions to be exposed to solvent and to incorporate more deuterium. These results are consistent with what was observed in gas-phase labeling experiments (see below).

UmuD3A incorporates more deuterium than UmuD

UmuD3A is a non-cleavable UmuD variant that has characteristics of the cleaved form.³³ As such, it was suggested that the N-terminal arms of UmuD3A may be less tightly bound to the globular domain than those of UmuD.³³ In order to determine if this were the case, we measured deuterium exchange in UmuD3A and compared it to that of wild-type UmuD at the level of individual peptides (Fig. 5b). HXMS data were obtained using the same methodology described above. Four out of 15 detected peptic peptides showed differences in the amount of deuterium incorporation: Peptic peptides 22–40 and 73–88 exhibited subtle changes, as shown in yellow (Fig. 5b-1 and Fig. 5b-4, respectively), while peptides 33–43 and 41–52 exhibited obvious differences between UmuD and UmuD3A, as shown in red (Fig. 5b-2 and Fig. 5b-3, respectively). In all of these peptides, UmuD3A showed more deuterium incorporation than UmuD implying less protection and/or structural organization in these regions in UmuD3A.

The peptides with obvious deuterium incorporation changes in UmuD3A compared to UmuD are located in or near the region containing the mutations (T14A L17A F18A, shown as spheres

in Fig. 5b). The deuterium uptake curves (Figs. 5b-2 and 5b-3) showed that these shoulder domains (residues 33–52) in UmuD3A are more flexible and/or solvent accessible than wild-type UmuD. Overall, from the comparison of HX data between UmuD and UmuD3A, we conclude that the mutations destabilize the binding of the N-terminal arm over the C-terminal globular domain and make the N-terminal arm of UmuD3A more flexible, thereby functioning as a mimic of UmuD'. While this difference in conformation was speculated to occur,³³ this is the first direct evidence that this is indeed the case. We note that these experiments in solution represent motions of the arm that are on a timescale longer than 10 s, the shortest labeling time used for these HXMS experiments.

Gas-phase HX of UmuD

To further probe the conformational properties of UmuD, UmuD3A and UmuD', particularly in very short timescales (< 1 ms), we performed gas-phase deuterium labeling experiments, as recently described.³⁵ Gas-phase HX experiments of UmuD proteins were performed by infusion of deuterated ND₃ gas into the transfer travelling wave ion guide of a customized synapt HDMS mass spectrometer. UmuD proteins were labeled within a few milliseconds after electrospray ionization, thus probing conformers that closely resemble those in solution.^{35, 43, 44, 45} Primarily labile side-chain hydrogens of UmuD were labeled in the short timescale of the gas-phase experiment.³⁵ As the exchange of protein side-chain residues was not measured by solution HXMS analyses, gas-phase labeling provides conformational information about sites in UmuD proteins not probed otherwise.

Gas-phase experiments on UmuD and UmuD' showed that UmuD' incorporated significantly more deuterium than UmuD under identical conditions (Fig. 6a). For instance, the 7+ charge state of the gaseous UmuD' protein exchanged 7 more hydrogens than the corresponding species of UmuD during 0.3 ms in the labeling gas (Fig. 6a and 6b). These results indicate that 7 exchangeable side-chain hydrogens were surface-exposed in UmuD' but protected from exchange in UmuD. Under similar conditions, UmuD3A exchanged an equal number of side-chain sites as UmuD, indicating that structural differences between these proteins do not involve the exposure of side-chain hydrogens to the labeling gas. The increased deuterium uptake of UmuD' was observed consistently for charge states 6+, 7+, and 8+, which correspond to folded gaseous forms of UmuD/UmuD' (only charge states 5+ to 8+ were observed under native ESI conditions, data not shown).

The gas-phase HX of UmuD' was further investigated by increasing the dwell time of UmuD proteins in the ND₃ gas (i.e. labeling time). Across gas-phase labeling times from 0.17 to 1 ms, UmuD' exchanged from 3 to 10 more sites, respectively, than UmuD or UmuD3A (Fig. 6c). The conformational differences between UmuD' and UmuD/UmuD3A were most pronounced at the longest labeling time, involving up to 10 labile side-chain hydrogens in the UmuD' protein.

