Introduction of a Tryptophan Reporter Group into the ATP Binding Motif of the Escherichia coli UvrB Protein for the Study of Nucleotide Binding and Conformational Dynamics

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The DNA-dependent ATPase activity of UvrB is required to support preincision steps in nucleotide excision repair in Escherichia coli. This activity is, however, cryptic. Elicited in nucleotide excision repair by association with the UvrA protein, it may also be unmasked by a specific proteolysis eliminating the C-terminal domain of UvrB (generating UvrB*). We introduced fluorescent reporter groups (tryptophan replacing Phe97 or Asn51) into the ATP binding motif of UvrB, without significant alteration of behavior, to study both nucleotide binding and those conformational changes expected to be essential to function. The inserted tryptophans occupy moderately hydrophobic, although potentially heterogeneous, environments as evidenced by fluorescence emission and time-resolved decay characteristics, yet are accessible to the diffusible quencher acrylamide. Activation, via specific proteolysis, is accompanied by conformational change at the ATP binding site, with multiple changes in emission spectra and a greater shielding of the tryptophans from diffusible quencher. Titration of tryptophan fluorescence with ATP has revealed that, although catalytically incompetent, UvrB can bind ATP and bind with an affinity equal to that of the active UvrB* form ($K_d$ of $-1$ m$m$). The ATP binding site of UvrB is therefore functional and accessible, suggesting that conformational change either brings amino acid residues into proper alignment for catalysis and/or enables response to effector DNA.

Two challenges to our understanding of nucleotide excision repair in Escherichia coli are 1) the remarkable breadth of structural damage to DNA recognized by the Uvr repair proteins and 2) the complexity and energetics of assembly of an incision complex at the damaged site (reviewed in Refs. 1–3). The extent of linkage between these may become more clear as the mechanisms of this multistep process are revealed in greater detail.

One of the greater enigmas remaining in our attempt to understand these mechanistic steps is that of the multiple roles of ATP binding and hydrolysis by the UvrA and, especially, UvrB proteins. Both recognition of damage and incision (an energetically favorable reaction) are accomplished by other, specific endonucleases without the need for ATP (1). UvrA is a DNA-independent ATPase, with two ATP binding sites, both essential in nucleotide excision repair (4–6). UvrB, although it contains one Walker type A ATP motif (7–9), is catalytically silent in isolation. A cryptic, DNA-dependent ATPase activity is elicited, however, by interaction with UvrA (10, 11), and this UvrB-associated activity is required for ensuing steps in damage location and in formation of the preincision complex.

The obligate nucleotide cofactor ATP has multiple roles in the succession of macromolecular association/dissociation reactions that lead to dual endonucleolytic incisions flanking a damaged site in DNA. The binding of ATP promotes dimerization of UvrA (12, 13) and the interaction of UvrA2 with UvrB and DNA (6, 14). It is significant that initial formation of this nucleoprotein complex, which is accompanied by a localized unwinding of the DNA (15), occurs at an undamaged site (13, 16). The ability to disengage from the nonspecific loading site, and thus to locate damage, is dependent on ATP hydrolysis by the UvrB component of the repair complex. Mutation of a conserved lysine in the UvrB ATP motif (K45A), which knocks out the UvrB ATPase, results in formation of a very stable, damage-insensitive UvrA$_2$-UvrB(K45A)-DNA complex (16). Once released from the loading site, ATP hydrolysis by UvrB may also be required to support the search for damage along DNA via a tracking mechanism (1) and/or to promote the conformational changes in DNA and/or in UvrB that lead to formation of the stable UvrB-damaged DNA preincision complex, from which UvrA dissociates (14, 17–22). With the addition of UvrC, dual incision ensues, with the 3’ cut apparently dependent on the binding although not on hydrolysis of ATP by UvrB (23–25).

The mechanistic role of the UvrB ATPase may be, at least in part, to power a UvrA$_2$-UvrB helicase activity. UvrB itself has the full complement of conserved helicase motifs (26, 27); yet strand displacement activity, 5’ to 3’ and specific for short oligomers or D-loops (28, 29), requires association with UvrA. The K45A mutation in motif I (ATP binding site), cited above, eliminates the strand displacement activity (16), as well as the ability of the UvrA$_2$-UvrB complex to generate supercoiling in plasmid DNA (30), suggesting a role in complex translocation. Mutations in other helicase motifs (V and VI) have substantiated the role of the strand displacement and ATPase activities associated with UvrB in formation of the preincision complex (27).

Conformational changes, to convey allosteric communications among binding and catalytic domains and to couple the energy of nucleotide binding or hydrolysis to physical displacements (work), are at the heart of proposed helicase mechanisms (31). To fulfill its role in repair, it is postulated that the UvrB
protein must undergo such multiple conformational changes: to transduce allosteric signals associated with UvrA or UvrC binding and association with double or single-stranded DNA (with or without damage), and to couple binding or hydrolysis to movement. In this paper, we begin to examine the conformational dynamics of UvrB by use of fluorescent reporter groups that provide a signal responsive to their local environment, and that can report on changes on the nanosecond time scale. The focus of this study is on the initial activation of the UvrB ATPase, using as a model the serendipitous activation by "reporter mutants" reveal conformational changes, localized to the binding site, that accompany proteolytic activation. Furthermore, the tryptophan signal may be titrated to characterize nucleotide binding properties of UvrB, information previously unobtainable.

