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

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Summary

UvrB is a central DNA damage recognition protein involved in bacterial nucleotide excision repair (NER). 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 solution behavior of UvrB, we have performed NMR studies on [methyl-¹³C]methionine-labeled UvrB from *B. caldotenax* (MW = 75 kD). 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-tetramethylpiperidyl-1-oxyl (TEMPOL). Methionine 632, 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 K_D value of 3.3 μ M 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.

Introduction

Nucleotide excision repair (NER) is a universal DNA repair mechanism capable of acting on a broad range of structurally diverse lesions¹. Structural modifications ranging from benzo[a]

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pyrene-guanine adducts to more subtle modifications such as guanine O6-methylation, can be recognized and corrected by the NER complex². 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, that are associated with increased incidences of cancer³.

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 briefly be summarized as follows: 1) damage recognition by the UvrA₂B or UvrA₂B₂ 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, re-synthesize 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 main focus of our laboratory.

UvrA/UvrB protein-DNA cross-linking studies suggest that the site of DNA damage is initially in close contact with UvrA⁷. 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 complex⁸ 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 (PDB 1T5L),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 place⁸. Despite the emergence of a general understanding of nucleotide excision repair outlined above, many fundamental aspects of the process remain unknown.

One current enigma is the role of 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 domain¹⁴. Both crystal¹⁵ 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 kD UvrB protein, we have introduced [methyl-¹³C]methionine into *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 groups¹⁹⁻²¹, 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 thirteen methionine methyl resonances using site-directed mutagenesis, utilized TEMPOL-induced shift and relaxation perturbations to evaluate the solvent accessibility of each methionine residue, and have 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 μ M) 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 Figure 1a. The assigned ^1H - ^{13}C HSQC spectrum of the methionine methyl region of the spectrum of UvrB labeled with [methyl- ^{13}C]methionine is shown in Figure 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 - ^{13}C HSQC spectrum of M607L is characterized by a single missing resonance, allowing the immediate assignment of the mutated residue (e.g., Figure 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 Figure 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 (Figures 1a & 1b). 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 carbons, taken from structure 1T5L¹¹.

The spectrum of the M256L mutant (Figure 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 (Figure 3b). The large shift perturbation for M297 in the M296L mutant is somewhat surprising, given the 10.9 Å distance between the methyl carbons of M296 and M297 (Figure 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 (Figure 3c). These results indicate that the four methionine residues clustered in domain 1b of the protein, M256, M296, M297, and M300, are 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 ssDNA in the recent crystal structure of a UvrB-DNA complex⁸, 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 structure¹⁷. A careful examination of the ^1H - ^{13}C 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 (Figure 4a). An expanded view of the ^1H - ^{13}C HSQC spectrum obtained for the M300L UvrB mutant also helps to reveal this broader resonance, which has a ^1H shift similar to that of M300 and a ^{13}C shift ~ 0.1 ppm upfield of the M300 peak (Figure 4b). The position of this resonance, $\delta(^1\text{H}, ^{13}\text{C}) \sim (2.09, 17.1)$ is fairly similar to the shifts assigned to the homologous residue, M33, in the spectrum of the *E. coli* UvrB domain 4 dimer: $\delta(^1\text{H}, ^{13}\text{C}) = (2.26, 17.30)$ ²². 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 (Figure 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 described below. The final unassigned resonance at $\delta(^1\text{H}, ^{13}\text{C}) = (2.09, 16.9)$, 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 Material-S1). 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).

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

Peak heights for the methionine methyl resonances, which correlate with transverse relaxation (T_2) values, were observed to exhibit a significant correlation with both solvent accessibility and crystallographic B-factors for the methionine C ϵ (Figure 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 observed²³. Interestingly, the analysis by Mittermaier et al.²³ of a set of eight proteins indicates that methionine appears to represent the single exception to this generalization. Thus, the data plotted in Figure 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 (MW = 75 kD) 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 Figure 5 presumably results from a similar dependence on packing density²⁴.

