NMR analysis of [methyl-13C]methionine UvrB from Bacillus caldotenax reveals UvrB–domain 4 heterodimer formation in solution

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UvrB is a central DNA damage recognition protein involved in bacterial nucleotide excision repair. Structural information has been limited by the apparent disorder of the C-terminal domain 4 in crystal structures of intact UvrB; in solution, the isolated domain 4 is found to form a helix–loop–helix dimer. In order to gain insight into the behavior of UvrB in solution, we have performed NMR studies on [methyl-13C]methionine-labeled UvrB from Bacillus caldotenax (molecular mass=75 kDa). The 13 methyl resonances were assigned on the basis of site-directed mutagenesis and domain deletion. Solvent accessibility was assessed based on the relaxation and chemical shift responses of the probe methyl resonances to the stable nitroxide, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL). M632, located at the potential dimer interface of domain 4, provides an ideal probe for UvrB dimerization behavior. The M632 resonance of UvrB is very broad, consistent with some degree of monomer–dimer exchange and/or conformational instability of the exposed dimer interface. Upon addition of unlabeled domain 4 peptide, the M632 resonance of UvrB sharpens and shifts to a position consistent with a UvrB–domain 4 heterodimer. A dissociation constant (K_D) value of 3.3 μM for the binding constant of UvrB with the domain 4 peptide was derived from surface plasmon resonance studies. Due to the flexibility of the domain 3–4 linker, inferred from limited proteolysis data and from the relaxation behavior of linker residue M607, the position of domain 4 is constrained not by the stiffness of the linking segment but by direct interactions with domains 1–3 in UvrB. In summary, UvrB homodimerization is disfavored, while domain 4 homodimerization and UvrB–domain 4 heterodimerization are allowed.

Keywords: UvrB; nucleotide excision repair (NER); NMR; surface plasmon resonance [methyl-13C]methionine UvrB

Introduction

Nucleotide excision repair (NER) is a universal DNA repair mechanism capable of acting on a broad range of structurally diverse lesions.1 Structural modifications ranging from benzo[a]pyrene–guanine adducts to more subtle modifications such as guanine O6-methylation can be recognized and corrected by the NER complex.2 In humans, the NER system is an important defense against many potentially genotoxic agents, including two major carcinogens, sunlight and cigarette smoke. Defects
in NER cause several diseases, most notably xeroderma pigmentosum, which are associated with increased incidences of cancer.9

The molecular components of the NER system in prokaryotes include three enzymes: UvrA, UvrB, and UvrC, collectively referred to as the UvrABC system, with UvrA and UvrB playing a critical role in damage recognition. The primary steps of the bacterial NER reaction pathway model can be briefly summarized as follows: (1) damage recognition by the UvrA–UvrB or UvrA–UvrB–UvrC complex, (2) damage verification whereby UvrA is released from the recognition complex and the damaged DNA remains bound to UvrB, and (3) recruitment of UvrC to the site of the lesion followed by the 3′ and 5′ incisions that excise the damage, resynthesize, and ligate the DNA.1,2,4–6

Elucidation of the intricate details involved in the damage recognition and verification steps prior to excision of the damage via systematic analysis of the different structural domains of each protein has been a major focus of our laboratory.

UvrA/UvrB protein–DNA cross-linking studies suggest that the site of DNA damage is initially in close contact with UvrA.7 Subsequent conformational changes in both the DNA and the proteins transfer the damaged DNA to UvrB for engagement by the damage recognition pocket. The first structure of a UvrB double-stranded DNA complex indicates the β-hairpin of UvrB bifurcates the two strands of DNA in close proximity to the lesion, confirming the model proposed by Theis et al.9,10 In the presence of damaged DNA, the flexible β-hairpin of UvrB adopts a different conformation from that of the apo enzyme crystal structure [Protein Data Bank (PDB) code 1TS[L]11 while maintaining key salt bridge and hydrophobic interactions between the tip of the β-hairpin and domain 1b to lock the inner DNA strand in place.9 Despite the emergence of a general understanding of NER outlined above, many fundamental aspects of the process remain unknown.

One current enigma is the role of the C-terminal domain (domain 4) in the mechanism of UvrB and its orientation relative to the rest of the protein. Truncated UvrB mutants lacking the C-terminal 43 residues are not able to form the UvrB–UvrC–DNA complex and are also strongly impaired in the 3′ incision reaction.12,13 Removal of domain 4 also has been shown to increase the affinity of UvrB for both undamaged and damaged DNA and to increase the ATPase activity by an order of magnitude, supporting an autoinhibitory role for this domain.14 Both crystal5 and solution state16 studies indicate that in isolation, domain 4 adopts a helix–loop–helix structure that forms a head-to-head dimer. However, crystal studies of intact UvrB have revealed a monomeric structure for domains 1–3, while domain 4 is not observed,9,11,17,18 presumably due to disorder.

