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The ATPase mechanism of UvrA2 reveals the distinct roles of proximal and distal ATPase sites in nucleotide excision repair

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ABSTRACT

The UvrA2 dimer finds lesions in DNA and initiates nucleotide excision repair. Each UvrA monomer contains two essential ATPase sites: proximal (P) and distal (D). The manner whereby their activities enable UvrA2 damage sensing and response remains to be clarified. We report three key findings from the first pre-steady state kinetic analysis of each site. Absent DNA, a P2ATP-D2ADP species accumulates when the low-affinity proximal sites bind ATP and enable rapid ATP hydrolysis and phosphate release by the high-affinity distal sites, and ADP release limits catalytic turnover. Native DNA stimulates ATP hydrolysis by all four sites, causing UvrA2 to transition through a different species, P2ADP-D2ADP. Lesion-containing DNA changes the mechanism again, suppressing ATP hydrolysis by the proximal sites while distal sites cycle through hydrolysis and ADP release, to populate proximal ATP-bound species, P2ATP-Dempty and P2ATP-D2ATP. Thus, damaged and native DNA trigger distinct ATPase site activities, which could explain why UvrA2 forms stable complexes with UvrB on damaged DNA compared with weaker, more dynamic complexes on native DNA. Specific coupling between the DNA substrate and the ATPase mechanism of each site provides new insights into how UvrA2 utilizes ATP for lesion search, recognition and repair.

INTRODUCTION

Nucleotide excision repair (NER) processes diverse lesions in DNA damaged by chemical modification (e.g. benzo[a]pyrene adducts) or UV radiation (e.g. cyclobutane pyrimidine dimers) (1–4). This multi-step pathway employs different proteins to scan the genome, distinguish damaged from undamaged (native) DNA, incise and remove the lesion-containing section of single-stranded DNA and, finally, mediate DNA synthesis using the undamaged strand as template (Figure 1A). Given the importance of NER for maintaining genome integrity, it is not surprising that these protein functions have been conserved through evolution, although, interestingly, the proteins themselves have not. Defects in eukaryotic NER are associated with cancer predisposition, UV sensitivity and premature aging among other conditions related to genome instability.

In bacteria, NER is initiated by UvrA2, which scans ds-DNA and binds to lesions with high affinity (5–8) (Figure 1A). Once a lesion is located, the DNA is handed off to UvrB, a helicase that translocates along single-stranded DNA and verifies the damage via contact with a β-hairpin (9–14). The timing and context of interaction between UvrA2 and UvrB is still under investigation. Biochemical and structural studies show that a UvrA2:B2 complex can form without DNA (15–17), and single molecule studies implicate this complex in the initial search (6), but also indicate that UvrA2 can find a lesion by itself and then recruit UvrB (5). The handoff to UvrB is accompanied by expulsion of UvrA2 from the damage-sensing complex (18). Lesion-bound UvrB recruits UvrC, a dual 5′ and 3′ nuclease, to nick the damaged strand at sites flanking the lesion (19–22). Subsequent strand displacement by UvrD helicase allows gap filling by DNA polymerase I, and finally DNA ligase completes repair (1).

During NER, UvrA2 binds DNA in a groove along the dimer interface, and has been captured in broadly defined ‘open’ and ‘closed’ conformations in crystal structures (15,23–26). Figure 1B shows the structure of Geobacillus stearothermophilus UvrA2 in an open conformation (26). It...
has been proposed that transient switching between these conformational states enables UvrA2 to scan DNA for lesions (15). UvrA2 is also an ATPase from the ATP-binding cassette (ABC) family of proteins, which have a distinctive composite nucleotide-binding site for coupling ATP binding and hydrolysis to conformational changes (Figure 1C) (27–31). UvrA2 is an unusual member of this family in that it has two ATPase sites per monomer instead of one, resulting in a total of four sites on the dimer (26,32). The two sites on each monomer are termed ‘proximal’ and ‘distal’, and each site comprises an ATP-binding domain with Walker A and B motifs and a signature domain with the ABC signature motif. The proximal site, which lies closer to the DNA-binding groove at the dimer interface, is formed by ATP-binding domain I and signature domain II (which also has residues critical for interaction with DNA and with UvrB) (15). The distal site is formed by ATP-binding domain II and signature domain I (which has the UvrB-binding domain and DNA-binding insertion domain) (Figure 1B and C).

Previous research on how UvrA2 utilizes ATP has shown that both proximal and distal ATPase sites are required for its function (25,32–34). Mutants of conserved Walker A and B motif residues have been employed to parse the role of each ATPase site; specifically, a Walker A lysine that hydrogen bonds with the β-phosphate and is important for nucleotide binding, and a Walker B glutamate that serves as a general base to activate water for ATP hydrolysis (31,35,36). Some key findings from these studies are: UvrA2 dimer is more stable in the presence of ATP (37,38), possibly as a mixed nucleotide-bound/free species in which the proximal site is empty and the distal site may be occupied by ATP or ADP (5,33,39); ATP promotes UvrA2 interaction with UvrB and its recruitment to the damage site, again possibly as a mixed species in which the distal site is ATP-bound (5,33,40); and, ATP hydrolysis facilitates UvrA2 dissociation from DNA (34). Based on structural, biochemical and in vivo single-molecule imaging data, it has been proposed that the proximal site is involved in regulating interactions among UvrA2, UvrB and DNA, and the distal site is involved in genome scanning (5,15). However, some studies have yielded discordant results; for example, the ATPase site mutants have been reported to suffer complete loss of ATP binding and hydrolysis or to maintain residual activity, which confounds interpretation of their function, and have been found either proficient or deficient in specific binding to lesions (5,32–34). These discrepancies, which could be due to differences in assays, reaction conditions and/or nucleotide or ATPase contaminants in the protein preparations, have hindered definitive understanding of the role of UvrA2 ATPase activity in NER. More importantly, the stoichiometry and kinetics of ATP binding and hydrolysis catalyzed by UvrA2 have not been determined thus far. Due to this fundamental gap in knowledge, questions about the proximal and distal site ATPase mechanisms, especially how they are coupled to each other and to UvrA2 interactions with DNA and UvrB, remain to be resolved.

In this study, we measured the rates of distinct steps in the ATPase reaction catalyzed by each site on UvrA2, from ATP binding to hydrolysis and product release, in the absence and presence of native and lesion-containing DNA. We chose *G. stearothermophilus* UvrA2 as the model protein, since crystal structures of this protein in different states are available to help interpret the kinetic data (15,26,41,42). We also leveraged information from the highest resolution UvrA2 structure (2.0 Å), reported here for the first time (*Thermotoga maritima* UvrA2; PDB code: 6N9L). Before initiating mechanistic analysis, we addressed the problem of potential nucleotide contamination by testing *G. stearothermophilus* UvrA2 dimer showing two ATPase sites on each subunit. (A) Minimal NER pathway depicting lesion recognition by UvrA2, verification by UvrB, nicking of damaged DNA by UvrC and removal by UvrD, followed by DNA resynthesis and ligation by polymerase and ligase. (B) *Geobacillus stearothermophilus* UvrA2 structure with one subunit colored by domains and the other in gray. The composite proximal (pink circles) and distal (green circles) ATPase sites made by ATP-binding domain I/signature domain II and ATP-binding domain II/signature domain I, respectively, are labeled on the gray subunit, as are Walker A and B residues in each site. The bound ADP is depicted as sticks (yellow) and the DNA binding groove by a dashed line (PDB code: 2R6F) (26). (C) Schematic of the composite ATPase sites, showing the two nucleotide-binding domains (NBD) in each subunit (chains A, B).
**mophilus** UvrA2 purified by a previously reported protocol (26). The results of a luciferase-based assay for ADP and ATP showed that the protein co-purifies with about one ADP bound per UvrA2. Adjustment of the purification protocol includes mild heat treatment that successfully removed all the ADP (Supplementary Figure S1). We also prepared a double Walker A mutant with the conserved lysines in both sites (K37 and K643) mutated to alanine, in order to detect any contaminating ATPas against a background of catalytically inactive UvrA2. ATPase assays with K37A-K643A UvrA2 found no detectable activity (Figure 6A). Pre-steady state kinetic analysis of wild-type and mutant UvrA2 proteins identified stark asymmetry in the ATP (6A). Pre-steady state kinetic analysis of wild-type and mutant UvrA2 proteins identified stark asymmetry in the ATP binding and hydrolysis activities of the proximal and distal sites, and showed that they are modulated differentially by native and lesion-containing DNA. The findings offer new ideas for understanding how UvrA2 utilizes ATP to discriminate between native versus damaged DNA and initiate NER.