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 resonances²⁵⁻²⁸ or by reductions in T_1 ²⁹. Although TEMPOL-induced broadening has previously been used to evaluate the solvent accessibility of methionine residues in [methyl- ^{13}C]methionine-labeled calmodulin³⁰, it was anticipated that the substantial variability of peak intensity in the labeled UvrB (Figure 2a) would limit the utility of this approach. 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 (Figure 6). In surface accessibility studies, TEMPOL-induced shifts were concluded to be small by Petros et al.²⁸. Shifts have been noted recently by Pintacuda and Otting in a study of ubiquitin, and these were ascribed to transient, specific TEMPOL-ubiquitin complexes²⁹. 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 (pseudo-contact) field due to some degree of orientational bias of the transient complex formed with the S-methyl group³¹ (see Supplementary Material-S2). Further investigations of this effect are currently in progress.

^1H - ^{13}C HSQC of [methyl- ^{13}C] UvrB M296L mutant before and after the addition of 20 mM TEMPOL are shown in Figure 6, and several 1D slices derived from this spectrum are included as Supplementary Material-S2. 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 ^1H 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 addition of the TEMPOL. The resonance corresponding to M256, which has about 7 % exposure, shows a very slight downfield shift in the ^{13}C 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, 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 Met17 residue in the structure of the *E. coli* dimer¹⁶. Of particular interest is the broad M632 resonance, which also appears to be shifted by the TEMPOL (Figure 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 Material-S2). 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 Figure 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 crystals³². 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 (Figure 7a). Additionally, the HSQC spectrum of [methyl- ^{13}C]methionine UvrB $\Delta 4$ reveals shift perturbations for methyl resonances corresponding to M350, M457, and M607. The shift for Met607 is consistent with the fact that the $\Delta 4$ 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^{11,14} and since positioning of domain 4 near Met350 would presumably occlude the DNA binding site. However, the TEMPOL-induced broadening of the M350 resonance (Figure 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.

A comparison of the HSQC spectra obtained for the $\Delta 4$ (Figure 7a) and the $\Delta 2\Delta 4$ double domain deletion constructs of UvrB (Figure 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 (Figure 7b) indicated the disappearance of a single resonance, which could then be directly assigned to M165, the only methionine residue in domain 2 (Figure 1a). In addition, the ^1H - ^{13}C HSQC spectrum reveals a large shift for M256, smaller but significant shifts for M0, M297, M300, as well as small shifts for Met457 and Met350. The shifts for Met256, Met297, and Met300 are consistent with the relative proximity of these residues to domain 2 (Figure 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 crystallographic¹⁵ and NMR¹⁶ studies have shown that the isolated UvrB domain 4 adopts a coiled-coil conformation which forms a stable dimer (Figure 1c). Nevertheless, the crystal structure of intact *Bca* UvrB does not reveal the position of domain 4 and does not provide evidence for dimerization^{9,11}. In order to further explore this apparent inconsistency, we studied an equimolar mixture of [methyl- ^{13}C]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^{14,16}. The helical nature of the peptide was confirmed by circular dichroism (Supplementary Material-S3). The M300L mutant was utilized in order to facilitate observation of the M632 resonance, which overlaps that of M300. The ^1H - ^{13}C HSQC spectrum of the UvrB-domain 4 shown in Figure 8 appears generally similar to the spectrum of M300L UvrB alone (Figure 4b), with the exception of a new resonance at $\delta(^1\text{H}, ^{13}\text{C}) = (2.27, 17.35)$. 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 - ^{13}C 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- ^{13}C]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 SPR

The interaction of UvrB and UvrB domain 4 peptide was evaluated more quantitatively by surface plasmon resonance³³, following a protocol in which the domain 4 peptide was immobilized on a CM5 sensor chip. A linear (Scatchard plot) analysis, as well as a more precise non-linear fit of the Biacore data, are shown in Figures 9a and b, respectively. The initial Scatchard analysis yielded a $K_D = 5.4 \mu\text{M}$. The sensorgram data were subsequently evaluated using several models for nonlinear binding included with the Biacore evaluative software. The best fit (lowest χ^2 value) was obtained using a heterogeneous ligand binding model which assumes parallel reactions with the chip (Figure 9b). This analysis yielded a $K_D = 3.3 \mu\text{M}$. The non-specific 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* UvrB¹⁶. 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*³⁴ and *T. thermophilus*¹⁸ 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 Material-S4).