Discussion

Conformation and dynamics are important for understanding protein function. The differences in the conformation and dynamics of UmuD and UmuD' play a key role in differentiating their functions. However, full-length UmuD₂ has not been amenable to crystallization or NMR analysis. Moreover, regions of UmuD, particularly in the N-terminal arm, in the UmuDD' heterodimer could not be assigned in NMR experiments.³⁰ Our results provide information about UmuD and UmuD' that is extremely difficult to obtain by other methods. Our observations suggest that UmuD is a flexible protein, especially the N-terminus. Our HXMS data showed that the N-terminus of UmuD is almost totally exposed to solvent at the first 30-s labeling time (Fig. 4, Fig. 5, and Supplementary Movie 1). The regions of the globular domain that are predicted to contact the N-terminal arm (including peptides 33–43 and 41–52) also

exhibit a high level of deuterium uptake. Indeed, a chemical probing and cross-linking study showed that single-Cys substitutions at residues 31–36 and 39–40 are solvent-accessible as determined by modification with iodoacetate, while those at residues 37 and 38 were weakly modified by iodoacetate.⁴⁶ Moreover, our data are consistent with the published homology model of UmuD₂.³³ Notably, the homology modeling experiments resulted in four different models of UmuD₂, which mainly varied in the conformations of the N-terminal arms,³³ suggesting a high degree of flexibility of the arms. The UmuD₂ C-terminal globular domain, including two sub-domains that are connected to each other by a three-stranded anti-parallel β sheet (residues 52–136),^{29, 30, 31, 47} were the most resistant to deuteration indicating that this region is the most stable and structured part of UmuD₂.

The NMR and X-ray crystal structures of UmuD' are substantially different.^{29, 30, 31, 32} Significantly, the conformation of the active site in the two different structures are dissimilar. In the NMR structure, the catalytic residues Ser60 and Lys97 are over 7 Å apart and are not positioned appropriately for catalysis,³⁰ while in the crystal structure these two residues are near each other and are poised for catalysis.²⁹ It has been suggested that the UmuD' crystal structure mimics the conformation of UmuD when it is bound to the RecA/ssDNA nucleoprotein filament,³⁰ particularly as several residues on the outer loops of UmuD facilitate interaction with RecA.^{48, 49} A low resolution structure of UmuD'₂C-RecA/ssDNA complex was determined by using cryo-electron microscopy.⁵⁰ UmuD'₂C binds in the deep helical groove of the RecA/ssDNA nucleoprotein filament, but no details of the orientation of UmuD' within the filament groove could be determined.⁵⁰ Cross-linking studies have implicated UmuD residues Val34, Ser57, Ser67, Ser81, and Ser112 in the interaction with RecA.⁴⁸ Moreover, Leu101 and Arg102 play an important role in proper positioning of the Ser/Lys active site dyad upon interaction with the RecA/ssDNA filament.⁴⁹ We find that the peptides that include these residues (89–125 and 95–128, Supplementary Fig. 4) are highly deuterated, and therefore are dynamic and/or exposed to solvent. Thus, the highly dynamic nature of UmuD in solution plays a major role in the regulation of its self-cleavage activity, which is facilitated by interactions with RecA/ssDNA.

A recent report suggests that UmuD is a member of the class of intrinsically disordered proteins.¹⁷ This could explain the difficulty in analyzing UmuD using traditional structural methods of NMR and X-ray crystallography. However, we note that some regions of UmuD incorporate only low levels of deuterium even at long time points, which suggests that these regions are not very dynamic. This is in contrast to another intrinsically disordered protein, *Herpesvirus saimiri* Tip, which exhibits substantially more deuterium uptake than UmuD.⁵¹ There are few known intrinsically disordered proteins that are dimeric. However, many of the residues predicted to be at the dimer interface of UmuD₂ are also predicted to be ordered,¹⁷ in general agreement with our observations. The equilibrium dissociation constant for the UmuD₂ dimer has been estimated to be less than 10 pM.¹⁷ Our thermal shift analysis suggests that UmuD₂ and UmuD'₂ are highly stable dimers, as the dimeric globular domain of each undergoes a single melting transition at approximately 60 °C (Fig. 2).