**MATERIALS AND METHODS**

**Proteins, DNA and other reagents**

Wild-type *G. stearothermophilus* UvrA2 was overexpressed in *Escherichia coli* Rosetta (DE3) pLysS cells (Millipore Sigma) from pET28a-NHis-UvrA plasmid and purified by a modified version of a previously described procedure (15). Briefly, cells were grown from a fresh colony in LB media at 37°C to 0.6 OD600, induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 3 h at 30°C and pelleted by centrifugation (all steps after growth were at 4°C unless noted otherwise). The cell pellet was resuspended and lysed by homogenization in buffer (50 mM Tris–HCl, pH 8, 0.5 M NaCl, 20% sucrose) containing a protease inhibitor cocktail and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Millipore Sigma). To remove DNA and nucleotide contaminants, the lysate was warmed at 55°C for 15 min and then cooled to 4°C, clarified by ultracentrifugation (50 000 g), brought to 1 M NaCl and treated with 0.5% polyethyleneimine (PEI), and then clarified again by centrifugation (25 000 g). Next, protein was precipitated overnight with 65% ammonium sulfate (43) and the pellet was suspended in buffer (50 mM Tris–HCl, pH 8, 0.5 M NaCl, 10 mM imidazole, 5 mM β-mercaptoethanol) for column chromatography. The protein solution was purified on a Ni-NTA agarose column (Qiagen) using a 10–150 mM imidazole gradient in the same buffer. Peak fractions were pooled, diluted with buffer (50 mM Tris–HCl, pH 7.4, 5 mM β-mercaptoethanol) to 0.2 M NaCl and further purified on a Heparin Sepharose 6 column (GE Healthcare) using a 0.2–1 M NaCl gradient in the same buffer. Finally, peak fractions were pooled, the protein was dialyzed against buffer (25 mM Tris–HCl, pH 7.4, 0.25 M NaCl, 20% glycerol, 1 mM DTT), and aliquots were flash frozen in liquid nitrogen for storage at −80°C (freshly thawed aliquots were used for each experiment). Preparation of overexpression clones for UvrA2 Walker A mutants, K37A-UvrA2, K643A-UvrA2, and K37A-K643A UvrA2, has been described previously (15), and clones for Walker B mutants, E512A UvrA2 and E554A UvrA2, were prepared using the QuikChange Lightning kit (Agilent Technologies); primer sequences: E512A forward: 5′-CGT GCT CGA CGC GCC GTC GAT CGG-3′; E512A reverse: 5′-CCG ATC GAC GGC GCG CGG TCG AGC ACG-3′; E854A forward: 5′-GCT CTA CAT TTT GGA CGC GCC GAC GAC C-3′; E854A reverse: 5′-GGT CGT CGG CGC GTCCAA AAT GTA GAG C-3′. All mutant proteins were purified as described for wild-type UvrA2 above. Protein samples were run on an agarose gel and stained with ethidium bromide to test for DNA contamination (none detected) and by a luciferase-based bioluminescence kit (Millipore Sigma) for nucleotide contamination (no significant level of ADP or ATP was detected in proteins purified by the above protocol; see Supplementary Data for method and results, Figure S1). *E. coli* phosphatase binding protein (PBp) was purified and labeled with MDCC as described (44). Preparation and crystallization of *T. maritima* UvrA Δ117-399 is described in Supplementary Data.

All DNAs were purchased from Integrated DNA Technologies. Unlabeled DNA strands were purified in-house by electrophoresis in 6 M urea/18% (w/v) polyacrylamide gels followed by electroelution and ethanol precipitation, and fluorescein-labeled strands were obtained by HPLC purified and desalted. The sequences are: template: 5′-TGG ATT ACT TAC GGC CAC ATT ACT GGA ACT CAG AAC GAG CTG ACA GG-3′; complement: 5′-CCT GTC AGC TCG TTC TGA GTT CCA GTA ATG TGG CCG TAA GTA ATC CA-3′; fluorescein lesion complement: 5′-CCT GTC AGC TCG TTC TGA GTT CCA G/fluor/T/A GTA ATG TGG CCG TAA GTA ATC CA-3′. Duplex DNA substrates were prepared by annealing complementary strands in 1:1 ratio by heating for 1 min at 95°C followed by slow cooling O/N to room temperature in buffer (20 mM Tris–HCl, pH 7.4, 0.1 M NaCl), and tested by non-denaturing PAGE to confirm >95% duplex. DNA binding to UvrA2 was measured by change in fluorescence anisotropy of fluorescein end-labeled (undamaged) or internally labeled (damaged) DNAs (see Supplementary Data for method and results, Figure S6). All nucleotides, ATP, ADP, mant-ATP and mant-ADP, were purchased from Millipore Sigma.

**Mant-nucleotide binding assays**

Mant-ATP and mant-ADP binding kinetics were measured by monitoring change in fluorescence of the mant fluorophore (λ<sub>ex</sub> = 352 nm, λ<sub>em</sub> > 420 nm) over time when increasing concentrations of the nucleotide were mixed with wild-type or mutant UvrA2 in the absence or presence of DNA in a stopped-flow instrument (KinTek Corp, Austin TX) in buffer (20 mM Tris–HCl, pH 7.4, 0.15 M NaCl, 5% glycerol, 5 mM MgCl<sub>2</sub>, 5 mM DTT) at 40°C (final concentrations: 0.1 μM UvrA2, ± 0.2 μM DNA and 2.5–10 μM mant-ATP or mant-ADP). Mant-ADP dissociation kinetics were measured directly by monitoring the change in fluorescence over time on mixing wild-type or mutant UvrA2 pre-incubated with mant-ADP, in the absence or presence of DNA, with excess unlabeled ADP or ATP on the stopped-flow (final concentrations: 0.1 μM UvrA2, ± 0.2 μM DNA, 10 μM mant-ADP and 2 mM ADP or
2 mM ATP). The signal from 3 to 5 traces was averaged for each experiment and corrected for background fluorescence from mant-nucleotide alone (mant-ADP photo-bleaching caused a slow, linear decrease in signal to 15% at most over the 150-s dissociation time scale). Association data were fit to a single exponential equation to determine the observed rate ($k_{obs}$), and linear dependence of this rate versus nucleotide concentration yielded the bimolecular rate constant, $k_{on}$, and the dissociation rate, $k_{off}$ ($k_{obs} = k_{on}[\text{mant-nucleotide}] + k_{off}$). Error bars report standard error of the mean (S.E.M.) from $N = 3$. Dissociation data were fit to a single exponential equation to determine $k_{off}$.