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^{15,16} (Figure 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 (Figure 1c). In the present study, we found that M632 yields a broad resonance near $\delta^1\text{H} = 2.09$ 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 ($> 100 \mu\text{M}$), UvrB is not primarily in the dimer form. This conclusion is further supported by the effect of TEMPOL addition on the M632 resonance. The apparent TEMPOL-induced shift of the M632 resonance (Figure 6) is inconsistent with the inaccessibility of M632 in the dimer structure (Figure 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 (Figure 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 (Figure 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 (Figure 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 (Figure 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 Material-S4). Data consistent with lability of the domain 3 - domain 4 linker have also been obtained for the *E. coli*³⁴, *T. thermophilus*¹⁸ and *B. subtilis*³² 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. caldotenax* 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 Material-S5). Structural comparisons for UvrB domains 1-3 and domain 4 based on available structural data for the *E. coli*, *B. caldotenax*, *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 Material-S6). Hence, the present study as well as previous work on UvrB from other sources are 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 (Figure 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 domain 1-3 in contact with a domain 4 dimer. In that structure (PDB code 2D7D) the long axis of domain 4 is roughly parallel to 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 Figure 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.³⁵ 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 competitive¹⁶.

Materials and Methods

Materials

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

Cloning, Expression, and Purification of *B. caldotenax* UvrB Proteins

Genes encoding *B. caldotenax* UvrB were subcloned in the pTYB1 vector of the T7 IMPACT system (New England Biolabs) as previously described³⁶. The domain 4/domain 2 truncation mutation of *B. caldotenax* UvrB {UvrB Δ 2 Δ 4, sequences between Pro612 to Gly658 (domain 4) and Leu157 and Pro245 (domain 2) deleted}, 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 bacteria³⁷. Protein expression was induced by IPTG (0.3 mM), and cell growth was continued for approximately 3-4 hours 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 previously⁹ 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, 0.5% Triton X-100) containing 5 μ g/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-CBD) began by flushing the column with one column volume of cleavage buffer (column wash buffer containing 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 μ M sodium azide, 100 μ M DSS as an internal chemical shift standard, in D₂O) and concentrated to approximately 0.5 mM. It was determined that these samples are stable and yield high quality nuclear magnetic resonance (NMR) data at 45 °C. Data was collected on Varian UNITY INOVA 500 and 600 MHz NMR spectrometers. ¹H-¹³C HSQC spectral data was collected utilizing the Varian gChsqc sequence³⁸. 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 5.0.4⁴⁰.

Domain 4 peptide

The UvrB domain 4 peptide, a 39mer - EREELIRTLE AEMKEAAKAL DFERAAQLRD IIFELKAEG, 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.¹⁶. Characterization of this peptide by circular dichroism yielded a spectrum qualitatively consistent with high α -helical content (Supplementary Material), consistent with the previously reported solution NMR results¹⁶.