There are two main differences between UmuD and UmuD' which could affect their functions. First, the N-terminal arm of UmuD is very flexible in solution (Fig. 4 and Fig. 5) and could adapt to interactions with other proteins. Second, binding the N-terminal arm over the globular domain forms an extended interface on the C-terminal globular domain of UmuD compared with UmuD'. At the same time, the binding of the arm over the globular domain masks some residues that would be exposed in UmuD'. Gas-phase exchange data show that when the arm is truncated, in UmuD', more side-chain sites can be labeled, reinforcing the idea that the arm protects part of the main body of the protein. Therefore, the flexible N-terminal arm and the extended binding interface are potential sites for UmuD to interact with other partner proteins.

Indeed, the β processivity clamp has been shown to interact with specific amino acids in both the N-terminal arm and the globular domain of UmuD.¹⁹

In our analysis of UmuD3A, conformational disturbances compared to wild-type UmuD were found. Differences in deuterium uptake were observed in the region of the C-terminal domain where the N-terminal arm binds. More residues in this area of UmuD3A underwent exchange compared with UmuD. This suggests that the mutations in UmuD3A disrupt the binding of the N-terminal arm on its C-terminal domain which results in this region of the protein incorporating more deuterium than wild-type UmuD. The disruption of binding likely prevents the N-terminal arm from approaching the Ser60 nucleophile, and hence UmuD3A is noncleavable. The similar functions of UmuD' and UmuD3A suggest that the extended interface formed by the binding of N-terminal arm to its C-terminus and the loops in the shoulder area (Fig. 5b) are important factors in UmuD-specific interactions. However, gas-phase labeling experiments, which sample the conformation in 0.3 ms, did not show differences between UmuD and UmuD3A. This suggests that the arm is still engaged with or protecting the body of the protein from labeling, or that motions that displace the arm are slower than 0.3 ms.

Both UmuD and UmuD' interact with the β processivity clamp, but UmuD binds more strongly to β than UmuD' does.¹⁶ It has also been shown that UmuD and UmuD3A bind to the β processivity clamp with approximately the same equilibrium dissociation binding constants.³³ Therefore, there may not be an exact parallel between the interactions of UmuD3A and UmuD' with their partner proteins based on the similar dynamic behavior of UmuD3A and UmuD'. However, the difference in relative binding affinity may simply be due to the fact that the reported binding affinities were determined using different methods (estimated by using affinity chromatography in the former case versus quantified by using tryptophan fluorescence in the latter case).^{16, 33} It is intriguing that both UmuD and UmuD' appear to adopt more defined structure upon interaction with known partner proteins, including the β processivity clamp.¹⁷

In this work, we carried out a detailed analysis of the deuterium exchange behavior of UmuD, UmuD', and the UmuD3A variant. We find that the proteins all possess some less dynamic regions as well as highly dynamic regions. Our observations are consistent both with the proposed model of UmuD³³ and with the finding that at least some regions of UmuD are relatively unstructured.¹⁷

Materials and Methods

Protein Expression and Purification

Wild-type UmuD, UmuD' and the UmuD3A variant were overexpressed in *E. coli* BL21(DE3) strain.^{12, 52} Frozen cells were thawed on ice at 4 °C and fresh phenylmethanesulfonyl fluoride (PMSF, 10 μ g/mL in isopropanol) and protease inhibitors (Mini Complete, Roche) were added. All subsequent steps were performed at 4 °C. Cells were lysed by sonication and then treated with lysozyme (0.3 mg/mL, Sigma) and DNase I (1 μ g/mL, Roche) for 30 min on ice. The lysate was clarified by centrifugation at 14,000 $\times$ g, 4 °C, for 1 h. UmuD was purified by weak anion exchange chromatography (HiTrap™ DEAE Sepharose™ Fast Flow, 5 mL, GE Healthcare). Protein was eluted with a gradient of 0–0.8 M NaCl in 20 mM HEPES, 0.1 mM EDTA and 1 mM DTT (pH 7.5). Fractions containing the protein of interest were pooled and diluted 1:1 (v/v) in Buffer A which contained 1 M ammonium sulfate, 20 mM HEPES, 0.1 mM EDTA and 1 mM DTT (pH 7.5). The solution was loaded onto a hydrophobic column [Phenyl Sepharose™ Fast Flow (low sub), 5 mL, GE Healthcare] and eluted with a 1.0 – 0 M ammonium sulfate gradient. The fractions containing the protein of interest were pooled and concentrated to ~2 mL, and were further purified by size exclusion chromatography on a