The stoichiometry of nucleotide binding was measured by Förster resonance energy transfer (FRET) between UvrA2 tryptophans (donor) and mant-ADP (acceptor). UvrA2 (3 μM) was titrated with mant-ADP (0–20 μM) in buffer (20 mM Tris–HCl, pH 7.4, 0.15 M NaCl, 5% glycerol, 5 mM MgCl$_2$, 5 mM DTT) at 40°C, and fluorescence intensity was measured after mixing and a 1-min incubation ($\lambda_{EX} = 290$ nm, $\lambda_{EM} = 305–400$ nm; Jobin-Yvon Horiba Fluoromax-3). Emission spectra were collected for UvrA2 alone (D), mant-ADP alone (A) and UvrA2 plus mant-ADP (DA), and the spectra were integrated using Grams/AI software (Thermo Scientific); the fluorescence intensity of A was subtracted from that of DA to correct for background mant-ADP excitation at 290 nm, and fluorescence intensity of D was aligned with the initial value for DA (at zero mant-ADP). FRET efficiency was calculated as $E_{\text{FRET}} = 1 - \left( \frac{F_{DA}}{F_{D}} \right)$; $F_{DA}$ and $F_{D}$ are the corrected fluorescence intensities of DA and D, respectively. $E_{\text{FRET}}$ values from three independent experiments were averaged and plotted versus mant-ADP concentration. The inflection point between initial and final slopes of the isotherm yielded the stoichiometry of mant-ADP binding to UvrA2 (error bars report S.E.M. from $N = 3$).

### Asymmetric nucleotide binding to proximal (weak) and distal (tight) ATPase sites on the UvrA2 dimer

We used ATP and ADP analogs with the ribose modified by 2′(3′)-O-(N-methylanthraniloyl) fluorophore (mant) (49) to measure the kinetics and stoichiometry of nucleotide binding to UvrA2. Mant-nucleotide fluorescence intensity increased on binding to UvrA2, as reported previously for other ATPases (50, 51). Monitoring the signal over time after mixing 0.1 μM UvrA2 with 10 μM mant-ATP on a stopped flow yields a binding rate of 0.6 s$^{-1}$ (Figure 2A; kinetic trace under pseudo first order conditions fit with a single exponential function). A titration with mant-ATP reveals linear concentration dependence of the binding rate, providing a bimolecular association rate constant from the slope and a rough estimate of the dissociation rate from the Y-intercept ($k_{on} = 0.4 \times 10^5$ M$^{-1}$ s$^{-1}$, $k_{off}$ (estimated) = 0.2 s$^{-1}$; Figure 2A inset; Supplementary Figures S2B, S2D, Table 1). A similar analysis of mant-ADP binding kinetics yields comparable rates ($k_{on} = 1.3 \times 10^5$ M$^{-1}$ s$^{-1}$, $k_{off}$ (estimated) = 0.06 s$^{-1}$; Figure 2B, Supplementary Figures S2C, S2E, Table 1). The dissociation rate was also measured directly by pre-incubating mant-ADP with UvrA2 and mixing with excess unlabeled ADP to prevent mant-ADP rebinding. In this experiment, mant-ADP fluorescence decreases as a single exponential over time and yields a slow $k_{off} = 0.03$ s$^{-1}$ (Figure 2C). The $k_{off}/k_{on}$ ratio yields a dissociation constant of 0.23 μM for mant-ADP, indicating a high affinity and stable interaction with UvrA2 ($K_{D1}$; Table 1). Mant-ATP dissociation was not measured directly, as this nucleotide is hydrolyzed in the time frame of the experiment (from the mant-ATP $k_{off}$ estimate above, $K_{D1} \sim$ 5 μM).

The next step was to determine how many of the four ATPase sites on UvrA2 bind nucleotides. We measured the stoichiometry by titrating UvrA2 (3 μM) at a concentration well above the measured dissociation constant (0.23 μM) with increasing amounts of mant-ADP. In this case, the reporter was UvrA2 tryptophan fluorescence quenching due to FRET to mant-ADP (52). Figure 2D shows distances.
Figure 2. Kinetics, affinity and stoichiometry of nucleotide binding to UvrA2. Nucleotide binding was monitored by increase in fluorescence on mixing UvrA2 with (A) mant-ATP or (B) mant-ADP (final: 0.1 μM UvrA2 and 2.5–10 μM mant-nucleotide; 10 μM trace shown here; see Supplementary Figure S2). Time traces fit to a single exponential yield rates that depend linearly on nucleotide concentration, and the slope yields $k_{on}$ = 0.4 and 1.3 $\times$ 10$^5$ M$^{-1}$ s$^{-1}$ for mant-ATP and mant-ADP, respectively (A and B, inset). (C) Nucleotide dissociation was measured by pre-incubating mant-ADP with UvrA2 and mixing with excess unlabeled ADP chase (final: 0.1 μM UvrA2, 10 μM mant-ADP and 2 mM ADP). The decrease in fluorescence fit to a single exponential yields $k_{off}$ = 0.03 ± 0.002 s$^{-1}$ ($k_{on}$/$k_{off}$ yields a tight $K_D$ = 0.23 μM for mant-ADP; Table 1). (D) UvrA2 structure showing distances between tryptophans W307, W609 (donor) and nucleotides (acceptor mant-ADP) (PDB code: 2R6F). (E) Nucleotide binding stoichiometry was measured by FRET on titrating UvrA2 (3 μM) with increasing mant-ADP; error bars report S.E.M. ($N$ = 3). The inset shows spectra for UvrA2 alone (donor, D) and with mant-ADP (donor + acceptor, D+A). The binding isotherm has an inflection point of 6.8 μM (2.3 mant-ADP per UvrA2 dimer).

Table 1. Measured parameters for the UvrA2 ATPase mechanism

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<th>mantADP $k_{on}$ (× 10$^5$ M$^{-1}$ s$^{-1}$)</th>
<th>mantADP $k_{off}$ (ADP chase; s$^{-1}$)</th>
<th>mantADP $K_D$ (μM)</th>
<th>mantADP $k_{off}$ (ATP chase; s$^{-1}$)</th>
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aS.E.M. range from 2 to 10% for all reported values from 2 to 4 independent measurements.
b$k_{cat}$ = linear slope/4 sites × [UvrA2] for wild-type, and slope/2 sites × [UvrA2] for mutants.
cND = not detectable.
between potential tryptophan donors and mant-ADP ac-
ceptors at the proximal and distal sites in G. streother-
mophilus UvrA2; tryptophan - mant R0 ∼25 Å (52,53). As
shown in Figure 2E. FRET efficiency increases linearly with
mant-ADP concentration until saturation, and the inflec-
tion point yields a ratio of 2.3 mant-ADP bound per UvrA2
(Figure 2E, inset shows the emission spectra of UvrA2,
alone and with mant-ADP). These results indicate asymme-
try in the nucleotide binding properties of UvrA2 since only
two of the four ATPase sites bind ADP with high affinity.