**EXPERIMENTAL PROCEDURES**

**Materials**—Acrylamide (ultrapear), NATA, and ATP were purchased from Sigma. Restriction enzymes and their buffers were obtained from New England Biolabs. The plasmid pTZuvrB, constructed by T. Seeley in this laboratory as described elsewhere (11), was used as a template for the wild-type gene and for protein expression (16). A SacI-PstI fragment of ~4.5 kilobase pairs from pUCuvrB (11), containing the endogenous promoters and complete coding sequence of the wild-type uvrb gene of *E. coli* (from strain AB1157), was cloned into the polynucleotide site of pTZ19R (obtained from Amersham Pharmacia Biotech), generating an Apo vec- tor of approximately 7.4 kilobase pairs. *E. coli* hosts for pTZuvrB included DH5a, DH5a, lacI, gyrA96, relA1, and SacI-PstI. Wild-type and mutant pTZuvrB plasmids, as resolved by agarose gel electrophoresis. Reaction mixtures containing diluted samples. The protein assay kit of Bio-Rad, based on the Bradford assay (37), or phosphoimager (Fujix BAS 1000), using the appropriate N364 (*D. radiodurans*)/pTZuvrB strain in late exponential growth. Cells in early exponential growth were irradiated at varying doses with ultraviolet light and assayed for survival by colony counts, as described by Seeley and Grossman (11).

**Proteins**—Mutant and wild-type UvrB proteins were purified from the appropriate N364 (Δuvrb)* pTZuvrB strain in late exponential growth. Cells, resuspended in lysis buffer (100 mM Tris-Cl (pH 7.5), 20% sucrose, 5 mM EDTA, 2 mM DTT) were lysed by a 1-h incubation with 0.25 mg/ml lysozyme and 20 U/ml RNase. Purification then followed the published protocol (33) with the following modifications: fast protein liquid chromatography (FPLC) using Butyl Sepharose 4 Fast Flow (Amersham Pharmacia Biotech) supplanted phenyl-agarose in the third chromatography step; Sephacryl S300 (Amersham Pharmacia Biotech) was used in place of Sephadex G-150; and a C18 Sepharose 6 Fast Flow column (Amersham Pharmacia Biotech) was added as a final step. The butyl-Sepharose column was packed and equilibrated in a loading buffer composed of 1 M ammonium sulfate, 50 mM Tris-Cl (pH 7.5), 25 mM KCl, 1 mM DTT, 5% glycerol. Ammonium sulfate was added to the fractions pooled from the DEAE-Sephalocolumn to a concentration of 1 M prior to loading. Protein was eluted with a linear gradient from 1 to 0 M ammonium sulfate, and other buffer components were unchanged. UvrB (or mutant)-containing fractions were concentrated by ultrafiltration (Amicon Diaflo YM30 membrane) before loading on a 4.9 cm × 100-cm Sephacryl S300 sizing column, equilibrated and eluted at 0.6–0.75 ml/min with a buffer composed of 50 mM Tris-Cl (pH 7.5), 300 mM KCl, 15% glycerol, 1 mM DTT. The Mono Q column (HR10/10) was equilibrated in a buffer of 1 M KCl in 20 mM Tris-Cl (pH 7.5), 5% glycerol, 1 mM DTT. Protein was loaded at this KCl concentration and eluted in a linear gradient from 1 to 25 mM KCl. The purified UvrB proteins (KCl concentration of the pooled Mono Q fractions was approximately 300 mM) were concentrated by ultrafiltration, supplemented with glycerol to 30%, and frozen in liquid nitrogen for storage at −80 °C. The purification of UvrA and UvrC proteins has been described (33).

The proteolytically truncated proteins, UvrB* and its mutant counterparts, were produced and purified as described (16), using the ompT-expressing *E. coli* strain UT5600/pML19 (34).

The concentration of protein in stock solutions was determined spectrophotometrically by absorbance at 280 nm in a buffer of 6 M guanidine hydrochloride, 20 mM phosphate, pH 6.5 (35). Molar extinction coefficients of 29,560 M−1 cm−1 for wild type and 35,250 M−1 cm−1 for both Trp reporter mutants were calculated from the amino acid sequence (36). The protein assay kit of Bio-Rad, based on the Bradford assay (37), using bovine serum albumin as a standard, was used for comparison among diluted samples.

**Infection Assay**—The comparative ability of mutant and wild-type UvrB proteins to complement UvrA and UvrC in the incision of UV-irradiated DNA was assessed by assay of supercoiled to nicked plas- mid, as resolved by agarose gel electrophoresis. Reaction mixtures included 25 ng of 3H-labeled plasmid (pHE6; 3996 base pairs at 105 cpm/μg, exposed to 750 J m−2 at 254 nm (11)) and UvrA and UvrC proteins at saturating concentrations in 40 mM KCl−MOPS (pH 7.6), 85 mM KCl, 1 mM EDTA, 1 mM DTT, 15 mM MgSO4, 2 mM ATP, and bovine serum albumin (10 μg/ml). UvrB was added at varying concentra- tions prior to a 5-min incubation at 37 °C. Reactions were stopped by the addition of sodium dodecyl sulfate (to 0.2%) and DNA species were separated by electrophoresis on a 0.8% agarose gel, 1 × TBE buffer. Ethidium bromide staining of outer lanes containing marker DNA was used to locate substrate and product bands. These were excised and solubilized in 21% hydrogen peroxide, 17% perchloric acid, and activity was measured by liquid scintillation counting (Biosafe II mixture, Research Products International). The extent of reaction was calculated by use of the Poisson distribution.