In order to characterize the solution behavior of the 75-kDa UvrB protein, we have introduced [methyl-13C]methionine into Bacillus caldotenax UvrB (Bca UvrB), thereby providing NMR probes that are distributed throughout the molecule. In general, introduction of labeled methionine residues provides an ideal NMR labeling strategy for studies of large biological molecules due to the three equivalent methyl protons and the very low order parameters that characterize methionine methyl groups,19–21 resulting in narrow linewidths even for very large macromolecules. Further, the position of highly conserved M632 (numbering for Bca UvrB) at the domain 4 dimer interface makes its chemical shift an ideal probe for the oligomeric state of the UvrB.

In the present study, we have definitively assigned each of the 13 methionine methyl resonances using site-directed mutagenesis, utilized 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL)-induced shift and relaxation perturbations to evaluate the solvent accessibility of each methionine residue, and evaluated the structural effects of domain 2 and 4 deletions. Finally, analysis of M632 at the domain 4 dimer interface suggests that UvrB is primarily monomeric, even at the relatively high (100–300 μM) concentrations used for the NMR studies. This result implies the existence of a structural constraint in the full UvrB molecule that disfavors dimerization.

Results

NMR studies of site-directed mutants

The 659-residue UvrB protein from B. caldotenax contains 12 methionine residues as well as an N-terminal methionine that can be used as probes to obtain information about structure, conformational changes, and interactions with DNA and with the other proteins of the NER complex. The distribution of the methionine residues in Bca UvrB is illustrated in Fig. 1a. The assigned 1H–13C heteronuclear single-quantum coherence (HSQC) spectrum of the methionine methyl region of the spectrum of UvrB labeled with [methyl-13C]methionine is shown in Fig. 2a. Assignments of most of the methyl resonances were made based on the construction of site-directed methionine to leucine mutants (Table 1). In general, the effects of these mutations fall into two categories: The 1H–13C HSQC spectrum of M607L is characterized by a single missing resonance, allowing the immediate assignment of the mutated residue (e.g., Fig. 2b). The M350L, M457L, and M616L mutants all showed similarly simple spectra characterized by a single missing resonance, allowing the direct assignment of M350, M457, and M616 (data not shown). Alternatively, mutation of M256, M296, M297, M300, M551, or M560 leads to more complex spectral changes in which at least one other resonance shifts position. For example, the shift of M551 in the M560L mutant is shown in Fig. 2c. There are three pairs of methionine residues in UvrB for which the methyl groups are in sufficient proximity so that the Met→Leu mutation is expected to perturb the shift of the nearby residue (Fig. 1a and b). These correspond to M256–M297 (5.65 Å), M296–M300 (4.70 Å), and M551–M560 (3.44 Å) where the values
in parentheses are the distances between methyl carbon atoms taken from the structure 1T5L.11

The spectrum of the M256L mutant (Fig. 3a) reveals the expected shift perturbation of the nearby M297 methyl resonance. For the M296L substitution, the methyl resonances of M256, M297, and M300 are all shifted (Fig. 3b). The large shift perturbation for M297 in the M296L mutant is somewhat surprising, given the 10.9-Å distance between the methyl carbon atoms of M296 and M297 (Fig. 1b, crystal structure 1T5L). Apparently, the effect of the mutation appears to involve a structural perturbation that is transmitted through the bond, rather than an alteration in the local microenvironment of the methionine methyl group. Larger shift perturbations of the methyl resonances corresponding to M256 and to M300 are observed in the M296L,M297L double mutant (Fig. 3c). These results indicate that the four methionine residues clustered in domain 1b of the protein, M256, M296, M297, and M300, are

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**Fig. 1.** Distribution of methionine residues in Bca UvrB. (a) Domains 1a (yellow), 2 (purple), 1b (green), 3 (brick red), and 4 (pink) are indicated in the top panel, along with the positions of methionine residues labeled in the present study. The distribution of methionine residues in the protein structure (based on PDB code 1T5L11) is indicated in the lower panel, with methionine C’s indicated as cyan spheres, and the domain structures color-coded as in the top panel. Methionine residues M607 (linker region), and M616 and M632 (domain 4) are not included in the crystal structure, and thus not shown in the lower panel. (b) Expanded view of methionine residues 256, 296, 297, and 300 in domain 1b illustrating their relative orientation. (c) Partial view of the *E. coli* UvrB domain 4 dimer (PDB file 1E5216) showing residues M33 and F42. These correspond to M632 and F641 in Bca UvrB. Formation of the dimer places M33 from one monomer next to the edge of F42 from the second monomer, implying a downfield shift due to the ring current effect.
able to sense the subtle structural perturbations that result from individual M→L substitutions. This group of residues, and particularly M296 and M300, are positioned near the bound single-stranded DNA in the recent crystal structure of a UvrB–DNA complex, suggesting a potential role in damage recognition.