Surface Plasmon Resonance (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 μ M 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 dissociation constant (K_D) corresponding to the binding of the Bca UvrB with the immobilized UvrB domain 4 peptide, 2-fold serial dilutions of UvrB (218.75 nM-28 μ M) 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 μ l/min at 25 °C. The dissociation constant (K_D) 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 structure⁸. 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

4-hydroxy-2,2,6,6-tetramethylpiperidiny-1-oxy (TEMPOL) was suspended in the appropriate NMR buffer at pH 7.4 and titrated in 20 mM increments with WT [methyl-¹³C]-Met 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 was 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 Protein Data Bank (PDB, entry 1T5L).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations and Textual Footnotes

ABC ATPase, ATP-binding cassette ATPase
 Bca, *B. caldopenax* or *Bacillus caldopenax*
 CBD, chitin-binding domain
 dsDNA, double-stranded DNA
 DSS, 2,2-dimethylsilapentane-5-sulfonic acid
 DTT, dithiothreitol
 EMSA, electrophoretic mobility shift assay
 HSQC, heteronuclear single-quantum coherence
 NER, nucleotide excision repair
 NMR, nuclear magnetic resonance
 IPTG, isopropyl-1-thio- β -D-galactopyranoside
 PDB, Protein Data Bank
 SPR, surface plasmon resonance
 TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidiny-1-oxyl
 UvrB₁₋₃, UvrB domains 1, 2 and 3
 Wt, wild type

References

1. Van Houten B, Croteau DL, DellaVecchia MJ, Wang H, Kisker C. 'Close-fitting sleeves': DNA damage recognition by the UvrABC nuclease system. *Mutat Res* 2005;577:92–117. [PubMed: 15927210]
2. Truglio JJ, Croteau DL, Van Houten B, Kisker C. Prokaryotic Nucleotide Excision Repair: The UvrABC System. *Chem Rev* 2006;106:233–252. [PubMed: 16464004]
3. Cleaver JE. Cancer in xeroderma pigmentosum and related disorders of DNA repair. *Nat Rev Cancer* 2005;5:564–73. [PubMed: 16069818]
4. Van Houten B. Nucleotide excision repair in *Escherichia coli*. *Microbiol Rev* 1990;54:18–51. [PubMed: 2181258]
5. Goosen N, Moolenaar GF. Role of ATP hydrolysis by UvrA and UvrB during nucleotide excision repair. *Res Microbiol* 2001;152:401–9. [PubMed: 11421287]
6. Croteau, DL.; Dellavechia, MJ.; Skorvaga, M.; Van Houten, B. Damage recognition by the bacterial nucleotide excision repair machinery. In: Wolfram, S.; Kow, YW.; Doetsch, PW., editors. *DNA Damage Recognition*. Taylor & Francis; Boca Raton, FL: 2005. p. 111-138.
7. DellaVecchia MJ, Croteau DL, Skorvaga M, Dezhurov SV, Lavrik OI, Van Houten B. Analyzing the Handoff of DNA from UvrA to UvrB Utilizing DNA-Protein Photoaffinity Labeling. *J. Biol. Chem* 2004;279:45245–45256. [PubMed: 15308661]
8. Truglio JJ, Karakas E, Rhau B, Wang H, DellaVecchia MJ, Van Houten B, Kisker C. Structural basis for DNA recognition and processing by UvrB. *Nat Struct Mol Biol* 2006;13:360–4. [PubMed: 16532007]
9. Theis K, Chen PJ, Skorvaga M, Van Houten B, Kisker C. Crystal structure of UvrB, a DNA helicase adapted for nucleotide excision repair. *EMBO J* 1999;18:6899–907. [PubMed: 10601012]
10. Theis K, Skorvaga M, Machius M, Nakagawa N, Van Houten B, Kisker C. The nucleotide excision repair protein UvrB, a helicase-like enzyme with a catch. *Mutat Res* 2000;460:277–300. [PubMed: 10946234]
11. Truglio JJ, Croteau DL, Skorvaga M, DellaVecchia MJ, Theis K, Mandavilli BS, Van Houten B, Kisker C. Interactions between UvrA and UvrB: the role of UvrB's domain 2 in nucleotide excision repair. *EMBO J* 2004;23:2498–509. [PubMed: 15192705]
12. Moolenaar GF, Franken KL, Dijkstra DM, Thomas-Oates JE, Visse R, van de Putte P, Goosen N. The C-terminal region of the UvrB protein of *Escherichia coli* contains an important determinant for UvrC binding to the preincision complex but not the catalytic site for 3'-incision. *J Biol Chem* 1995;270:30508–15. [PubMed: 8530482]