**ATPase Assays**—Comparisons of mutant and wild-type UvrB ATPase properties, in association with UvrA protein, or of the proteolyzed form of UvrB in isolation were performed essentially as described (11, 16). The reaction, using [γ-32P]-labeled ATP, in 50 mM KCl−MOPS (pH 7.5), 100 mM KCl, 15 mM MgCl2, 1 mM DTT and bovine serum albumin (to 50 μg/ml), was monitored by thin layer chromatography. Chromato- grams were scanned by a phosphor imager (Fujix BAS 1000), using MacBas version 2.0 for intensity quantitation. UvrB was added to varying concentrations either to 70 μM UvrA in assays omitting DNA or to 15 μM UvrA in the presence of UV-irradiated plasmid DNA (pTZ18R) at 750 J m−2. The kinetic properties of UvrB* and the protelyzed mutants, in the absence of UvrA, were determined using single-stranded DNA (ssDNA) as effector ([α-32P]dATP at 200 μM).

† The abbreviations used are: DTT, dithiothreitol; NATA, N-acetyltryptophanamide; FPLC, fast protein liquid chromatography; MOPS, 4-morpholinopropanesulfonic acid; ssDNA, single-stranded DNA.
Fluorescent Reporters of UvrB Conformation and ATP Binding

Steady State Fluorescence—Steady state emission spectra and intensity measurements were obtained with a Perkin-Elmer LS-50 luminescence spectrometer, with cuvette temperature controlled by a Lauda circulating bath. All spectra and intensity measurements were corrected for background emission and scatter and, where appropriate, inner filter effects. Spectra are not corrected for wavelength-dependent instrument effects (output and photomultiplier response), but their stability was monitored during all assays by use of a p-terphenyl standard (available from Perkin-Elmer embedded in polymethylmethacylate; excitation at 295 nm, emission at 340 nm). Quantum yields were estimated by direct comparison of emission intensities of protein samples to equimolar aqueous solutions of tryptophan, using a value of 0.14 for the quantum yield of tryptophan in water (38).

Quenching experiments with acrylamide were performed in stirred cells, at 25 °C, titrating from a stock of 3 mM acrylamide. UvrB Trp reporter mutants were at 1 μM concentration in 25 mM HEPES (pH 7.5), 100 mM KCl. Tryptophan emission, monitored at 340 nm, was selectively observed using 295-nm excitation. Intensity data following quencher additions were averaged over a 10-s collection and corrected for background emission (paired control lacking protein), dilution, and inner filter effect (calculated from acrylamide absorbance at 295 nm as described by Lakowicz (39)). Intensities, $I$, at given quencher concentration, $[Q]$, were then analyzed using the Stern-Volmer equation,

$$F_0/F = 1 + K_{SV} \cdot [Q]$$  \hspace{1cm} (Eq. 1)

where $F_0$ is the emission intensity of the protein in the absence of quencher, and $K_{SV}$ is the Stern-Volmer constant for quenching, given by the slope when data are plotted as $F_0/F$ versus $[Q]$ (40). Where there is an apparent static component to the quenching mechanism, a modified form of the Stern-Volmer equation is used to describe total quenching and estimate the contributions of dynamic and static interactions (41),

$$F_0/F = (1 + K_{SV} \cdot [Q]) \exp(V \cdot [Q])$$  \hspace{1cm} (Eq. 2)

where the additional term, $V$, is a descriptor of the probability of an instantaneous interaction with a nearby quencher.

Fluorescence emission was similarly titrated for study of ATP binding. Protein, at 1 μM, was assayed in 25 mM HEPES (pH 7.5), 85 mM KCl, 15 mM MgCl$_2$, 1 mM DTT, 10% glycerol in stirred cells at the indicated temperature. Excitation was at 295 nm, with emission and spectra collected in triplicate, and averaged for each addition of ATP. A paired titration substituting N-acetyltryptophanamide (NATA) for protein was performed to empirically correct for the significant inner filter effects arising from ATP absorbance as well as from dilution (at the maximum ATP concentration, 8 mM, the measured reduction in light intensity, $I_0 - I_{max}$, as gauged by the decrease in NATA emission, $F_0/F_{max}$, was 0.35 ± 0.02).

To obtain binding data from the ATP titrations, data were fit to the model,

$$\Delta F = \Delta F_{max} - K_d \cdot \Delta F/L$$  \hspace{1cm} (Eq. 3)

where $\Delta F$ is the observed change in fluorescence intensity (observed minus initial, corrected), $K_d$ is the association constant, and $L$ is the concentration of free ligand (approximated by the total ATP concentration). This model assumes a single binding site per protein molecule, with no interaction among proteins, and represents a trivial derivation from a standard binding expression (Eadie). In this model, simple yet appropriate to the published characteristics of UvrB, fluorescence quenching from $\Delta F_0$ (no ligand bound) to $\Delta F_{max}$ (100% of protein binding) is equivalent of ATP is linearly related to occupancy of the binding site.

Fluorescence Lifetime Measurements—Time-resolved fluorescence measurements were performed in collaboration with Drs. Ludwig Brand and Dmitri Toptygin (Johns Hopkins University). Instrumentation, data collection, and analysis have been described (42, 43). Briefly, excitation was by the pulse method, using a picosecond dye laser pumped by a mode-locked Nd:YAG laser (Spectra-Physics 3000), with dye laser output frequency doubled to provide 295-nm pulses. Emission data, at 340 nm, were collected in duplicate using single photon counting detected by a Hamamatsu R1564U-06 microchannel plate photomultiplier. Parallel collections were made with a buffer control and a Lodox scattering solution, the latter for characterization of lamp output. Protein concentrations and buffer, which varied, are described in Table II and accompanying text. Data collection was at 25 °C.