As noted above, the behavior of domain 4 is of interest since its position is not revealed in the crystal structure. A careful examination of the 1H–13C HSQC spectrum obtained for the M632L mutant reveals a broad resonance that is largely obscured by the resonances of M300, M607, and M0, which is eliminated or greatly reduced in this mutant (Fig. 4a). An expanded view of the 1H–13C HSQC spectrum obtained for the M632L UvrB mutant also helps to reveal this broader resonance, which has a 1H shift similar to that of M300 and a 13C shift ~0.1 ppm upfield of the M300 peak (Fig. 4b). The position of this resonance, δ(1H,13C) ~ (2.09,17.1) is fairly similar to the shifts assigned to the homologous residue, M33, in the spectrum of the E. coli UvrB domain 4 dimer: δ(1H,13C) = (2.26,17.30). The methyl group of residue M33 in the domain 4 structure (corresponding to M632 in Bca UvrB) is positioned near the edge of F42 (F641 in Bca UvrB) in the symmetry-related monomer (Fig. 1c), so that dimer formation is predicted to contribute to the downfield 1H shift. Broadening of the M632 methyl resonance observed for UvrB could result from a monomer–dimer exchange process or from instability of the exposed hydrophobic surface of the monomer.

Two additional methionine resonances, corresponding to M165 and M616, were assigned from the domain 2 and 4 deletions, respectively, described below. The final unassigned resonance at δ(1H,13C) ~ (2.09,16.9) was presumed to correspond to the N-terminal methionine. A comparison of the shift with spectra obtained for free methionine and for the Met–Val dipeptide corresponding to the two N-terminal residues of UvrB shows very similar shift values, consistent with the proposed assignment (spectra provided as Supplementary Data). Interestingly, although this residue is not observed in the crystal structure, presumably due to disorder, the NMR spectrum indicates that it apparently has not been processed off the enzyme by methionine aminopeptidase. N-terminal sequencing by Edman degradation was consistent with the presence of the N-terminal methionine residue (data not shown).

**Fig. 2.** Assigned 1H–13C HSQC spectrum of [methyl-13C]methionine-labeled Bca UvrB obtained at 45 °C. (a) Assignments of methionine methyl resonances based on studies of site-directed M→L mutants. Note that at the threshold used for the figure, M560 is visible as only a single contour. (b) Overlaid 1H–13C HSQC spectra of wild-type (black) and M607L UvrB (red). (c) Overlaid 1H–13C HSQC spectra of wild-type [methyl-13C]methionine UvrB (black) and the M560L mutant (red).

**Correlation of peak height with solvent accessibility, crystal B-factor**

Peak heights for the methionine methyl resonances, which correlate with transverse relaxation (T2) values, were observed to exhibit a significant correlation with both solvent accessibility and crystallographic B-factors for the methionine Cε (Fig. 5). Although the expectation that the motion of more solvent-exposed residues should be less
constrained, and therefore characterized by longer relaxation times and greater disorder (lower order parameters) seems intuitive, correlation of solvent accessibility with $S_2$ axis for methyl side chains generally has not been observed.\textsuperscript{23} Interestingly, the analysis by Mittermaier et al. of a set of eight proteins indicates that methionine appears to represent the single exception to this generalization.\textsuperscript{23} Thus, the data plotted in Fig. 5a are consistent with and extend the results of their study. It may be that the larger size of the protein used in the present study (molecular mass = 75 kDa) enhances this correlation since the relative significance of protein tumbling as a determinant of $T_2$ is increased.

Although the static disorder represented by the crystallographic B-factors does not necessarily correlate well with the dynamic order parameter derived from NMR studies, the consistency of the two plots shown in Fig. 5 presumably results from a similar dependence on packing density.\textsuperscript{24}

### Solvent accessibility—response to TEMPOL

Typically, solvent accessibility of enzyme residues has been evaluated based on the addition of relaxation reagents, which is monitored by broadening of the resonances\textsuperscript{25–28} or by reductions in $T_1$.\textsuperscript{29} Although TEMPOL-induced broadening has previously been used to evaluate the solvent accessibility of methionine residues in [methyl-$^{13}$C] methionine-labeled calmodulin,\textsuperscript{30} it was anticipated that the substantial variability of peak intensity in the labeled UvrB (Fig. 2a) would limit the utility of this approach. Esposito et al. have observed TEMPOL-induced $^1$H shifts in hen egg white lysozyme.\textsuperscript{26} Unexpectedly, addition of 20 mM TEMPOL to the UvrB M296L mutant (used due to its availability) resulted in both $^{13}$C shift and relaxation perturbations (Fig. 6). In surface accessibility studies, TEMPOL-induced shifts were concluded to be small by Petros et al.\textsuperscript{28} Shifts have been noted recently by Pintacuda and Otting in a study of ubiquitin, and these were ascribed to transient, specific TEMPOL–ubiquitin complexes.\textsuperscript{29} However, studies using a group of methyl-labeled compounds suggest that the shift perturbation is a general phenomenon that does not require protein binding and probably results from an incompletely averaged dipolar (pseudocontact) field due to some degree of orientational bias of the transient complex formed with the S-methyl group\textsuperscript{31} (see Supplementary Data). Further investigations of this effect are currently in progress.