The above experiments do not reveal which two sites
bind ADP tightly and whether asymmetry exists within a
monomer (between each proximal and distal site) or be-
tween monomers (between the pairs of sites across the
dimer) (Figure 1B and C). We addressed this question us-
ing ATPase mutants in which the conserved Walker A lysine
was replaced with alanine to disrupt ATP binding (proxi-
mal: K37A; distal: K643A; Figure 3A) (54), and the con-
served Walker B glutamate was replaced with alanine to
disrupt ATP hydrolysis while (likely) retaining ATP bind-
ing (proximal: E512A; distal: E854A; Figure 3A) (36). The
kinetics of ATP and ADP binding to the mutants was mea-
sured as for wild-type UvrA2 (Figure 2A). As shown in Fig-
ure 3B and C, both Walker B mutants, E512A UvrA2 (prox-
imal) and E854A UvrA2 (distal), exhibit similar mant-ATP
and mant-ADP binding kinetics as wild-type (Figure 2A),
with koff on the order of 105 M−1 s−1. Thus, this mutation
does not disrupt nucleotide binding to UvrA2 (Supple-
mentary Figures S2D, S2E and Table 1); a slightly higher signal
for mant-ATP-bound E854A UvrA2 may indicate a change
in the local environment of the nucleotide, although this
difference is not observed with mant-ADP. The Walker A
mutant K37A UvrA2, in which proximal ATP binding is dis-
rupted while the distal site is intact, also exhibits a simi-
lar nucleotide binding rate. In contrast, the complementary
Walker A mutant K643A UvrA2, in which distal ATP bind-
ing is disrupted while the proximal site is intact, shows no bind-
ing at nucleotide concentrations tested on the stopped flow
(Figure 3B and C; Supplementary Figures S2D, S2E). Ac-
cording to these results, distal sites bind mant-nucleotides,
but proximal sites do not. Mant-ADP dissociation measure-
ments show that the three mutants, K37A UvrA2, E512A UvrA2
and E854A UvrA2, bind mant-ADP with comparable high
affinity and stability to wild-type (koff ∼0.05 s−1, KD ∼0.2
µM; Figure 3D, Table 1), whereas K643A UvrA2 does not
show any change in the baseline signal, consistent with its
inability to bind mant-ADP under these conditions (Figure
3D). Moreover, the FRET-based assay used to measure
mant-ADP binding stoichiometry (Figure 2E) reports
weak interaction at best for K643A UvrA2 (Supplementary
Figure S2F). Together, these results clearly indicate that dis-
tal sites on UvrA2 bind ATP and ADP tightly, whereas
proximal sites have weaker affinity. This finding is consis-
tent with the detection of [γ-32P]ATP bound to K37A UvrA2
but not K643A UvrA2 in a nitrocellulose filter binding assay
(15). Thus, we can conclude that nucleotide binding asym-
metry exists between the proximal and distal ATPase sites
on each monomer in UvrA2, and the stoichiometry of two
mant-ADP per UvrA2 reflects occupancy of the two high-
affinity distal sites on the dimer (Figure 1B and C).

Structural analysis of asymmetric nucleotide binding by
UvrA2
To gain additional insights into the finding that nucleotides are held more tightly by the distal ATPase sites than prox-
imal sites, we interrogated atomic models of several UvrA2
orthologs for underlying sources of the differential affin-
ity. These included a newly determined high resolution 2.0
Å crystal structure of UvrA2 from T. maritima (Supple-
mentary Figure S3A, Supplementary Table S1; PDB code:
6N9L) as well as published structures of G. streother-
mophilus, T. maritima, Deinococcus radiodurans and My-
cobacterium tuberculosis UvrA2 (15,23–26) (discussion of

![Figure 3](image-url)

Figure 3. The distal ATPase sites on UvrA2 bind nucleotides with high affinity. (A) Aligned proximal and distal ATPase sites depicting Walker A and B residues (PDB code: 2R6F). Kinetics of (B) mant-ATP and (C) mant-ADP binding to Walker A (K37A UvrA2, K643A UvrA2, E512A UvrA2, and E854A UvrA2) mutants of the proximal and distal sites were measured as described for wild-type UvrA2 in Figure 2 (final: 0.1 µM UvrA2, 2.5–10 µM mant-nucleotide; 10 µM trace shown; Supplementary Figure S2). All mutants yielded binding rate constants similar to wild-type, except for K643A UvrA2, which did not bind either nucleotide (Table 1). (D) mant-ADP dissociation was measured as for wild-type in Figure 2 (final: 0.1 µM UvrA2, 10 µM mant-ADP and 2 mM ADP). All mutants had similar dissociation rates as wild-type, except for K643A UvrA2, which did not bind mant-ADP (Table 1).
the new *T. maritima* UvrA2 structure is restricted to aspects relevant to this kinetic study; the reader is referred to the cited reports for detailed structural descriptions of UvrA2). Our analysis examined the number and types of interactions between the ATPase sites and the bound nucleotides, which was simplified by the high degree of sequence and structural conservation in both proximal and distal sites in these orthologs. Indeed, the eight ATPase sites in question could be easily superimposed using the Walker A sequence (Supplementary Table S2), highlighting the overall similarity in these domains among currently available structures. The largest deviation in the superpositions was found in the ABC signature and the Q-loop motifs.

Detailed examination of the complete set of contacts between UvrA2 and nucleotide at all the sites revealed 21 polar and 28 hydrophobic contacts per site, on average (both proximal and distal sites make 21 polar contacts, and 29 and 27 hydrophobic contacts, respectively). These contacts are highly conserved between the two types of sites and between orthologs, with one notable exception. The proximal site has a glutamine residue that precedes the ABC signature motif by five residues (Q821, *G. stearothermophilus* residue numbering; Figure 4A). In contrast, the distal ATPase site of every UvrA2 ortholog features an arginine in the equivalent position, which is involved in a pi–cation interaction with the aromatic base of adenine (R480; Figure 4B). While both residues stack on the adenine base, the more extensive interaction and pi–cation stacking by R480 implicate this residue in the higher affinity of distal sites for nucleotides. Both R480 and Q821 are absolutely conserved in a large set of UvrA structures (PDB ID: 2R6F, 2VF7 and 2VF8) among transparent R480 residues from allother distal ADP-bound UvrA structures). R480 is highlighted in solid red among transparent Q821 residues from all proximal ATPase sites showing K37, E512 and ADP, as well as Q821 highlighted in solid red among transparent Q821 residues from all other proximal ATPase sites showing K643, E854 and ADP (ADP from 2R6F is also shown, demonstrating a ring flip observed in this site in some UvrA structures). *T. maritima* R480 is highlighted in solid red among transparent R480 residues from all other distal ATPase-bound UvrA structures (PDB ID: 2R6F, 3UX8, 2VF7 and 2VF8) (15,25,26). The insets show sequence alignment of several UvrA proteins (Figure 4 insets), and are located at the end of a region previously noted as the ‘structurally diverse region’ (SDR) in ABC importers (55,56).

To determine if our findings about the potential roles of the arginine and glutamine residues in UvrA generalize to the larger ABC family of ATPases, we interrogated the Protein Data Bank using the ScanProsite (ExPASy) tool and search patterns that include these residues in the SDR regions of UvrA: R-X(4)-L-S-G(2)-X and Q-X(4)-L-S-G(2)-X. The search revealed 13 entries containing both patterns, all of which were ABC ATPases. Three of these entries corresponded to a heterodimeric ABC ATPase, the ABCE1 RNase L inhibitor (PDB codes: 3J16, 4CRM and 5LL6), and the remaining 10 were other UvrA proteins. Separate searches with one or the other pattern revealed that the Energy-coupling factor ABC ATPase importer retained an arginine in a similar position to R480 at one nucleotide binding site and an alanine in a similar position to Q821 at the second site (PDB codes: 4HLU and 4ZIR) (57,58). It would be interesting to determine whether this difference confers asymmetry in nucleotide binding (and catalytic activity, as shown below) to other dimeric ABC ATPases, as observed with UvrA2.