Superdex™ 75 column (26 mm × 70 cm, GE Healthcare) that had been equilibrated with 20 mM HEPES, 50 mM NaCl, 0.1 mM EDTA and 1 mM DTT, pH 7.5. The final purity and mass of all proteins were verified by electrospray mass spectrometry (LCT premier, Waters) and each theoretical mass matched the measured mass to within 0.4 Da (Supplementary Fig. 1).

Melting Analysis using ThermoFluor Assay

Samples consisting of assay buffer (50 mM Hepes, pH 7.5, 100 mM NaCl), 1 μL of 400x Sypro Orange (Invitrogen), and 22.5 μM protein (dimer concentration) were assembled directly in the wells of a 96-well plate (Applied Biosystems). The final volume was adjusted to 16 μL with sterile water and the plate was then sealed with an optical adhesive film (Applied Biosystems). The iCycler iQ5 Real Time PCR (Bio-Rad) was used to increase the temperature from 25 °C to 75 °C with an increment of 0.1 °C and a 10 s dwell time per cycle. The fluorescence intensities detected by the built-in CCD camera were plotted versus temperature. In order to calculate the T_m , the Microsoft Excel XLfit 5 add-on program was used to fit the data to a Boltzmann model represented by the equation:

$$Intensity = \left(A + \frac{B - A}{1 + e^{\frac{C - Temperature}{D}}} \right) \quad (1)$$

where C is the T_m , A and B are pre- and post-transitional intensities, respectively, and D is a slope factor. The data shown are representative of several independent experiments, each with similar observations and consistent T_m values.

Hydrogen Deuterium Exchange Analysis

Deuterium Labeling of Proteins—A protein stock solution (~25 pmol/μL, dimer concentration) in equilibration buffer (20 mM HEPES, 0.1 mM EDTA, 50 mM NaCl, and 1 mM DTT, pH 7.5) was diluted 17-fold (v/v) with D₂O buffer (20 mM Hepes, 0.1 mM EDTA, 50 mM NaCl, and 10 mM DTT, pH 7.5) at 21 °C. We note that this temperature is near the melting temperatures of the N-terminal arms of UmuD₂ and UmuD3A₂ (Fig. 2), and therefore the high degree of local solvent exposure is not unexpected. However, this temperature is within the range of conditions under which biochemical analyses of UmuD₂ and UmuD3A₂ have been carried out and is low enough to allow us to detect fairly subtle differences in the dynamics of these proteins.^{12, 33} At selected times (ranging from 30 s to 8 h), approximately 75 pmol of protein were removed from the exchange reaction and the labeling was quenched by addition of 45 μL of phosphoric acid buffer (phosphoric acid and monobasic sodium phosphate, pH 2.1, containing 6 M Guanidine HCl) to reduce the pH to 2.6 (final concentration of GdHCl was 1.8 M). The quenched samples were immediately frozen on dry ice and stored at −80 °C until analysis.

Intact Protein Analysis—To measure the mass of deuterated intact proteins, HPLC-MS was used.⁵³ Deuterium labeled proteins were rapidly thawed at 0 °C and 75 pmol of protein were injected onto a self-packed protein trap (2×20 mm, Alltech, Deerfield, IL) containing POROS 20 R2 media (Applied Biosystems, Foster City, CA) flowing 0.05% TFA in H₂O at a flow rate of 50 μL/min. The injector, sample loop, column and all transfer lines were immersed in an ice bath to minimize deuterium back-exchange.⁵⁴ Proteins were eluted with a gradient of 15–75% acetonitrile in 3 min and directed into an LCT Premier mass spectrometer (Waters, Milford, MA) equipped with a standard electrospray ionization source. The instrument was calibrated by infusing 500 fmol/mL myoglobin at the end of each run. Mass spectra were transformed using Waters Masslynx software. The mass of undeuterated protein was subtracted from the mass of the deuterated protein at each time point and plotted. No

adjustment was made for deuterium back-exchange during analysis, and therefore all results are reported as relative deuterium level.³⁹