The $^1$H-$^{13}$C HSQC spectrum of [methyl-$^{13}$C]UvrB M296L mutant before and after the addition of 20 mM TEMPOL is shown in Fig. 6, and several 1-D slices derived from this spectrum are included as Supplementary Data. In order to facilitate comparison of the extensively overlapped resonances, the spectrum of the TEMPOL-containing sample has been offset by 0.15 ppm in the $^1$H dimension only. It is immediately apparent that the resonances of the three buried methionine residues, M297, M551, and M560, are almost completely unaffected by the

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addition of the TEMPOL. The resonance corresponding to M256, which has about 7% exposure, shows a very slight downfield shift in the 13C dimension, consistent with a small degree of solvent exposure. Resonances arising from M350, M457, and probably M616 are broadened below the threshold of the spectrum, consistent with a large degree of solvent exposure. Resonances corresponding to M0, M165, M300, and M607 show both relaxation and shift perturbations. For these resonances, the degree of broadening is insufficient to reduce the peak height below the threshold of the plot. Additionally, the reduction in \( T_1 \) resulting from the interaction with TEMPOL will result in intensity increases if the spectra are overpulsed, which is typically the case for methionine methyl resonances. This is particularly true for the M0 and M607 peaks, which are the most intense in the spectrum. For this group of residues, the shift is thus a more readily observed indicator of solvent exposure than intensity or broadening.

Based on the response to TEMPOL, two residues not observed in the crystal structure, M607 and M616, exhibit a significant level of solvent exposure in intact UvrB. M607 is predicted to sit in the linker segment connecting domains 3 and 4, while M616 probably aligns with the solvent-exposed M17 residue in the structure of the Escherichia coli dimer.16 Of particular interest is the broad M632

Fig. 3. Conformational perturbations of domain 1b residues resulting from M→L mutations. (a) Overlay of the 1H-13C HSQC spectra of wild-type enzyme (black) and M256L (red). (b) Spectra of wild-type enzyme (black) overlaid with M296L UvrB (red). The shift of M300 is more clearly visible in the expanded region shown as an inset. (c) Spectra of wild-type enzyme (black) overlaid with doubly mutated M296L-M297L UvrB (red).

Fig. 4. Assignment of M632. (a) Expanded region of the 1H-13C HSQC spectra of M632L mutant (red) overlaid with that of the wild type [methyl-13C]methionine UvrB (black). (b) Expanded region of the M300L mutant (red) overlaid with that of the wild-type [methyl-13C]methionine UvrB (black). Elimination of the M300 resonance reveals the nearby M632 resonance more clearly.
resonance, which also appears to be shifted by the TEMPOL (Fig. 6). Due to the importance of understanding the behavior of domain 4, we also performed TEMPOL-based solvent-exposure studies on the methionine-labeled M607L UvrB mutant (Supplementary Data). This mutant was selected to allow better identification of the perturbed M632 resonance. Although there is still substantial overlap with the M300 resonance in this mutant, the TEMPOL study of M607L UvrB clearly reveals a significant shift and/or broadening of the M632 peak, consistent with a substantial degree of solvent exposure. As is apparent from Fig. 1c, in the dimer structure M33 (M632) is largely shielded from the solvent. Consequently, this is consistent with the conclusion that in solution, domain 4 of intact UvrB is not primarily dimeric.

Effects of domain deletions

Domain 4 is of particular interest as it is not observed in the Bca UvrB crystal structure but is nevertheless present according to SDS-PAGE gel analysis and mass spectrometry of the dissolved crystals. As expected from the assignment work outlined above, deletion of domain 4 eliminated both the sharper resonance assigned to M616, as well as the broader resonance corresponding to M632 (Fig. 7a). Additionally, the HSQC spectrum of [methyl-13C]methionine UvrBΔ4 reveals shift perturbations for methyl resonances corresponding to M350, M457, and M607. The shift for M607 is consistent with the fact that the Δ4 construct is terminated at residue K611, so that a perturbation of the immediate environment of M607 is predicted. The shift for M350 is of particular interest in light of data indicating that domain 4 acts as an autoinhibitory domain to inhibit ATP hydrolysis and DNA binding and since positioning of domain 4 near M350 would presumably occlude the DNA-binding site. However, the TEMPOL-induced broadening of the M350 resonance (Fig. 6) indicates that M350 is solvent accessible and hence not completely blocked by domain 4. The observed shift perturbation may therefore result from a more indirect, conformation-mediated perturbation.

Fig. 5. Correlations of methionine peak intensity with solvent accessibility (left) and with crystallographic B-factor (right). Crystallographic B-factors were taken from structure 1D9Z. Data for residues M0, M607, M616, and M632 are not included, since these residues are not present in the crystal structure.