**Asymmetric and linked ATPase activities of the proximal (slow) and distal (rapid burst) sites on UvrA2**

The discovery of differential nucleotide binding by the proximal and distal sites raised the question whether these sites also hydrolyze ATP differentially. If true, this possibility has profound implications for understanding how UvrA2 utilizes the ATPase reaction to drive NER. We addressed this question by analyzing UvrA2-catalyzed ATP hydrolysis and phosphate (Pi) release under pre-steady state conditions, which can reveal the stoichiometry of ATP hydrolysis and rate-limiting steps in the mechanism. We used an assay developed by the Webb group in which rapid (10⁸ M⁻¹ s⁻¹) and high affinity (*K₅₀ = 0.1 μM) binding of Pi by phosphate-binding protein (PBP) labeled with

![Figure 4. UvrA structures showing interactions of ADP with R480 and Q821 (*G. stearothermophilus* residue numbers). (A) Representation of the *T. maritima* proximal ATPase site showing K37, E512 and ADP, as well as Q821 highlighted in solid red among transparent Q821 residues from all other proximal ADP-bound UvrA structures (PDB ID: 2R6F, 2VF7 and 2VF8). (B) *T. maritima* distal ATPase site showing K643, E854 and ADP (ADP from 2R6F is also shown, demonstrating a ring flip observed in this site in some UvrA structures). *T. maritima* R480 is highlighted in solid red among transparent R480 residues from all other distal ATPase-bound UvrA structures (PDB ID: 2R6F, 3UX8, 2VF7 and 2VF8) (15,25,26).](image-url)
7-diethylamino-3-(((2-maleimidyl)ethyl)amino)carbonyl)coumarin (MDCC) leads to a large increase in MDCC fluorescence (44). Due to these properties, MDCC-PBP reports any free Pi in solution effectively at the rate at which it is released upon ATP hydrolysis by UvrA2, thus enabling transient kinetic measurements.

The reaction was initiated by mixing UvrA2 and MDCC-PBP with ATP in a stopped-flow apparatus and Pi formation was monitored over time (Figure 5A). The kinetic trace for 2 μM UvrA2 mixed with 1 mM ATP shows a slight lag phase and then a burst of Pi followed by a linear phase. The lag indicates at least two steps in the reaction leading to fast ATP hydrolysis and Pi release (which can be described by a double exponential function), and then a slow step limits steady state turnover (which can be described by a linear function) (59). Fitting the trace to a double exponential + linear function yields a rate of 0.2 s⁻¹ preceding the burst indicates that a slow step(s) occurs before/after ATP hydrolysis as well and is followed by Pi release. This experiment was repeated at varying concentrations of UvrA2 and constant ATP (1 mM) to accurately determine the stoichiometry of ATP hydrolysis from the burst amplitude of the first turnover (Supplementary Figure S4B). In addition, complementary experiments were performed at constant UvrA2 (2 μM) and increasing ATP concentrations to determine how the different nucleotide binding affinities of proximal (weak) and distal (tight) sites impact ATPase activity. As shown in Figure 5B, the burst of Pi release increases with ATP concentration, approaching half-maximum at ~350 μM and maximum at >1.5 mM. The need for such high ATP concentrations implies that the low affinity proximal sites play an important role in the ATPase activity of UvrA2. The data from all these experiments were analyzed by global fitting to a model ATPase mechanism, as shown in Scheme 1 and explained below.

A minimal kinetic model for UvrA2 ATPase activity

The goal was to develop a kinetic mechanism with the minimal number of steps and parameters required to simultaneously fit all the ATPase data described above. The measured nucleotide binding (Figures 2 and 3) and ATPase rates (Figure 5 and Supplementary Figure S4B) were used as initial estimates and allowed to float during data fitting, as was the number of ATPase sites catalyzing the burst of hydrolysis. The raw data, together with details of model development and data fitting by KinTek Explorer, are available as Supplementary Data at NAR Online (46,47). The best fit model is shown in Scheme 1A, and the corresponding rate constants are shown in Table 2 and Scheme 1B, which also includes findings from experiments with UvrA2 mutants described below. The fits are shown as dashed lines overlaid on the corresponding experimental data in Figure 5A,B and Supplementary Figure S4B. The reaction begins with ATP binding rapidly to the two high affinity distal sites (step 1; $K_{D1} = 1 \mu M$; UvrA2•ATP₂D) and two low affinity proximal sites (step 2; $K_{D2} = 350 \mu M$; UvrA2•ATP₂D₂P). ATP binding is followed by a slow step that has been designated as ATP hydrolysis (step 3; $k = 2.5 \text{s}^{-1}$), and then another step designated as Pi release (step 4; $k_4 = 20 \text{s}^{-1}$). The final step in the reaction, which limits the steady state turnover rate, is designated as ADP release (step 5; $k_{5a} = 0.5 \text{s}^{-1}$ or $k_{5b} = 0.2 \text{s}^{-1}$ depending on the number of active sites per turnover, as explained further below).

These are the minimal number of steps required to simultaneously fit all the ATPase data described above and obtain well constrained parameters for each step (error analysis by FitSpace Explorer is described in Supplementary Data, and the resulting limits on rate constants are shown in Table 2 and Supplementary Figure S4C) (48). The model mechanism shows that: (i) the burst rate is determined by at least two steps, which we propose are associated with ATP hydrolysis at 2.5 s⁻¹ and subsequent Pi release at 20 s⁻¹ (note that these two rates likely reflect slow protein conformational dynamics that enable relatively fast ATP hydrolysis and Pi release events); (ii) the best fit burst amplitude ($n$) of two ATP molecules per UvrA2 (Supplementary Figure S4B) confirms that only two of the four ATPase sites catalyze fast ATP hydrolysis and Pi release. Important questions that remain unresolved are: which two sites on UvrA2, proximal or distal, have burst activity, and what is the slow step that limits steady state turnover. These are addressed in the following sections.

The high ATP concentration required for a maximal burst either implicates the low affinity proximal sites in rapid ATP hydrolysis or implies that ATP binding to these sites is required for rapid ATP hydrolysis by the distal sites. These two possibilities were investigated by analyzing the UvrA2 ATPase mutants. The Walker A mutant K37A UvrA2, in which ATP binding to proximal sites is disrupted while distal sites are intact, shows no burst and suffers near complete loss of activity (Figure 6A), consistent with an important role for the proximal sites in rapid ATP hydrolysis by UvrA2. However, the Walker B mutant E512A UvrA2, in which proximal ATP binding remains intact but hydrolysis is disrupted, exhibits a burst of ATP hydrolysis and Pi release by the wild-type distal sites followed by linear steady state ($k_{cat} = 0.3 \text{s}^{-1}$; linear slope/2 sites × [E512A UvrA2]), similar to wild-type UvrA2 (Figure 6A, Table 1). The data fit to the same mechanism in Scheme 1 yield $k_3 = 2 \text{s}^{-1}$, $k_4 = 42 \text{s}^{-1}$, $k_{5a} = 0.6 \text{s}^{-1}$ and $n = 1.3$ Pi/E512A UvrA2, which differ at most 2-fold from wild-type parameters (Table 2). The corresponding distal site Walker A mutant K643A UvrA2, in which distal ATP binding is disrupted while proximal sites are intact, shows no burst and has very low ATPase activity (Figure 6B). Moreover, the Walker B mutant E534A UvrA2, in which distal ATP binding remains intact but hydrolysis is disrupted, exhibits a lag and no burst activity by the wild-type proximal sites. A faster $k_{cat} = 0.6 \text{s}^{-1}$ (linear slope/2 sites × [E512A UvrA2]) suggests that the proximal ATPase is stimulated slightly when distal sites are ATP-bound (note: the proximal ATPase is inhibited when distal sites are ADP-bound; Supplementary Figure S5). Together these results confirm that the distal sites are responsible for the initial burst of ATP hydrolysis, and that ATP binding to the proximal sites is required for this activity.
Figure 5. Two sites on UvrA2 catalyze a burst of ATP hydrolysis and phosphate (Pi) release. Pre-steady state kinetics of Pi release were measured by mixing UvrA2 and MDCCPBP reporter with ATP at (A) increasing UvrA2 (final: 0.125–2 μM UvrA2, 1 mM ATP and 15 μM MDCCPBP; 2 μM UvrA2 trace shown; Supplementary Figure S4) or (B) increasing ATP (final: 2 μM UvrA2, 10 μM to 2 mM ATP and 15 μM MDCCPBP). After a slight lag, Pi is released rapidly in a burst phase followed by a slow linear phase that yields $k_{cat} = 0.2 \pm 0.03 \text{ s}^{-1}$ (slope/4 × [UvrA2]; Table 1). Simultaneous fitting of data at all UvrA2 and ATP concentrations to a minimal ATPase mechanism (Scheme 1) yields $k_3 = 2.45 \pm 0.04 \text{ s}^{-1}$ that limits the burst rate, and a maximum burst amplitude $n = 1.9 \pm 0.03$ ATP molecules hydrolyzed rapidly per UvrA2 per turnover (Table 2); gray dashed lines show the fit.