RESULTS

Preparation of “Tryptophan Reporter” Mutants and Proteins—Residues within the ATPase motif of UvrB targeted for replacement by the fluorescent amino acid tryptophan included phenylalanine 47 and asparagine 51. It has been demonstrated that the latter position could be mutated without significant phenotypic effect, either by conservative or nonconservative substitutions (N51A, N51K, Ref. 11). A calculated risk was taken with the Phe47 position, due to the conservative nature of the substitution and because the residue at this position within the motif is not conserved (see Fig. 1 in Ref. 11).

Employing cassette mutagenesis generated the desired mutants with 75–100% efficiency. The sequences of selected mutant clones were verified by reading at least 10 nucleotides beyond each ligation junction. We note that a commonly used oligo-directed mutagenesis approach (44) was not effective with template DNA produced from the full-length pTZuvrB construct. We have since been able to use oligo-directed mutagenesis following the cited protocol, by cloning only a fragment of the $uvrB$ gene (EcoRI to BglIII) into the pTZ19R vector. The same gene fragment, inserted into an M13 phage vector, was used previously in this laboratory for mutagenesis (11). We have also encountered difficulty in sequencing the 5′ terminus of the gene and regions upstream of the promoters from the ssDNA template; these problems were obviated by performing sequencing reactions at elevated temperatures. We speculate that, when unpaired, DNA sequence(s) 5′ to the $uvrB$ gene may adopt stable secondary structures that could interfere with hybridization of the probe or provide alternative initiation sites for DNA replication.

An additional peripheral note arises from a discrepancy noted among published sequences of the E. coli $uvrB$ gene. We find, for the $uvrB$ gene derived originally from E. coli strain AB1157, an A rather than a G at nucleotide position 1875.
(sequence published in Ref. 8). Our result is in agreement with the sequence of Backendorf et al. (9) and with that recently published for the E. coli genome (45). This suggests that amino acid residue 477 is a histidine (CAC codon) rather than an arginine (CGC). Histidine occupies this position in the UvrB sequences of Micrococcus luteus (46), Streptococcus pneumoniae (47), and Neisseria gonorrhoeae (48).

Each of the Trp reporter mutants, expressed from the pTZ19R-derived phagemids, could fully complement deletion of the E. coli host uvrB gene (strain N364). Survival (Fig. 2) following UV irradiation did not differ significantly from that accorded by plasmid encoding the wild-type gene (D37 estimates, the dose at which survival is reduced to 37%, are 27 J m⁻² for the wild-type uvrB plasmid, 25.8 J m⁻² for uvrB-F47W, and 34.7 J m⁻² for uvrB-N51W). With the finding that the reporter mutants exhibit no apparent change in phenotype, although based on expression from high copy number plasmids, we proceeded to purify the mutant and wild-type proteins for further characterization. No difference in binding or elution behavior was observed among the proteins in any purification step. The final column (Mono Q) was added as a routine precaution because it quantitatively eliminated a tryptophan-containing contaminant that co-purified with wild-type UvrB on one occasion (notable for a low level of UvrB expression). Purity of all preparations exceeded 97%, as judged from densitometric scans of Coomassie Brilliant Blue-stained SDS-polyacrylamide gel electrophoresis gels, loaded with 5 μg of total protein.

Molecular Phenotype of Trp Reporter Mutant Proteins—Assays to characterize function of the purified reporter proteins included incision of UV-irradiated plasmid DNA, association of UvrB with UvrA protein as evidenced by inhibition of the DNA-independent ATPase activity of UvrA, the DNA-dependent ATPase activity of UvrA-UvrB complex (manifestation of the cryptic UvrB ATPase), and kinetics of the ssDNA-dependent ATPase of UvrB activated by the specific ompT proteolysis (UvrB*).

In concert with UvrA and UvrC proteins, the Trp reporter mutants execute ATP-dependent incision of damaged DNA at levels comparable with that with wild-type UvrB (Fig. 3a). At the greatest level of addition of UvrB (24 ng, or 315 fmol), the UV-irradiated plasmid was nicked to 81 ± 7% completion. This represents the mean for wild-type and mutant proteins, among which differences were not statistically significant (analysis of variance, p = 0.338). The data do suggest, however, that the F47W substitution may have led to a slight reduction in activity. This can be seen more clearly in a time course for incision (Fig. 3b), with UvrB-F47W slower early on in the introduction of nicks (rate from the 1- to the 4-min time point was 47% that of the wild-type protein). In either assay design, the N51W protein supported incision equally to wild-type UvrB.

As described above, dual incision is the ultimate product of multiple enzymatic activities and a succession of macromolecular interactions, some of which are moderated by nucleotide binding. An alteration in a specific and essential UvrB function, resulting from mutation, could conceivably go undetected in an incision or repair assay if that activity were not diminished to the extent that it became rate-limiting. We therefore continued characterization of the Trp reporter mutants by looking at earlier steps in the pathway and specific enzymatic activities.

An early step preceding incision is the association of UvrA and UvrB proteins. It is unclear whether this protein-protein interaction occurs first or if UvrB is recruited to a UvrA-DNA complex, but it has been shown that an ATP-dependent association of UvrA and UvrB can occur in solution (14). In such an

(a) UV exposure J/m²

(b) Survival, %

Fig. 2. UV survival of plasmid-encoded Trp reporter mutants in a ΔuvrB E. coli host. Mean survival is plotted, expressed as percentage of zero-exposure control, calculated from three experiments. Cells in exponential growth were resuspended in M9 salts prior to UV irradiation and then plated in darkness on rich agar. ●, N364/pF47W; ○, N364/pN51W; ×, N364/pUvrB; ■, N364/pTZ18R (vector control).