Fig. 6. Solvent accessibility based on TEMPOL-induced resonance perturbations. Overlay of 1H-13C HSQC spectra of M296L [methyl-13C]methionine UvrB in standard buffer (black) and after the addition of 20 mM TEMPOL (red). The spectrum of the TEMPOL-containing sample has been offset upfield in the 1H dimension by 0.15 ppm in order to facilitate spectral comparison. The 1H axes in the absence and presence of TEMPOL are shown in black and red, respectively. Continuous blue lines connect resonances that are largely unchanged; dashed blue lines indicate resonances that are shifted in the 13C dimension, and in some cases broadened below the threshold of the plot.
A comparison of the HSQC spectra obtained for the Δ4 (Fig. 7a) and the Δ2Δ4 double-domain deletion constructs of UvrB (Fig. 7b) indicates that the latter results in more widely distributed shift perturbations. Nevertheless, for most of the methionine residues the magnitudes of these perturbations are fairly small, suggesting that the overall structure of domains 1 and 3 remains largely intact in the absence of domains 2 and 4. A comparison of the UvrBΔ4 with the UvrBΔ2Δ4 spectrum (Fig. 7b) indicated the disappearance of a single resonance, which could then be directly assigned to M165, the only methionine residue in domain 2 (Fig. 1a). In addition, the 1H–13C HSQC spectrum reveals a large shift for M256, smaller but significant shifts for M0, M297, and M300, as well as small shifts for M457 and M530. The shifts for M256, M297, and M300 are consistent with the relative proximity of these residues to domain 2 (Fig. 1a). In particular, the side chain of Q180 in domain 2 extends into a hydrophobic pocket containing M256, with Q180 Nε2 positioned 6.3 Å from the M256 methyl carbon.

Domain 4-UvrB interaction

As noted above, both X-ray crystallographic and NMR studies have shown that the isolated UvrB domain 4 adopts a coiled-coil conformation that forms a stable dimer (Fig. 1c). Nevertheless, the crystal structure of intact Bca UvrB does not reveal the position of domain 4 and does not provide evidence for dimerization. In order to further explore this apparent inconsistency, we studied an equimolar mixture of [methyl-13C]methionine M300L UvrB with unlabeled domain 4 peptide, corresponding to residues E620–G658. This peptide is somewhat shorter than the conventional domain boundary at P612 and was selected based on homology with the folded E. coli UvrB domain. The helical nature of the peptide was confirmed by circular dichroism (Supplementary Data). The M300L mutant was utilized in order to facilitate observation of the M632 resonance, which overlaps that of M300. The 1H–13C HSQC spectrum of the UvrB–domain 4 shown in Fig. 8 appears generally similar to the spectrum of M300L UvrB alone (Fig. 8).
4b), with the exception of a new resonance at δ(1H,13C)=(2.27,17.35). The shifts for this newly observed resonance are extremely close to those reported by Alexandrovich et al.22 for residue M33 in the E. coli domain 4 dimer, strongly supporting the conclusion that the new resonance arises from the labeled M632 residue of UvrB when it has formed a UvrB–domain 4 heterodimer. Additional support for this conclusion was obtained from a 1H–13C HSQC spectrum obtained on the unlabeled domain 4 peptide. Further confirmation of this interpretation was derived from a study of a sample containing the domain 4 peptide and [methyl-13C] methionine-labeled M632L UvrB. For this mixture, the newly identified resonance observed for the domain 4-UvrB sample was not present. We note that a broad resonance at the initial position of M632 is also observed for the domain 4-M300L UvrB mixture. Presumably, this results from the fact that some of the domain 4 peptide added forms a homodimer rather than a heterodimer with the UvrB.

**Quantitation of the domain 4–UvrB binding interaction by surface plasmon resonance**

The interaction of UvrB and UvrB domain 4 peptide was evaluated more quantitatively by surface plasmon resonance (SPR),33 following a protocol in which the domain 4 peptide was immobilized on a CM5 sensor chip. A linear (Scatchard plot) analysis and a more precise nonlinear fit of the Biacore data are shown in Fig. 9a and b, respectively. The initial Scatchard analysis yielded a dissociation constant (K_D) value of 5.4 μM. The sensorgram data were subsequently evaluated using several models for nonlinear binding included with the Biacore evaluative software. The best fit (lowest χ² value) was obtained using a heterogeneous ligand-binding model that assumes parallel reactions with the chip (Fig. 9b). This analysis yielded a K_D of 3.3 μM. The nonspecific binding inherent in this model probably arises from interactions between UvrB and non-optimally oriented domain 4 peptide, which has been covalently linked to the CM5 chip via lysine amino group(s), instead of, or in addition to, the N-terminal amino group. As an important control, no evidence of binding was observed if UvrBΔ4 rather than UvrB was used in the study, indicating that domain 4 on UvrB was necessary for the observed UvrB–domain 4 binding interaction (data not shown). This result is consistent with the conclusion that the UvrB–domain 4 complex involves a domain 4 dimer, similar to that observed in NMR16 and crystallographic15 studies of isolated domain 4. The values obtained are somewhat above the previously determined K_D range of 0.2–0.6 μM reported for the isolated domain 4 dimer from E. coli.