Peptic Digestion and Localized Analysis—To localize deuterium to short stretches of amino acid sequence, deuterated proteins (as prepared above) were digested off-line with pepsin prior to UPLC separation and mass analysis.⁵⁴ Each deuterated and quenched sample was thawed in ice and porcine pepsin (Sigma) was added at a 1:10 ratio (protein:pepsin by mass) and allowed to incubate for 5 min at 0 °C. Approximately 5 pmol of peptic digest was injected into a Waters UPLC HX system held at 1.0 °C⁵⁵ where the peptides were trapped with a VanGuard Pre-Column (2.1 mm × 5 mm, ACQUITY UPLC BEH C8, 1.7 μm) for 4 min of desalting. The trap was then placed in line with an ACQUITY UPLC BEH C8 1.7-μm 1.0 mm × 100 mm column (Waters Corp.) and a 5–40% gradient of acetonitrile over 7 min at a flow rate of 40 μL/min was used to separate the peptides and elute them into the mass spectrometer (QToF-Premier, Waters Corp.). All spectra were calibrated with lock-mass correction using Glu-fibrinogen B peptide as the lock-mass standard. Mass spectra were acquired over the range 100–1700 m/z. Pepsin fragments were identified in undeuterated control samples using a combination of exact mass and MS^E, aided by Waters Identity software.⁵⁶ The amount of deuterium in each peptic peptide was determined by subtracting the mass of the unlabeled peptide from the mass of the labeled peptide, at each deuterium exchange time.⁵⁷ The deuterium levels were not corrected for back-exchange and are therefore reported as relative.³⁹ Back-exchange in this experimental setup averaged 15–20% as determined by analysis of completely deuterated control peptides.³⁹

Gas-phase HX experiments

Gas-phase deuterium labeling of proteins was performed in a custom-modified Synapt HDMS mass spectrometer described previously.^{35, 58} Electrospray ionization was performed in positive ion mode with the electrospray ion source operated with a capillary voltage of 3.5 kV, a sample cone of 45 V, an extraction voltage of 3V, a source-block temperature of 75 °C and a N₂ desolvation gas flow of 600 L/h at 250 °C. External mass calibration was performed in MS/MS mode using 400 fmol/μL Glu-fibrinogen peptide B. Mass spectra were acquired over an *m/z* range of 200–2000. Deuterated ND₃ gas was gradually infused into the transfer travelling wave ion guide (TWIG) of the instrument via a needle valve as described elsewhere.³⁵ The instrument was operated in TOF-MS mode using a constant T-wave velocity of 300 m/s and a T-wave height of 3V for the source, trap and mobility TWIG. At these settings, protein ions produced in the ESI ion source reached the labeling environment of the transfer TWIG in less than 1.5 ms.³⁵ The transfer TWIG harboring the ND₃ gas was operated at variable T-wave speeds but a constant T-wave height of 6V to ensure that protein ions were retained in the potential wells of the travelling wave thus obtaining equal residence times in the labeling gas.³⁵ The latter is paramount if structural information is to be deduced from gas-phase deuterium uptake profiles of protein ions in a travelling ion guide.

UmuD, UmuD3A or UmuD' samples were infused into the ESI source at 5 μL/min by a syringe pump at a concentration of (a) 20 μM in 0.1 M ammonium acetate solution at pH 6.0 or (b) 5 μM in a 50% acetonitrile, 0.05% trifluoroacetic acid solution at pH 2.5, containing 2 μM Leucine enkephalin (internal gas-phase HX control peptide). UmuD proteins were retained in the potential wells of the travelling voltage wave of the transfer TWIG and labeled as a function of the partial ND₃ gas pressure (1×10^{-4} to 4.2×10^{-3} mbar) in the gas-filled ion guide or the speed of the travelling wave (T-wave velocities from 600 to 100 m/s corresponded to deuterium labeling times of proteins from 0.17 ms to 1 ms). Replicate gas-phase labeling experiments on UmuD' indicated a standard deviation of 1 Da (n=3) of measuring the deuterium uptake of deuterated protein species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

HXMS	hydrogen-deuterium exchange mass spectrometry
HX	hydrogen exchange
TLS	translesion DNA synthesis

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Fig. 1.