13. Moolenaar GF, Franken KL, van de Putte P, Goosen N. Function of the homologous regions of the *Escherichia coli* DNA excision repair proteins UvrB and UvrC in stabilization of the UvrBC-DNA complex and in 3'-incision. *Mutat Res* 1997;385:195–203. [PubMed: 9506888]
14. Wang H, DellaVecchia MJ, Skovvaga M, Croteau DL, Erie DA, Van Houten B. UvrB domain 4: an autoinhibitory gate for regulation of DNA binding and ATPase activity. *J Biol Chem* 2006;281:15227–37. [PubMed: 16595666]
15. Sohi M, Alexandrovich A, Moolenaar G, Visse R, Goosen N, Vernede X, Fontecilla-Camps JC, Champness J, Sanderson MR. Crystal structure of *Escherichia coli* UvrB C-terminal domain, and a model for UvrB-UvrC interaction. *FEBS Lett* 2000;465:161–4. [PubMed: 10631326]
16. Alexandrovich A, Czisch M, Frenkiel TA, Kelly GP, Goosen N, Moolenaar GF, Chowdhry BZ, Sanderson MR, Lane AN. Solution structure, hydrodynamics and thermodynamics of the UvrB C-terminal domain. *J Biomol Struct Dyn* 2001;19:219–36. [PubMed: 11697728]
17. Machius M, Henry L, Palnitkar M, Deisenhofer J. Crystal structure of the DNA nucleotide excision repair enzyme UvrB from *Thermus thermophilus*. *Proc Natl Acad Sci U S A* 1999;96:11717–22. [PubMed: 10518516]
18. Nakagawa N, Masui R, Kato R, Kuramitsu S. Domain structure of *Thermus thermophilus* UvrB protein. Similarity in domain structure to a helicase. *J Biol Chem* 1997;272:22703–13. [PubMed: 9278428]
19. Prabhu NV, Lee AL, Wand AJ, Sharp KA. Dynamics and entropy of a calmodulin-peptide complex studied by NMR and molecular dynamics. *Biochemistry* 2003;42:562–70. [PubMed: 12525185]
20. Bose-Basu B, DeRose EF, Kirby TW, Mueller GA, Beard WA, Wilson SH, London RE. Dynamic characterization of a DNA repair enzyme: NMR studies of [methyl-¹³C]methionine-labeled DNA polymerase beta. *Biochemistry* 2004;43:8911–22. [PubMed: 15248749]
21. Mittermaier A, Davidson AR, Kay LE. Correlation between ²H NMR side-chain order parameters and sequence conservation in globular proteins. *J Am Chem Soc* 2003;125:9004–5. [PubMed: 15369343]
22. Alexandrovich A, Sanderson MR, Moolenaar GF, Goosen N, Lane AN. NMR assignments and secondary structure of the UvrC binding domain of UvrB. *FEBS Lett* 1999;451:181–5. [PubMed: 10371161]
23. Mittermaier A, Varani L, Muhandiram DR, Kay LE, Varani G. Changes in side-chain and backbone dynamics identify determinants of specificity in RNA recognition by human U1A protein. *J Mol Biol* 1999;294:967–79. [PubMed: 10588900]
24. Ming D, Bruschweiler R. Prediction of methyl-side chain dynamics in proteins. *J Biomol NMR* 2004;29:363–8. [PubMed: 15213434]
25. Improta S, Molinari H, Pastore A, Consonni R, Zetta L. Probing protein structure by solvent perturbation of NMR spectra. A comparison with photochemically induced dynamic nuclear polarization techniques applied to native alpha-lactalbumin. *Eur J Biochem* 1995;227:78–86. [PubMed: 7851445]
26. Esposito G, Lesk AM, Molinari H, Motta A, Niccolai N, Pastore A. Probing protein structure by solvent perturbation of nuclear magnetic resonance spectra. Nuclear magnetic resonance spectral editing and topological mapping in proteins by paramagnetic relaxation filtering. *J Mol Biol* 1992;224:659–70. [PubMed: 1314901]
27. Molinari H, Esposito G, Ragona L, Pegna M, Niccolai N, Brunne RM, Lesk AM, Zetta L. Probing protein structure by solvent perturbation of NMR spectra: the surface accessibility of bovine pancreatic trypsin inhibitor. *Biophys J* 1997;73:382–96. [PubMed: 9199802]
28. Petros AM, Mueller L, Kopple KD. NMR identification of protein surfaces using paramagnetic probes. *Biochemistry* 1990;29:10041–8. [PubMed: 2176860]
29. Pintacuda G, Otting G. Identification of protein surfaces by NMR measurements with a paramagnetic Gd(III) chelate. *J. Amer. Chem. Soc* 2002;124:372–373. [PubMed: 11792196]
30. Yuan T, Ouyang H, Vogel HJ. Surface exposure of the methionine side chains of calmodulin in solution. A nitroxide spin label and two-dimensional NMR study. *J Biol Chem* 1999;274:8411–20. [PubMed: 10085072]
31. Waterton J, Sanders J. Radical-induced Pseudcontact Shifts. *J. Amer. Chem. Soc* 1978;100:1295–1296.