1A

$\text{Scheme 1. A minimal kinetic model of the UvrA2 ATPase mechanism. (1A) All ATPase kinetic data obtained for wild-type UvrA2 were fit to this model, with rate and affinity constants measured in mann-nucleotide binding/release and ATP hydrolysis/Pi release experiments serving as initial estimates for global fitting by KinTek Explorer (Supplementary Data). The best fit rate constants and stoichiometry are listed in Table 2 and shown in bold in (1B), which also includes ATPase site specific information from mutant analysis. The reaction starts with (1) rapid ATP binding by the high affinity distal sites (ATP2D), and then (2) the low affinity proximal sites (ATP2D-2P). A subsequent slow step (3) that we speculate is associated with ATP hydrolysis by the distal sites (ADP2D,Pi2D) is followed by (4) fast Pi release. Finally, a slow step involving (5a) ADP release from distal sites, or additionally (5b) ATP hydrolysis and product release by proximal sites, limits the catalytic turnover rate ($k_{cat}$).}$

1B

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<th>Table 2. Best fit parameters for the UvrA2 ATPase mechanism</th>
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Parameters in bold were linked during global fitting and confidence contour analysis.

bStandard errors are shown for parameters allowed to float during data fitting.

cUpper and lower limits for parameters from confidence contour analysis are in parentheses.
measurements revealed that UvrA2 binds fluorescein lesion—a good model substrate for bacterial NER) (20, 61, 62). Our centrally located fluorescein adduct (a fluorescein lesion is (native) duplex and one with an identical sequence plus a mechanism (the same alone, indicating significant changes in the reaction mechanism (the same is about 4- and 7-fold in the presence of native and lesion DNA in the UvrA2 ATPase mechanism. In many ATPases, the slow step following Pi release is accelerated by native DNA (step 5), it remains slow enough to limit turnover. Figure 7A also shows that in the presence of fluorescein lesion DNA, UvrA2 ATPase kinetics change again, showing no burst activity, just a fast linear phase following a short lag. This result means that in the UvrA2–lesion complex, the slow step after ATP hydrolysis and Pi release has been accelerated and does not limit the turnover rate; instead, a step before or at ATP hydrolysis has become rate limiting.

Differential effects of native and lesion DNA on the proximal and distal site ATPase mechanisms

A key question driving this study is how UvrA2 integrates its ATPase and DNA-binding activities to initiate NER. To address this question, we measured the ATPase kinetics of UvrA2 bound to two types of DNAs: a 50 bp undamaged (native) duplex and one with an identical sequence plus a centrally located fluorescein adduct (a fluorescein lesion is a good model substrate for bacterial NER) (20, 61, 62). Our measurements revealed that UvrA2 binds fluorescein lesion-containing DNA ~4-fold tighter than native DNA (KD = 12 nM versus 45 nM; Supplementary Figure S6), which is in line with earlier reports (24, 63–68). Neither type of DNA appears to affect the nucleotide binding kinetics (Supplementary Figure S7; Table 1). However, both DNAs stimulate the steady state ATPase rate (linear phase in Figure 7A, Table 1) with kcat = 0.8 s−1 and 1.4 s−1 for the native and the lesion DNA, respectively, compared with 0.2 s−1 for UvrA2 alone, indicating significant changes in the reaction mechanism (the same kcat values were obtained from malachite green-based steady state experiments) (60); note: to avoid saturation of the MDCCPBP reporter due to this high ATPase rate, pre-steady state measurements were performed at a lower UvrA2 concentration (0.25 μM) than in the absence of DNA (2 μM; Figure 5). In the presence of native DNA, UvrA2 again shows a short lag, a burst of ATP hydrolysis and Pi release, and then a linear steady state phase; however, the burst amplitude is higher than for protein alone (Figure 7A). The data were fit to the mechanism shown in Scheme 1, and in this case allowing the number of ATPase sites to float during fitting yields a burst amplitude of ~5 ATP hydrolyzed per UvrA2 (k3 = 2.4 s−1, k4 = 30 s−1, k5 = 1 s−1, n = 5.4 ± 0.3 Pi/UvrA2). We interpret this result to mean that all four sites in the UvrA2–native DNA complex hydrolyze ATP rapidly (versus only the two distal sites in UvrA2 alone); the overestimate of five sites instead of four may arise from fitting error given the relatively small difference between the burst and linear rates. Also, while the step following Pi release is accelerated by native DNA (step 5), it remains slow enough to limit turnover. Figure 7A also shows that in the presence of fluorescein lesion DNA, UvrA2 ATPase kinetics change again, showing no burst activity, just a fast linear phase following a short lag. This result means that in the UvrA2–lesion complex, the slow step after ATP hydrolysis and Pi release has been accelerated and does not limit the turnover rate; instead, a step before or at ATP hydrolysis has become rate limiting.

Additional information on these rate-limiting step(s) is needed in order to understand what changes are induced by DNA in the UvrA2 ATPase mechanism. In many ATPases, catalytic turnover is associated with and limited by ADP release, and we hypothesized this might be the case for UvrA2 as well (also note that ADP co-purifies with UvrA2 and multiple ADP-bound UvrA2 structures have been solved, indicating high affinity). We expected ADP release rates to increase by about 4- and 7-fold in the presence of native and
lesion DNA, respectively, corresponding to the increases in ATPase turnover rate noted above ($k_{cat} = 0.2 s^{-1}$, 0.8 s$^{-1}$ and 1.4 s$^{-1}$ for UvrA2 alone, and with the native and lesion DNAs, respectively; Figure 7A, Table 1). However, as shown in Figure 7B, the rate of mant-ADP dissociation from distal sites on DNA-bound UvrA2 remains unchanged at 0.03 s$^{-1}$, as observed in the absence of DNA (Figure 2C). Note that in these experiments we used excess unlabeled ADP as a passive chase to prevent mant-ADP rebinding to UvrA2 after dissociation. But, since the proximal and distal sites exhibit asymmetric nucleotide occupancy and allosteric communication, we also tested ATP as chase, wondering if ATP binding by the proximal sites might affect the mant-ADP bound at distal sites. Indeed, when excess unlabeled ATP is added to the mant-ADP–UvrA2 complex in the absence of DNA, we observe ~70-fold faster release of mant-ADP at 2 s$^{-1}$ (Figure 7C). The presence of native and lesion DNA further stimulates mant-ADP release by 4- and 6-fold to 9 and 13 s$^{-1}$, respectively (Figure 7C). While the absolute release rates of the mant-ADP analog are faster than the ATPase $k_{cat}$ values (Table 1), the match between the relative increases in both rates induced by DNA strongly indicates that native and lesion DNA binding to UvrA2 alters the rate of ADP release following ATP hydrolysis and Pi release.