Fig. 3. In vitro incision assays. The nicked products of incision were resolved from the substrate supercoiled plasmid (tritium-labeled, UV-irradiated to 720 J m⁻²) by agarose gel electrophoresis, followed by liquid scintillation counting of excised bands. UvrA and UvrC proteins were in excess, with ATP provided at 2 mMs. a, titration with wild-type and mutant UvrB proteins, incubated for 10 min at 37 °C. Means from four experiments are expressed as the total yield of incisions (above that seen in UvrA plus UvrC-only controls), calculated as the product of plasmid present (15 fmol) and the average nicks per plasmid, derived from a Poisson distribution. UvrB proteins are F47W (●), N51W (○), and UvrB wild-type (×). b, time course of incision with UvrB proteins, present at 150 fmol. Assay components minus UvrC and ATP were preincubated 5 min at 37 °C; the addition of ATP and UvrC defined time 0. Means from three experiments are shown, with proteins as identified in a. The initial level of nicks in plasmid ranged from 5 to 17% (0.75–2.0-fmol nicks); the maximum adventitious nicking seen in controls lacking only UvrB was 1.0 fmol.
interaction, the DNA-independent ATPase activity of UvrA is partially inhibited, while that of UvrB remains suppressed (10, 12, 16). In Fig. 4a, it can be seen that both Trp reporter UvrB mutants inhibit the ATPase activity associated with UvrA (in the absence of DNA) and to an extent that is indistinguishable from that effected by wild-type UvrB. Fitting this data to an exponential decay model (16), it can be estimated that 28 nM wild-type UvrB was sufficient for 50% inhibition ($I_{50}$), with saturable inhibition to 12% of the initial activity. The corresponding calculations for the Trp reporter mutants are $I_{50} = 39$ and 29 nM, with saturation at 22 and 15% initial activity for the F47W and N51W species, respectively.

With the addition of UV-irradiated DNA to UvrA and UvrB proteins in an ATPase supporting buffer, the overall ATPase activity of the complex is stimulated to levels exceeding that of UvrA alone. This reflects an unmasking of the cryptic activity of UvrB (10–12). This characteristic behavior by wild-type and Trp reporter UvrB constructs is shown in Fig. 4b. The addition of wild-type UvrB (to excess) leads to a 3.5-fold stimulation of ATP hydrolysis over that with UvrA alone. With all UvrB species at saturating concentrations, the rate of hydrolysis observed with the F47W mutant is close to that manifested by unaltered UvrB, at 86%. Surprisingly, activity with UvrB-N51W surpasses that seen with wild-type, by 2.0-fold.

Caron and Grossman (10) first reported that the cryptic ssDNA-dependent ATPase of UvrB could be activated by a specific proteolytic hydrolysis of the C terminus. The truncated protein, UvrB*, may serve as a model for the active conformation of the protein and allow a more direct examination of the consequences of tryptophan substitutions within the ATPase motif. The kinetic parameters of the UvrB* species, presented in Table I, were obtained from assays done within 24 h of ompT-mediated proteolysis and subsequent purification. Although UvrB is a stable protein, known to withstand prolonged storage in the freezer (49), it has been our experience (not yet formally addressed) that UvrB* is prone to loss in activity ($I_{50}$ remains stable) with freeze-thaw cycles or extended storage on ice.

The two fluorescent derivatives of UvrB do show statistically significant deviations from wild-type, and each other, in kinetic parameters of the B* fragment. The F47W protein exhibits a slight decrease in apparent affinity for substrate, with $K_{m}$ increasing from 0.8 mM for the wild type to 2.2 mM. The effect on function is compounded by a decrease in $k_{cat}$ increasing to 65% of that of wild-type. An increase is also seen in the $K_{m}$ for UvrB-N51W, to 3.1 mM, but the effect on turnover number is nearly negated by a 3.6-fold acceleration in the catalytic rate. We may conclude that the tryptophan substitutions, for phenylalanine 47 and asparagine 51, do result in measurable alterations in the catalytic properties of UvrB. These changes, however, can be viewed as minor ones, resulting in no substantial loss in function. Gross changes in the structure of the active site would therefore be considered to be improbable.

**Table I**

| UvrB*          | $K_{m}$ | $k_{cat}$ | $k_{cat}/K_{m}$ | $k_{cat}/K_{m}$ (relative) |
|---------------|--------|----------|-----------------|--------------------------|
| Wild type     | 0.8 ± 0.12 | 263 ± 26  | 329             | 1.0                      |
| F47W          | 2.2 ± 0.53 | 171 ± 30  | 78              | 0.24                     |
| N51W          | 3.1 ± 0.33 | 959 ± 84  | 309             | 0.94                     |

**Fluorescence Characterization of the Inserted Tryptophan Environments**—Tryptophan has now been engineered into the ATPase motif of functional UvrB proteins, and specific proteolysis of these proteins leads to activation of an ssDNA-dependent ATP hydrolyase, as in the wild-type protein. What can we now learn from diverse fluorescence parameters about the tryptophan environments, and by inference, the binding/catalytic site? More specifically, what is the structural basis for the inactivity of intact UvrB, and does proteolytic activation lead to measurable conformational changes at the presumed active site?