32. Eryilmaz J, Ceschini S, Ryan J, Geddes S, Waters TR, Barrett TE. Structural insights into the cryptic DNA-dependent ATPase activity of UvrB. *J Mol Biol* 2006;357:62–72. [PubMed: 16426634]
33. Wilson W. Tech. Sight. Analyzing biomolecular interactions. *Science* 2002;295:2103–5. [PubMed: 11896282]
34. Caron PR, Grossman L. Involvement of a cryptic ATPase activity of UvrB and its proteolysis product, UvrB* in DNA repair. *Nucleic Acids Res* 1988;16:10891–902. [PubMed: 2974538]
35. Verhoeven EE, Wyman C, Moolenaar GF, Goosen N. The presence of two UvrB subunits in the UvrAB complex ensures damage detection in both DNA strands. *EMBO J* 2002;21:4196–205. [PubMed: 12145219]
36. Skorvaga M, Theis K, Mandavilli BS, Kisker C, Van Houten B. The beta -hairpin motif of UvrB is essential for DNA binding, damage processing, and UvrC-mediated incisions. *J Biol Chem* 2002;277:1553–9. [PubMed: 11687584]
37. Muchmore DC, McIntosh LP, Russell CB, Anderson DE, Dahlquist FW. Expression and nitrogen-15 labeling of proteins for proton and nitrogen-15 nuclear magnetic resonance. *Methods Enzymol* 1989;177:44–73. [PubMed: 2691846]
38. John BK, Plant D, Hurd RE. Improved Proton-Detected Heteronuclear Correlation Using Gradient-Enhanced Z and Zz Filters. *Journal of Magnetic Resonance Series A* 1993;101:113–117.
39. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR* 1995;6:277–93. [PubMed: 8520220]
40. Johnson BA, Blevins RA. Nmr View - a Computer-Program for the Visualization and Analysis of Nmr Data. *Journal of Biomolecular Nmr* 1994;4:603–614.

Figure 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 1T5L - ¹¹) is indicated in the lower panel, with methionine C ϵ '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 1E52 ¹⁶) 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.

Figure 2.

Assigned ^1H - ^{13}C HSQC spectrum of [methyl- ^{13}C]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 - ^{13}C HSQC spectra of wild type (black) and M607L UvrB (red). c) Overlaid ^1H - ^{13}C HSQC spectra of wild type [methyl- ^{13}C]methionine UvrB (black) and the M560L mutant (red).

Figure 3.

Conformational perturbations of domain 1b residues resulting from M $\rightarrow$ L mutations. a) Overlay of the ^1H - ^{13}C 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).