**Fig. 9.** SPR studies of UvrB–domain 4 peptide interaction. (a) Scatchard plot analysis of the sensorgram data using a linear regression fit. The slope of the line corresponds to K_D=5.44 μM. (b) Set of SPR sensorgrams corresponding to the interaction of Bca UvrB with immobilized UvrB domain 4 peptide. The curves correspond to a series of twofold dilutions of purified UvrB, which were injected and allowed to interact with the immobilized domain 4 peptide for 60 s at a flow rate of 35 μl/min at 25 °C. Curves a through h correspond to UvrB concentrations of 28 μM, 14 μM, 7 μM, 3.5 μM, 1.75 μM, 875 nM, 437.5 nM, and 218.75 nM, respectively, so that each curve was obtained by diluting the UvrB solution by a factor of 2. The global nonlinear regression fit (black lines) corresponds to the heterogeneous ligand (parallel reactions) model for the interaction of UvrB with UvrB domain 4 peptide.
UvrB. This variability may be related to the different conditions of the studies, the difference in species, or to the limitation on dimer formation by the domain when it is linked to the rest of the UvrB molecule, as discussed below.

**Limited proteolysis of Bca UvrB**

As noted above, the intensity of the M607 resonance is consistent with its location in a flexible loop connecting UvrB domains 3 and 4. Previous limited proteolysis studies of both the *E. coli* and *Thermus thermophilus* UvrB with trypsin have demonstrated rapid cleavage of domain 4, consistent with a highly exposed and flexible loop connecting these two domains. Analogous studies performed on Bca UvrB demonstrate a qualitatively similar lability of this enzyme, indicative of the lability of domain 4 and the apparent flexibility/accessibility of the domain 3–domain 4 linker segment (Supplementary Data).

**Discussion**

Since the process of DNA repair by the UvrABC system involves formation of a series of complexes with the damaged DNA, characterization of the oligomeric structures that form during NER is central to understanding this process. This characterization has been constrained by the apparent inconsistency that although UvrB domain 4 in isolation is observed to dimerize (Fig. 1c), there is currently no crystallographic evidence for dimeric UvrB. M632 represents an ideal probe for domain 4 dimerization, since it sits at the dimer interface and is positioned at the edge of F641 of the symmetry-related domain (Fig. 1c). In the present study, we found that M632 yields a broad resonance near δ^1H=2.09 ppm. The broad linewidth could result from a monomer–dimer exchange process or from the fact that the hydrophobic interface region of the isolated monomer is conformationally unstable and subject to significant conformational exchange broadening. However, the shift and linewidth results indicate that even at the relatively high concentrations of the NMR studies (>100 μM), UvrB is not primarily in the dimer form. This conclusion is further supported by the effect of TEMPO adduction on the M632 resonance. The apparent TEMPO-induced shift of the M632 resonance (Fig. 6) is inconsistent with the inaccessibility of M632 in the dimer structure (Fig. 1c), also supporting the conclusion that domain 4 is not primarily in the dimeric state. These observations are thus not consistent with a model in which domain 4 sits at the end of a flexible linker that would allow dimer formation (Fig. 10a).

More direct information about the dimerization of UvrB and the effect of domain 4 was obtained in the study of the effect of added domain 4 peptide on the UvrB HSQC spectrum. Addition of unlabeled domain 4 peptide resulted in the appearance of a new resonance at the expected position of M632 in the domain 4 dimer. Hence, a UvrB–domain 4 heterodimer is apparently formed (Fig. 10b). This result indicates that there is some type of structural barrier inhibiting UvrB dimerization, but apparently not limiting the ability of UvrB to form a heterodimer with the domain 4 peptide. The obvious implication of this study is that UvrB dimerization involving domain 4 is in some way limited by a steric conflict that involves some portions of domains 1a, 1b, 2, and/or 3 (Fig. 10c). This in turn requires that the position of domain 4 relative to domains 1–3 must be constrained. If, alternatively, domain 4 were
positioned at the end of a fully flexible linker, there should be no impediment to dimerization (Fig. 10a). There are two ways that this constraint might be achieved: (1) the linker is not completely flexible, but is sufficiently stiff to constrain the position of domain 4; (2) the linker is fully flexible, but the position of domain 4 is partially constrained due to direct interactions with domains 1–3. The NMR data obtained for M607, which is positioned in the linker, show a high degree of solvent exposure (TEMPOL-induced shift and relaxation rate enhancements) and mobility (M607 resonance is relatively sharp and intense), consistent with the conclusion that the linker is highly flexible and does not limit the position of domain 4.