The steady state fluorescence emission spectra of the introduced tryptophans are shown in Fig. 5. Wild-type UvrB, as expected, exhibits tyrosine emission only (at 305 nm) with excitation at 280 nm (Fig. 5a) and is “dark” with 295-nm excitation (Fig. 5b), a wavelength at which there is significant absorbance by tryptophan but not tyrosine. The spectra obtained with 280-nm excitation are shown primarily to note an unusual property of the F47W and N51W UvrB derivatives, that being the persistence of tyrosine emission in a Trp-containing protein. Quantitative quenching of tyrosine emission in proteins with as few as one Trp residue is observed with few exceptions (50). The intensity of the tyrosine emission in the UvrB derivatives is not only relatively strong, but it varies in relation to that of Trp between the two reporter mutants and in both cases is abolished by proteolysis to the B* form (no tyrosine residues are eliminated in cleavage).

As noted above, it is feasible to selectively observe the tryp-
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Fluorescence emission spectra of UvrB and the Trp reporter mutant proteins. Spectra are shown for wild-type UvrB (wt), the intact Trp reporter derivatives UvrB-F47W and UvrB-N51W, and the ompT-proteolized reporter proteins (F47W* and N51W*) in 10 mM TrisCl (pH 7.5), 85 mM KCl, 1 mM DTT, 10% glycerol at 10 °C. All spectra were corrected for scatter and background at 280 nm. Proteins were at 1 μM, with 3 nm excitation and emission slits, a, spectra obtained with excitation at 280 nm. Proteins were at 1 μM, with 3 nm excitation and emission slits. b, spectra obtained with excitation at 295 nm. Proteins were at 1.4 μM, with 5-nm excitation and emission slits.

Trp47 and 6.1 ns for Trp 51. Tryptophan in water, for comparison, 6.4 ns, with intensity-weighted average lifetimes of 5.3 ns for proteins, more so in N51W, and the relatively long lifetime, 5.8–6.1 ns for trypotaphan in water, 0.14 (38), and comparable with the difference seen in emission intensity between native and guanidine-denatured UvrB-F47W (12% greater in native buffer). Though not as blue-shifted as the emission from Trp57, that of Trp57 is significantly more intense, with an estimated quantum yield of 0.24. These data suggest that, although both environments are relatively shielded from polar solvent, Trp47 may be partially quenched by contact with neighboring amino acid side chains. With proteolysis, small shifts in λmax of emission can be seen in both proteins, although in opposite directions, from 338.5 to 340 nm in UvrB-F47W and from 345 to 342 nm in UvrB-N51W. Emission intensities also change for both proteins with proteolysis, increasing in both cases, by 42% with proteolysis of UvrB-F47W, and by 19% for UvrB-N51W.

We were able, in the laboratory of Dr. Ludwig Brand, and under the direction of Dr. Dmitri Toptygin (Department of Biology and the McCollum-Pratt Institute, The Johns Hopkins University), to compare the fluorescence decay characteristics of the two reporter proteins. The fluorescence decay of Trp47 is shown in Fig. 6. Four decay times were required, for both proteins, to provide an adequate fit to the data, free of systematic error as indicated by the residual and autocorrelation plots. The multiple decay parameter values, presented in Table II, are suggestive of heterogeneity in either tryptophan environment, although the two shorter lifetimes contribute little to the average lifetime calculation (because the contribution to intensity was minor (0.6–1.5%) and because it is unlikely that such lifetimes, if real, could be resolved given the timing calibrations of 13.301 ± 0.0085 ps/channel. Neither the average lifetime, 4.8 ns for UvrB-F47W and 4.9 ns for UvrB*-F47W, nor the distribution of lifetimes changed significantly with proteolysis.

Accessibility of the Trp reporters to small, neutral molecules in solution was directly addressed by steady state fluorescence quenching experiments with acrylamide. Quenching occurred without shifts in the wavelength maxima for emission (spectra not shown). Stern-Volmer plots (Fig. 7) reveal that the tryptophans of both derivatives of UvrB are less susceptible to quenching than the model compound NATA (in neutral aqueous solution) and that quenching is further and significantly reduced for both proteins after proteolysis. Upward curvature in the plots for NATA and the intact proteins indicates an apparent static component to the quenching mechanism (40). A modification of the Stern-Volmer equation (see “Experimental Procedures”) was therefore used to obtain the estimates tabulated (Table III) for the dynamic (Ksv) and static (V) quenching constants for these species. After proteolysis, the Stern-Volmer plots were linear, with a decrease in slope for both proteins. Plots for B*-F47W and B*-N51W were virtually superimposable, with Ksv values of 2.96 and 2.86 s−1, respectively, as compared with 5.05 and 6.34 s−1, respectively, for the intact

![Fig. 5. Fluorescence emission spectra of UvrB and the Trp reporter mutant proteins.](http://www.jbc.org/)

![Fig. 6. Fluorescence decay of UvrB-F47W, showing fit to four exponential terms.](http://www.jbc.org/)

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Quenching by acrylamide of the steady state fluorescence of UvrB derivatives F47W and N51W

Exponential decay parameters calculated from time-resolved data are given for two comparisons: between UvrB-F47W and UvrB-N51W and between UvrB*-F47W and UvrB-F47W, the latter comparison done in a glycerol and MgCl₂-supplemented buffer. Proteins were at 5 μM, in 10 mM Tris · Cl (pH 7.5)/85 mM KCl/1 mM DTT, at 25 °C. χ², goodness of fit parameter (reduced χ²-square); χ and α, lifetime (ns) and associated fractional contribution to total emission intensity, respectively; τsv, intensity-weighted average fluorescence lifetime (52):*  

| UvrB   | τ | α | UvrB | τ | α |
|--------|---|---|------|---|---|
| F47W   | 2.018 | 0.53 | 0.67 | N51W | 2.018 | 0.53 | 0.67 |
| F47W² | 5.61 | 0.48 | 1.028 | F47W² | 5.61 | 0.48 | 1.028 |

F47W τsv, 5.3 ns  
F47W² χ², 0.481  
F47W² χ², 0.561  

Comparison of F47W versus F47W² was performed in buffer supplemented with glycerol (15%) and MgCl₂.
Fluorescent Reporters of UvrB Conformation and ATP Binding

fluorescence with nucleotide has allowed, for the first time, a direct demonstration of the binding of ATP to UvrB. Estimates of the binding constants, $K_d$, do not differ significantly between UvrB and UvrB* forms of the protein or between the different reporter constructs, with a mean value of $1.0 \pm 0.1 \text{ mM}$. These values are close to the $K_m$ values determined for ssDNA-dependent ATPase activity of the proteolytically activated proteins (2.2–3.1 mM).