Figure 4. Assignment of Met632. a) Expanded region of the ^1H - ^{13}C HSQC spectra of M632L mutant (red) overlaid with that of the wild type [methyl- ^{13}C]methionine UvrB (black). b) Expanded region of the M300L mutant (red) overlaid with that of the wild type [methyl- ^{13}C]methionine UvrB (black). Elimination of the M300 resonance reveals the nearby M632 resonance more clearly.

Figure 5. Correlations of methionine peak intensity with solvent accessibility (A) and with crystallographic B-factor (B). Crystallographic B-factors were taken from structure 1D9Z⁹. Data for residues M0, M607, M616, and M632 are not included, since these residues are not present in the crystal structure.

Figure 6.

Solvent accessibility based on TEMPOL-induced resonance perturbations. Overlay of ^1H - ^{13}C HSQC spectra of [methyl- ^{13}C]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. Solid blue lines connect resonances which are largely unchanged; dashed blue lines indicate resonances which are shifted in the ^{13}C dimension, and in some cases broadened below the threshold of the plot.

Figure 7. Effect of domain deletions. A) ^1H - ^{13}C HSQC spectrum of [methyl- ^{13}C]methionine UvrBΔ4 (red) relative to the spectrum of wild type UvrB (black). B) ^1H - ^{13}C HSQC spectrum of [methyl- ^{13}C]methionine UvrBΔ2Δ4 (blue) relative to the spectrum of UvrBΔ4 (red). Several of the shifts that result from the domain deletions are indicated with blue arrows.

Figure 8.

Effect of added domain 4 peptide. Overlay of the ^1H - ^{13}C HSQC spectrum of [methyl- ^{13}C] methionine M300L UvrB (black) with the spectrum obtained in the presence of a stoichiometric concentration of domain 4 peptide (green). Addition of domain 4 results in a new resonance at the position expected for M632 in a dimeric structure.

Figure 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 $K_D = 5.44 \mu\text{M}$. b) Set of SPR sensorgrams corresponding to the interaction of *B. caldotenax* UvrB with immobilized UvrB domain 4 peptide. The curves correspond to a series of 2-fold 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 $35 \mu\text{L}/\text{min}$ at 25°C . Curves a through h correspond to UvrB concentrations of $28 \mu\text{M}$, $14 \mu\text{M}$, $7 \mu\text{M}$, $3.5 \mu\text{M}$, $1.75 \mu\text{M}$, 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.

Figure 10.

UvrB dimerization. a) Dimerization of UvrB based on a model that includes a flexible linker, and no additional conflicting interactions. According to this model, UvrB should be able to form a stable dimer in solution at concentrations exceeding the equilibrium dissociation constant. b) Schematic representation of the heterodimer formed from UvrB and the domain 4 peptide. The unlabeled M632 residue on the peptide is shown as an open circle, and the labeled M632 and M607 residues on the UvrB are purple. This structure is supported by the NMR study shown in Figure 8. c) Dimerization of UvrB is disfavored by interactions of domain 4 with UvrB's domains 1-3 which position domain 4 in an orientation that is inconsistent with dimerization. The interactions between domains 3 and 4 indicated by the dotted lines could include salt bridge interactions analogous to those that appear to position domain 4 in the reported structure of UvrB* from *B. subtilis* (PDB code 2D7D). Dimer formation results in steric conflict of UvrB's domains 1-3, as indicated.

Table 1

Methionine Assignment Strategy

	M256L	M296L	M296L, M297L	M300L	M350L	M457L	M551L	M560L	M607L	M616L	M632L	UvrBA4	UvrBA4 Δ2	MET	MET-VAL
M0														X	X
M1165															
M256	X	X	X	X									X		
M296	X	X	X	X											
M297	X	X	X	X											
M300	X	X	X	X											
M350					X										
M457						X									
M551							X	X							
M560							X	X				X			
M607									X						
M616										X					
M632				X							X				
Methionine Residue															