This conclusion is consistent with the lability of the linker demonstrated by the limited proteolysis study (Supplementary Data). Data consistent with lability of the domain 3–domain 4 linker have also been obtained for the E. coli,34 T. thermophilus,18 and Bacillus subtilis32 enzymes. In the case of the B. subtilis UvrB* crystal structure, a 31-residue segment connecting domains 3 and 4 that includes the site of proteolysis is not present in the recently reported crystal structure (2D7D).32 The B. subtilis enzyme, which has 82% sequence identity with the B. caldopenax enzyme, exhibits a high degree of structural homology that is illustrated, for example, by the agreement between the 1H shifts observed for the methionine methyl resonances of Bca [methyl-13C] methionine-labeled UvrB and the 1H shifts calculated from the crystal structure of the B. subtilis enzyme (Supplementary Data). Structural comparisons for UvrB domains 1–3 and domain 4 based on available structural data for the E. coli, B. caldopenax, B. subtilis, and T. thermophilus enzymes suggest that the domain 3–linker and linker–domain 4 boundaries occur at similar, if not identical positions (Supplementary Data). Hence, the present study as well as previous work on UvrB from other sources is consistent with a structure in which a highly flexible linker probably does not constrain the position of domain 4. We thus conclude that the inhibition of UvrB dimerization results instead from a direct interaction of domain 4 with domains 1–3 of UvrB, such that dimer formation leads to steric conflict of the latter (Fig. 10c).

In combination, the NMR and crystallographic studies of UvrB indicate that domain 4 may be weakly tethered to domains 1–3, sufficient to limit dimer formation, but not so rigidly as to be inconsistent with the disorder that apparently characterizes this domain in the crystalline state. The above analysis may be further elaborated in the context of recent structural data obtained for B. subtilis UvrB*, which shows domains 1–3 in contact with a domain 4 dimer. In that structure (PDB code 2D7D) the long axis of domain 4 is roughly parallel with the long axis of UvrB1–3 and is apparently positioned by two salt bridges. This interface between domains 3 and 4 is quite insubstantial; however, such a tenuous interface would be consistent with the crystal structures in which domain 4 is generally not observed. Further, if we try to introduce a second UvrB molecule by assuming that the paired domain 4 also is involved in analogous salt bridge interactions, we run into steric conflicts of the type illustrated in Fig. 10c.

To summarize, the NMR results obtained for [methyl-13C]methionine UvrB indicate that (1) it is substantially monomeric, with the interface residue M632 broadened due to some degree of monomer–dimer exchange and/or conformational instability; (2) accessibility of M632 to TEMPOL is generally not consistent with a UvrB dimer structure, and hence supports conformational instability as the more likely explanation for the observed broadening; (3) the position of domain 4 appears not to be constrained by the stiffness of the linking residues, but by direct interactions with domains 1–3 of UvrB; (4) the orientational constraint on domain 4 does not inhibit formation of a UvrB–domain 4 heterodimer, but does disfavor dimerization of the full UvrB molecule, presumably due to steric conflict of domains 1–3 in the dimer structure. The observed UvrB–domain 4 heterodimer may be structurally similar to the recently observed B. subtilis structure of UvrB domains 1–3 plus a domain 4 dimer, although this remains to be demonstrated. Finally, the direct interaction of domain 4 with domains 1–3 of UvrB suggests that the autoinhibitory effect of this domain may not require the linker, and hence could be produced by addition of domain 4 to domains 1–3 of UvrB. In our view, the tendency of isolated domain 4 and, as shown here, the UvrB–domain 4 mixture to dimerize is consistent with a functional role for a UvrB dimer in some part of the NER process, but in order for this to occur, additional factors must intervene to release the structural constraints on domain 4. For example, Verhoeven et al. find evidence for a UvrB2–DNA complex, suggesting that the presence of DNA may, at least under some circumstances, be sufficient to allow UvrB dimerization to occur. Additionally, the limited ability of UvrB to form a homodimer may facilitate UvrB–UvrC heterodimer formation, particularly as these reactions appear to be at least partially competitive.

Materials and Methods

Materials

Oligonucleotides used as PCR primers for site-directed mutagenesis were purchased from Sigma-Genosys (Woodland, TX) and from IDT DNA (Coralville, IA). Unlabeled L-amino acids were obtained from Sigma (St. Louis, MO). IPTG was obtained from Gold Biotechnology, Inc. (St. Louis, MO).

Cloning, expression, and purification of Bca UvrB proteins

Genes encoding Bca UvrB were subcloned in the pTYB1 vector of the T7 IMPACT system (New England Biolabs) as
Previously described. The domain 4/domain 2 truncation mutation of Bca UvrB [UvrBA2A4, sequences between Pro612 to Gly658 (domain 4) and Leu157 and Pro245 (domain 2) deleted]Int as well as each [ε-13C]methionine to leucine point mutations were constructed following the protocol of the QuikChange site-directed mutagenesis kit (Stratagene). Synthetic oligonucleotides were designed to replace the ATG codons (Met) with CTG (Leu). DNA sequencing confirmed the presence of the desired deletion and absence of additional mutations in the entire amplified uvrB gene. All UvrB proteins were expressed in BL21-CodonPlus®(DE3)-RIL cells (Stratagene). All of the mutant proteins utilized in this study are listed in Table 1.