Positioning of the Fluorescent Probe and Its Phenotypic Effects—Scant structural information was available to guide introduction of a tryptophan reporter to an ideal site, i.e. one that would not disrupt structure nor function but yet would be responsive to both conformational change at the presumed ATP binding site and to the presence of substrate. It had been shown in the laboratory of Ref. 39) that residues of a hydrophobic sheet adjoining the conserved phosphate binding loop of the Walker ATP binding motif could be mutated without a discernible effect on the overall repair capacity in in vivo UV challenges. The one such mutant protein selected for further study, UvrB-N51A, also performed at wild-type efficiency in in vitro incision assays, although its ATPase activity was reduced to 65% of wild-type levels for the DNA-dependent activity of the UvrAB complex, and to 4% of the wild-type turnover number for the proteolyzed protein. In the present study, the two Trp reporter mutants, likewise, functioned at the wild-type level in the in vivo complementation assays. And likewise, the ATPase activities of both Trp reporter mutants were diminished, but to a lesser extent than seen with N51A. The F47W substitution appears to be the more taxing of the two Trp substitutions, with a turnover number ($k_{cat}/K_m$) reduced to 24% that of wild type for the $B^*$ assay, while that of the N51W construct was close to wild type (94%). An unexpected observation, given the decrease in $k_{cat}$ reported for N51A, is the apparent stimulation of the catalytic rate in N51W, counterbalanced to a degree by a roughly 3-fold increase in the $K_m$. Opinion in general is that helicase motif I mutations affect substrate binding, with catalysis governed by motif II (26, 53). Recent structure determinations for proteins with the conserved helicase motifs described by Gorbalenya and Koonin (26), Bacillus stearothermophilus PerA (54) and E. coli Rep (55), and located in UvrB by Moolenaar et al. (27) have led to the prediction that motifs I and II would be closely apposed in UvrB (56), together creating the binding/catalytic site. A mutation such as N51W in motif I could conceivably alter the structure of, or the structure supporting that of, motif II as well. Another possibility could involve rate acceleration by facilitating product (ADP) release.

The utility of the Trp reporter mutants may most prudently be dictated by the level of detail asked of them. They function in repair at the cellular level indistinguishably from wild type and at near wild-type efficiency in in vitro incision assays. The slightly reduced efficiency of the F47W construct in incision serves to underscore the dependence of the repair pathway on expression of the UvrB ATP hydrolase. These properties and others (unaltered expression and purification behavior, interaction with UvrA) indicate that no gross alteration of structure or function resulted from the introduction of the tryptophans at positions 47 and 51. With these reporters proximal to the active site, information was obtained regarding solvent accessibility to the binding site in the unactivated protein and regarding conformational changes at this site accompanying activation. This information would probably be unobtainable from a distant probe that responded to more global conformational changes. The cost for such information is set by the alterations, up to severalfold, in apparent kinetic constants for ATPase activity. This would place limitations on the degree of detail to which one could examine binding, catalytic site geometry, and perhaps substrate specificity.

Tryptophan substitutions in UvrB have a lesser impact on ATPase activity when judged by assay of the UvrA-UvrB-ssDNA complex rather than by the activity of the proteolyzed UvrB with ssDNA. We can speculate that the association with UvrA could both stabilize the active conformation of UvrB and impose greater constraints on the structure that would not exist in the proteolytically activated protein. It is expected that use of the Trp reporter mutants of UvrB for structural or nucleotide binding studies of the UvrA–UvrB complex would be limited by the fact that UvrA contains three tryptophan residues (4). Preliminary work (not shown) has shown that the emission of Trp$^\gamma$ is discernible in the spectra of UvrA–UvrB complexes. A more promising approach, however, could employ tryptophan analogues incorporated biosynthetically into the UvrB protein (57).

The steady state fluorescence data taken together provide a description of the tryptophan reporters’ environments as being moderately hydrophobic, or partially shielded from polar solvent. The blue shifts in emission maxima are significant but do not approach those reported for Trp residues known to be buried within a hydrophobic core (to $310–320 \text{ nm}$; Ref. 39). The inten-
sity of emission also increases in the native protein, slightly for Trp$^{47}$ and to a greater extent in Trp$^{51}$. Some degree of Trp quenching occurs in the folded protein, by its accessibility to polar solvent, and, more likely in the case of Trp$^{47}$, by interaction with neighboring side chains. Accessibility to solvent was unambiguously demonstrated by the vulnerability of both Trp reporters to quenching by acrylamide, a polar and uncharged solute. Again, accessibility is moderate but sufficient for there to be a static as well as dynamic component. The estimated bimolecular quenching constants ($k_Q$) are intermediate between values reported (41) for a deeply buried residue ($\ll 0.05 \times 10^{-9}$ M$^{-1}$ s$^{-1}$, in azurin) to near maximum exposure as in a randomly coiled peptide, such as adrenocorticotropic (4.2 $\times 10^{-9}$ M$^{-1}$ s$^{-1}$), or for the free model compound NATA (5.6 $\times 10^{-9}$ M$^{-1}$ s$^{-1}$; this study). It is unlikely, therefore, that the inactivity of UvrB as an ATPase results simply from positioning of the C terminus (or any other domain or loop) as a steric block to substrate access.