To summarize the results thus far with wild-type UvrA2: (i) ATP-bound proximal sites trigger ADP release from distal sites; (ii) in the absence of DNA, ADP release limits catalytic turnover following ATP hydrolysis by distal sites; (iii) when UvrA2 binds native DNA, ADP release from distal sites is accelerated following hydrolysis by all four sites, but is still slow enough to limit the turnover rate and finally, (iv) when UvrA2 binds a lesion, further acceleration of ADP release means an earlier step in the reaction (prior to/at ATP hydrolysis) becomes rate limiting instead of ADP release. In order to examine these findings in more detail and determine the effects of DNA on each ATPase site, we also analyzed the Walker A and B mutants in the presence of DNA as described below.

Figure 8A shows results from pre-steady state ATPase experiments with wild-type and mutant UvrA2 in the presence of native DNA. The Walker A mutant K37A UvrA2, in which proximal ATP binding is disrupted while distal sites are intact, suffers near complete loss of activity, as seen in the absence of DNA (Figure 6A). The Walker B mutant E854A UvrA2, in which proximal ATP binding is preserved but hydrolysis is disrupted, exhibits a burst of ATP hydrolysis and Pi release by the wild-type distal sites, followed by the linear steady state ($k_{cat} = 0.6 s^{-1}$), again as seen in the absence of DNA (Figure 6A). These results show that in the UvrA2–native DNA complex, ATP binding to the weak proximal sites remains necessary for ATP hydrolysis by the distal sites, and that native DNA does not fundamentally alter the distal site ATPase mechanism, except to speed up the $k_{cat}$ to some extent (likely by accelerating ADP release; Figure 9). The corresponding distal site mutants, K434A UvrA2 and E854A UvrA2, in which proximal sites are intact, do not exhibit any burst activity with native DNA, just as in the absence of DNA but, notably, the Walker A mutant K37A UvrA2, in which distal ATP binding is disrupted, has a >10-fold faster ATPase rate (1.4 s$^{-1}$ with DNA versus 0.1 s$^{-1}$ without DNA; Figures 8A and 6B, respectively). This result shows that native DNA stimulates ATP hydrolysis and ADP release by the proximal sites on UvrA2 (when the distal sites are empty).

Next, Figure 8B shows results from experiments in the presence of fluorescein lesion-containing DNA. Again, the proximal site Walker A mutant K37A UvrA2 shows near com-
Figure 8. Native and fluorescein lesion DNAs have different effects on proximal and distal site ATPase mechanisms. Pre-steady state Pi release kinetics were measured for DNA-bound UvrA2 Walker A and B mutants as described for wild-type in Figure 7 (final: 0.25 μM UvrA2, 1 μM DNA, 1 mM ATP and 15 μM MDC-PBP). (A) With the native duplex, K37A UvrA2 shows no burst and has little activity, while E512A UvrA2 exhibits a burst of ATP hydrolysis and Pi release followed by a slow linear phase at $k_{\text{cat}} = 0.6 \text{s}^{-1}$ (slope/2 × [E512A UvrA2]), as in the absence of DNA (Figure 6A, green trace). K643A UvrA2 and E854A UvrA2 do not exhibit burst activity, and the linear rates differ than in the absence of DNA at $k_{\text{cat}} = 1.4 \text{s}^{-1}$ and 0.3 s$^{-1}$, respectively. (B) With lesion DNA, K37A UvrA2 shows no burst and has little activity, while E512A UvrA2 exhibits a slight burst and faster $k_{\text{cat}} = 2 \text{s}^{-1}$ (slope/2 × [E512A UvrA2]). Both K643A UvrA2 and E854A UvrA2 show no burst and have low activity at $k_{\text{cat}} = 0.3 \text{s}^{-1}$. The double Walker A mutant K37A-K643A UvrA2 has little detectable activity (gray trace).

DISCUSSION

The critical question of how UvrA2 ATPase activity is mechanochemically coupled to the search for lesions and initiation of DNA repair has been tackled previously (reviewed in (1)). However, UvrA2 presents a complex case as an ABC-type ATPase with two distinct active sites per monomer, and the workings of the two pairs of sites on the dimer are not resolved. The goal of this study was to determine, for the first time, the transient kinetics of ATP binding, hydrolysis and product release catalyzed by each site in three physiological contexts—free of DNA, and bound to native or lesion-containing DNA. Our results provide new information about the ATPase mechanism of each site, and how the sites are influenced by each other and by DNA. Based on these findings, and information from prior biochemical and structural studies, we propose a model of how each ATPase site contributes to UvrA2 function in NER (Figure 10).

UvrA2 ATPase mechanism in the absence of DNA

We determined that the two distal sites on UvrA2 bind nucleotides tightly and hydrolyze ATP rapidly, whereas the two proximal sites bind nucleotides weakly and hydrolyze ATP slowly. This striking asymmetry is evident in the high affinity nucleotide binding and the exclusive burst of ATP hydrolysis and Pi release by the distal sites in the absence of DNA; as described earlier, inspection of a high resolution UvrA2 structure offers a molecular explanation for the differential activities of the two sites. We also found evidence of allosteric communication between the sites, the most striking being that ATP binding by the proximal sites...
Figure 9. DNA stimulates ADP release from the distal site, only on ATP binding to the proximal site. Mant-ADP dissociation with ATP chase was measured for UvrA2 proximal and distal site Walker A and B mutants as described for wild-type in Figure 7C (final: 0.1 μM UvrA2, 0.2 μM DNA, 10 μM mant-ADP and 2 mM ATP). (A) K37AUvrA2 exhibits slow mant-ADP release with or without DNA as it cannot bind the ATP needed to stimulate ADP release from distal sites. (B) K643AUvrA2 exhibits no signal since proximal sites do not bind mant-ADP at the low concentration tested (Figure 3). (C) E512AUvrA2 and (D) E854AUvrA2 exhibit proximal site ATP and DNA binding-induced stimulation of mant-ADP release from distal sites as observed with wild-type UvrA2 (Figure 7C).

is necessary for ATP hydrolysis. ADP release and catalytic turnover by the distal sites (note: the reverse is not true, but nucleotide occupancy at distal sites does alter proximal site activity to some extent as well). Interactions across the dimer linking Walker A and B sub-structures have been proposed previously as a conduit for communication between proximal and distal sites (26). Figure 10A shows the ATPase mechanism for apo UvrA2, wherein high affinity distal sites remain ATP-bound until ATP binding to the > 300-fold lower affinity proximal sites (A2) triggers fast hydrolysis and Pi release (A3) followed by slower ADP release (A4), which limits catalytic turnover (this step also requires ATP-bound proximal sites). Meanwhile proximal sites hydrolyze ATP at a slower rate and release both products rapidly (A5, A6). It is unclear if UvrA2 has an obligate sequential ATPase mechanism, i.e. if proximal sites must hydrolyze ATP after each turnover of the distal sites, or if they hydrolyze ATP stochastically at a low frequency while the distal sites continue cycling (two pathways lead back to A2 in Figure 10A). In either case, P_{2ATP-D2ADP} (A3) accumulates in the reaction during steady state. Note that the low nucleotide affinity proximal sites may empty often, resulting in transient P_{empty-D2ADP} species, but at high ATP concentration the equilibrium should favor P_{2ATP-D2ADP}.