For the purpose of NMR analysis, Bca UvrB proteins were expressed at 37 °C using an enriched medium containing all 19 standard, unlabeled amino acids, plus [ε-13C]methionine (CIL, Cambridge, MA), which is expected to repress endogenous synthesis of methionine by the bacteria. Protein expression was induced by IPTG (0.3 mM), and cell growth was continued for approximately 3–4 h at 30 °C. Cells were harvested by centrifugation (5000 g, 5 min) and stored at −80 °C. All proteins were purified using the IMPACT CN system (New England Biolabs) as described previously with slight modification. Briefly, pelleted cells were quick-thawed (37 °C, 2 min) and resuspended in column wash buffer (20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 0.1 mM EDTA, 0.8% Triton X-100) containing 5 μg/ml leupeptin followed by sonication. Upon centrifugation (20000 g, 30 min), the supernatant was incubated overnight with the chitin resin at 4 °C. The resin/supernatant mixture was transferred to a column and eluted with 10 column volumes of column wash buffer. The cleavage of the fusion protein (UvrB–intein–chitin-binding domain) began by flushing the column with one column volume of cleavage buffer (20 mM Tris–HCl, pH 8.0, 300 mM NaCl, 30 mM DTT) and incubating overnight at 4 °C. Fractions were collected and analyzed by SDS-PAGE gel electrophoresis. Proteins used in this study are more than 95% pure as judged by staining of SDS-PAGE protein gel with SimplyBlue™ SafeStain (Invitrogen).

NMR spectroscopy

Prior to NMR analysis, UvrB samples were exchanged into NMR buffer [40 mM Tris–HCl, pH 7.4, 150 mM KCl, 200 μM sodium azide, 100 μM 2,2-dimethylsilapentane-5-sulfonic acid (DSS) as an internal chemical shift standard, in D2O] and concentrated to approximately 0.2 mM. For the NMR studies, the concentration of each UvrB mutant was between 100 and 200 μM. It was determined that these samples are stable and yield high-quality NMR data at 45 °C. Data were collected on Varian UNITY INOVA 500- and 600-MHz NMR spectrometers. 1H-13C HSQC spectral data were collected utilizing the Varian gChsqc sequence. Experimental data were processed using a squared cosine bell apodization function in both dimensions. Spectra were processed using NMRPipe version 2.139 and analyzed with NMRview software, version 5.0.40

Domain 4 peptide

The UvrB domain 4 peptide, a 39-mer EREELIRL AEMKEAAKAL DFERAQLR DDIFELKAEQ, was obtained from Global Peptide, Inc. (Ft. Collins, CO). This peptide, corresponding to residues E620–G658, lacks several residues at the N terminus typically included with domain 4, but alignment with the sequence of the previously studied E. coli domain 4 suggested that it should be sufficient to form the coiled-coil structure previously observed by Alexandrovich et al. Characterization of this peptide by circular dichroism yielded a spectrum qualitatively consistent with high α-helical content (Supplementary Data), consistent with the previously reported solution NMR results.16

SPR studies

The binding kinetics of UvrB domain 4 peptide with UvrB were determined by SPR analysis using a Biacore 3000™ biosensor system (Biacore, Uppsala, Sweden). UvrB domain 4 peptide was covalently immobilized on a carboxymethylated dextran matrix (research CM5 sensor chip) at a concentration of 10 nM in 10 mM sodium acetate (pH 4.0), using the amine coupling kit [1-ethyl-3-(dimethylaminopropyl)carbodiimide, N-hydroxysuccinimide supplied by the manufacturer]. The unreacted moieties on the surface were blocked with ethanolamine. To evaluate the Kd value corresponding to the binding of the Bca UvrB with the immobilized UvrB domain 4 peptide, twofold serial dilutions of UvrB (218.75 nM–28 μM) in running buffer [10 mM Hepes, 150 mM NaCl, and 0.005% (v/v) surfactant P-20 at pH 7.4] flowed over the peptide chip at a rate of 35 μl/min at 25 °C. The dissociation constant (Kd) was first evaluated by the Scatchard plot. A more precise fit of the data was also determined by a global nonlinear regression fit to a heterogeneous ligand (parallel reactions) model using the BIAevaluation 4.1 software (Biacore).

Nomenclature

The UvrB methionine residues are numbered beginning with residue V1 in order to be consistent with the numbering in the crystal structure. Since we observed a resonance attributed to the N-terminal methionine, and based on Edman sequencing results, according to which the N-terminal sequence is M-V-E-G-R-F-Q, the N-terminal methionine is present and is numbered as M0.

Surface accessibility studies

TEMPOL was suspended in the appropriate NMR buffer at pH 7.4 and titrated in 20 mM increments with wild-type [methyl-13C]methionine UvrB to a final concentration of 100 mM. It was found that the 1H resonance of the HDO solvent peak shifted significantly with the addition of the TEMPOL, but that no 1H shifts of the solute resonances were observed if DSS was used as an internal 1H shift standard. Solvent accessibility of each methionine observed in the crystal structure of UvrB was calculated using the Profiles-3D Verify function of Discovery Studio (Accelrys, Inc., San Diego, CA) from the coordinates listed in the PDB (code 1TSL).

Note added in proof

While this manuscript was in press, the following article appeared which strongly supports our findings that in solution and in the absence of UvrA or DNA, UvrB is...

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2007.07.045

References