Several lines of evidence indicate that conformational changes accompany the proteolytic elimination of the C terminus and associated activation of the UvrB DNA-dependent ATPase. Tyrosine emission, seen in both of the intact UvrB-Trp mutants when excited at 280 nm, is uncommon in Trp-containing proteins and is quenched in the B$^+$ peptides. This provides a ready spectroscopic handle to distinguish the B$^+$ forms but yields little information as to the nature of the structural alteration. The UvrB to UvrB$^+$ transition, in that it does lead to suppression of the tyrosine signal, may be of future interest to spectroscopists in the continuing effort (39) to understand intrinsic protein emission, if the structure of UvrB can be solved. Changes in the tryptophan emission with proteolysis provide information characterizing the conformational changes accompanying activation that can be better localized to the active site. Shifts in emission maxima are small but significant, and in the opposite direction for the two reporters. These wavelength shifts are accompanied by increases in emission intensity that may reflect a greater degree of shielding from solvent. This was more directly shown by the decreases in susceptibility to quenching by acrylamide, with the dynamic quenching rate constants decreasing for both proteins to identical values and with static components apparently eliminated. Together, these data indicate that both residues 47 and 51 are shielded from solvent to a greater extent in the activated conformation and with a position in such a cleft. We further predict that the tryptophan reporter environments would be fully consistent with coincidental or owing to greater structural constraints on the binding site in the B$^+$ form.

Looking in greater detail at the tryptophan environments, the necessity to invoke a multienzyme conformational model for both proteins may suggest a considerable degree of heterogeneity or flexibility in the vicinity of the putative binding site. Both tryptophans were described best by four lifetimes, but each was dominated by the two longer terms. Of these, the greater term (68–84.5% of total intensity for F47W and N51W, respectively) was roughly twice the lifetime of tryptophan in water, with the second term (contributing 24–12.5% of total intensity) similar or equal to the value expected in aqueous solution. The interpretation of multiple lifetimes is still not clear (58, 59), and one cannot, with assurance, simply associate each lifetime with a discrete conformation. However, it would be prudent to bear in mind that the steady state results may represent the averaged behavior of multiple conformers. And although we noted above an increase in similarity of the tryptophan environments in the proteolyzed proteins, if there were greater constraints on the architecture of the site, they do not appear to impose greater homogeneity on the distribution of lifetimes, at least for B$^+$ F47W (lifetimes were not determined for B$^+$ N51W).

**Binding of Nucleotide—Quenching of tryptophan fluorescence** by acrylamide revealed that residues near the presumed binding site were accessible to solvent. Quenching by ATP further revealed that the binding site is not only accessible in the intact protein but also functional. The conformational change that accompanies ompT proteolysis reduces the extent of quenching by ATP, as it did toward acrylamide, but the apparent affinity of binding between B and B$^+$ forms is altered little if at all.

The mechanism of quenching of tryptophan emission by ATP is not clear. Without a change in the wavelength of emission, there is no evidence that binding itself induces a conformational change. The addition of ATP in one preliminary experiment, to 1 mM, in fact did not alter the average lifetime or the distribution of decay terms of UvrB-F47W or of UvrB$^*$-F47W (data not shown). This suggests that the substrate may be close enough to the tryptophans to quench by a static mechanism.

It remains to be seen whether ATP binding in itself alters or regulates any relevant behavior of UvrB, as it does with UvrA (6, 13, 60), or whether the binding site undergoes further conformational adjustments. Studies on the UvrB-DNA precision complex suggest that ATP may be bound at an apparent affinity in the micromolar range (20). A conformational change has been postulated as necessary to confer upon UvrB the ability to stably bind DNA at a damaged site, following the departure of UvrA from the complex (17). A concomitant refinement of the ATP binding site would not be unreasonable.

We referred above to recent successes in structure determination for other proteins with the helicase motif manifold found in UvrB. As seen initially for RecA, complexed with ADP (61), the ATP binding motif is likely to be found at the base of a deep cleft between domains, closely linked to a juncture that could transduce conformational changes resulting from ATP binding and/or hydrolysis to adjoining DNA binding domains. Recently, a multidomain structure for the UvrB protein of *Thermus thermophilus* was described (56). Two domains, N (residues 2–105, containing the Walker ATP binding motif) and C1 (residues 456–590) were required for ATPase activity, with C1 also required for DNA binding. Based on the domain structure and location of the helicase motifs, a model structure similar to those of PrA and Rep was proposed. Our description of the tryptophan reporter environments would be fully consistent with a position in such a cleft. We further predict that the conformational change noted with proteolytic activation is correlated with a rearrangement of UvrB domains to complete the architecture of the catalytic site and/or to permit interaction with the ssDNA co-effector or transduction of this signal to the catalytic site. Recalling an early observation (10) that the velocity of ATP hydrolysis in UvrB$^+$ increased without saturation in response to increasing ssDNA concentration, it would seem likely that responsiveness to effector DNA is coupled to catalytic efficiency via such a co-dependence on conformational change.

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Introduction of a Tryptophan Reporter Group into the ATP Binding Motif of the Escherichia coli UvrB Protein for the Study of Nucleotide Binding and Conformational Dynamics
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