UvrA2 ATPase activity has been linked to significant changes in its conformation and dynamics, as detailed below. UvrA2 structures from different organisms have been determined in nucleotide-free, P_{2ADP-D2ADP} or P_{empty-D2ADP} state, with most in open conformation (including the T. maritima UvrA2 structure reported here; Supplementary Figure S3A) with a shallow and wide DNA binding surface (23–26), and one structure (in complex with UvrB) in closed conformation with a deep and narrow DNA binding surface (15). Open UvrA2 can accommodate both native and
damaged DNA, whereas the closed form appears to preclude binding of DNA distorted by damage (15). One important feature of ABC ATPase sites is the conserved signature domain (Figure 1B and C), which changes conformation in concert with ATP binding, hydrolysis and product release, coupling these events to changes in protein structure and interactions (69–72). In UvrA2, the proximal site signature domain II is notable as it presents cationic residues on the DNA-binding surface in both open and closed UvrA2 forms, and contributes to the UvrA2–UvrB binding interface as well (15,23). Nucleotide-bound/free UvrA2 structures reveal signature domain II in different conformations, suggesting it could affect ATPase-driven changes in UvrA2 interactions with DNA and UvrB. Such dynamism has not been reported for distal site signature domain I. A C-terminal zinc-binding hairpin within the proximal site signature domain II also adopts different conformations and is implicated in ATPase-modulated interactions of UvrA2 with damaged DNA (the UvrA2 structure reported here shows the hairpin in a new, intermediate position; Supplementary Figure S3B) (7,42). These movements depend more on distal site ATPase activity, illustrating an asymmetric allosteric effect on the distal site reaction on proximal site structure (42). Prior studies also indicate that UvrA2 dimerizes more effectively in the presence of ATP than non-hydrolyzable ATPγS or ADP (39), and that an ATPase-active distal site favors dimerization while the proximal site may be empty (33). UvrA2 also binds more specifically to lesions in the presence of ATP than ATPγS, ADP or no nucleotide (33,39). All of these results indicate the need for a mixed ATP/ADP/nucleotide-free UvrA2 species to initiate NER, and provide functional validation for our finding that the protein cycles through rapid ATP hydrolysis by the distal sites and produces a significant fraction of mixed P2ATP-D2ADP (or Pempty-D2ADP) dimer in steady state, ready to interact with DNA and locate a lesion either via 3D or combined 3D and localized 1D diffusion (5,6).

**UvrA2 ATPase mechanism in the presence of DNA**

Interaction between UvrA2 and native DNA has an asymmetric allosteric effect on the ATPase mechanism, with proximal sites undergoing significant change and distal sites less so. The proximal sites still bind nucleotide weakly, but ATP hydrolysis is accelerated when distal sites can hydrolyze ATP or are empty. Meanwhile, the distal sites still bind nucleotide tightly and require ATP binding by proximal sites for rapid ATP hydrolysis and Pi release. DNA stimulates ADP release from distal sites resulting in faster

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**Figure 10.** A summary of the differential effects of DNA on the UvrA2 ATPase mechanism. (A) Absent DNA, all ATPase sites on UvrA2, distal (D, tight binding = dark color) and proximal (P, weak binding = light color), binds ATP and only distal sites hydrolyze ATP and release phosphate rapidly. Rate limiting proximal ATP-induced ADP release from distal sites leads to accumulation of a P2ATP-D2ADP species in steady state (A3). The proximal sites also hydrolyze ATP, but at a relatively slow rate especially when distal sites are empty, and perhaps not in every turnover. (B) When UvrA2 is bound to native DNA, both distal and proximal sites hydrolyze ATP and release Pi rapidly, resulting in a distinct, fully ADP-bound species, P2ADP-D2ADP (B3); weak proximal affinity for ADP can lead to Pempty-D2ADP (B4); ADP release from distal sites remains rate limiting. (C) When UvrA2 is bound to a lesion, distal sites hydrolyze ATP and release both Pi and ADP rapidly; however, ATP hydrolysis by proximal sites is suppressed, thus these sites remain ATP-bound in the reaction through P2ATP-D2ATP, P2ATP-D2ADP, P2ATP-Dempty species (C2–C4), with P2ATP-D2ATP (C2) having the longest lifetime. Drawing key is shown top right; pink numbers highlight UvrA2 species that may affect differential interactions with DNA.
catalytic turnover, but this step remains rate-limiting. Thus, DNA-bound UvrA2 has the ATPase mechanism outlined in Figure 10B, wherein the distal sites remain ATP-bound until proximal ATP binding (B2) triggers fast hydrolysis and Pi release by all four sites (B3). The weak proximal sites release ADP (B4), and subsequent ATP binding (B5) enables ADP release from distal sites (B6). This in turn promotes ATP hydrolysis by proximal sites (B7) and fast ADP release to continue the reaction cycle. Notably, due to DNA-induced changes in the mechanism, UvrA2 transitions more rapidly to the turnover rate. Thus, lesion-bound UvrA2 must orchestrate its hand-off to UvrB for lesion verification. As noted earlier, outward movement of signature II domain in the Pempty-D2ADP open UvrA2 form can disrupt interactions with DNA and UvrB, implying that ATP binding by proximal sites promotes these interactions (15). Consistent with this interpretation, the proximal Walker A mutant has low affinity for UvrB and is defective in loading UvrB on the lesion, whereas the distal Walker A mutant is less affected (15,33).

We propose that changes in the UvrA2 ATPase mechanism on lesion binding, i.e. stabilization of the ATP-bound proximal site and ongoing catalytic turnover by the distal site allows UvrA2 to remain localized at the lesion and recruit UvrB (38). Subsequent ATP hydrolysis and ADP release by the proximal site would reset the signature II domain, promoting UvrA2 dissociation and UvrB access to the lesion. The only crystal structure of UvrA2 bound to DNA containing a pair of opposing fluorescein lesions is in open form (23), with all four ATPase sites empty and signature II domain in a distinct conformation compared with P2ADP-D2ADP and Pempty-D2ADP structures (15,26). Based on the ATPase mechanism of lesion-bound UvrA2 described here, this structure could reflect an early state, before lesion specific interactions promote ATP-bound proximal sites and ATP hydrolysis by the distal sites for recruiting UvrB. Alternately, the structure could reflect a late state after ATP hydrolysis and ADP release, ready to dissociate from DNA and make room for UvrB at the lesion.

A mechanochemical model of ATPase-driven UvrA2 actions during NER

The ATPase kinetic mechanism determined in this study offers a view of the mechanochemical coupling in UvrA2 as it searches for damage lesions in DNA and initiates NER. As seen in other well-studied ATPases, including ABC transporters (30), each step is closely linked to the next in both the mechanical and chemical cycles. For UvrA2, we posit that ATP binding increases affinity for DNA, and DNA binding in turn promotes UvrA2 closure around a native duplex, which triggers ATP hydrolysis, followed by opening of ADP-bound/nucleotide-free UvrA2 with weakened contacts and lower affinity for the duplex, until an ADP–ATP switch resets the cycle and the protein can continue scanning DNA. Lesion binding would block UvrA2 closure, which in turn would alter the ATPase mechanism and maintain UvrA2 in proximal ATP-bound form with higher affinity for DNA and UvrB, setting the stage for UvrB entry. Presumably, a subsequent event would trigger ATP hydrolysis and ADP release, resulting in lesion hand-off to UvrB.
and UvrA2 exit. While many of the transient events in this model have not been explicitly measured, the UvrA2 ATPase mechanism presented here will facilitate more specific interpretation of structural and kinetic data on its interactions with DNA and UvrB, and related conformational dynamics, to understand how this protein uses ATP to initiate NER.

**DATA AVAILABILITY**

Atomic coordinates and structure factors for the reported crystal structure have been deposited with the Protein Data Bank under the accession number 6N9L.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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