Cleavage of UmuD removes the N-terminal 24 amino acids and dictates different cellular functions. (a) A homology model of the UmuD₂ dimer³³ which functions in a primitive DNA damage checkpoint and (b) NMR structure of UmuD'₂ dimer (PDB code: 1I4V31) which facilitates UmuC-dependent TLS. One monomer is in blue; the other is in red in both images. The first 24 residues at the N-termini are shown in yellow. The residues mutated to alanines in UmuD3A [Thr14 (purple), Leu17 (cyan), and Phe18 (green)] are shown in space-filling rendering.

Fig. 2. Melting curves of UmuD (blue), UmuD' (green), and UmuD3A (pink) show the distinctive unfolding transitions of each protein. The T_m s of UmuD and UmuD' are each 60 °C. The T_m of UmuD3A is 59 °C. Additionally, the UmuD melting curve shows a second melting transition at a T_m of approximately 30 °C.

Fig. 3.

Relative deuterium uptake as a function of time for intact UmuD (blue), UmuD3A (pink) and UmuD' (green). Intact protein exchange analyzed at different deuterium exchange time points between 0 and 480 min. The data shown here are the average of two experiments. The total number of exchangeable backbone amide hydrogens in intact UmuD and UmuD3A is 128 and in UmuD' is 108.

Fig. 4.

Deuterium incorporation of UmuD and UmuD' as a function of time. (a) Deuterium incorporation information for UmuD is mapped on to its homology model³³. (b) Deuterium incorporation data for UmuD' are mapped on the "filament" dimer of UmuD' crystal structure²⁹. For both UmuD and UmuD', the protein is shown in two orientations, with the top orientation turned 90 degrees toward the observer on the y-axis. The relative percentage deuterium incorporation for all monitored residues of UmuD (see also Supplementary Fig. 4) is shown at the times indicated. The color code is shown at the bottom of the figure. The UmuD data in this figure represents each frame of Supplementary Movie S1. The UmuD' data in this

figure represents each frame of Supplementary Movie S2. Regions colored gray represent residues where deuterium levels were not determined.

Fig. 5. Comparison of deuterium exchange in (a) UmuD and UmuD' and in (b) UmuD and UmuD3A. The deuterium uptake curves for representative peptides (the complete dataset is in Supplementary Fig. 4) are shown at the top (UmuD in blue, UmuD3A in pink, and UmuD' in green). The location of each peptide, according to the labels a1-3 and b1-4, is shown on the homology model of UmuD³³ at the bottom, in two orientations: from the “front” and from the “top”. Obvious changes in red were defined as a difference between deuterium exchange of 1.0 Da or greater, observed in at least one time point. Subtle changes in yellow were 0.4–1.0 Da. No changes, shown in gray, were defined as differences of 0.0–0.4 Da. The first N-terminal

24 residues are shown in (a) in blue. In (b), the residues mutated to alanines in UmuD3A [Thr14 (purple), Leu17 (cyan) and Phe18 (green)] are shown as spheres.

Fig. 6. Gas-phase HX of UmuD proteins. (a) Mass spectra recorded of UmuD (spectra i and ii) and UmuD' (spectra iii and iv) in the absence and presence of labeling ND₃ gas (4.2×10^{-3} mbar). (b) Bar chart of the deuterium uptake during 0.3 ms of charge states 6+, 7+, and 8+ of UmuD' (green) UmuD3A (pink) and UmuD (blue) in the presence of ND₃ gas (4.2×10^{-3} mbar). The deuterium uptake of a gas-phase HX control peptide (Leucine enkephalin), which was co-infused with the UmuD proteins, is shown in white bars. The error bars represent the standard deviation of triplicate measurements for UmuD'. Data for UmuD and UmuD3A were acquired in duplicate. (c) Probing sub-millisecond conformational dynamics of UmuD proteins by time-resolved gas-phase deuterium uptake of the $[M+7H]^{7+}$ charge state of UmuD' (green triangles), UmuD3A (pink squares) and UmuD (blue diamonds) upon exchange with ND₃ gas (1.6×10^{-3} mbar). Note, gas-phase HX at such short timescales primarily occurs at labile and exposed hydrogens in the side-chain positions of the protein.³⁵