Flap endonucleases catalyze cleavage of single-stranded DNA flaps formed during replication, repair, and recombination and are therefore essential for genome processing and stability. Recent crystal structures of DNA-bound human flap endonuclease (hFEN1) offer new insights into how conformational changes in the DNA and hFEN1 may facilitate the reaction mechanism. For example, previous biochemical studies of DNA conformation performed under non-catalytic conditions with Ca^{2+} have suggested that base unpairing at the 5′-flap:template junction is an important step in the reaction, but the new structural data suggest otherwise. To clarify the role of DNA changes in the kinetic mechanism, we measured a series of transient steps, from substrate binding to product release, during the hFEN1-catalyzed reaction in the presence of Mg^{2+}. We found that whereas hFEN1 binds and bends DNA at a fast, diffusion-limited rate, much slower Mg^{2+}-dependent conformational changes in DNA around the active site are subsequently necessary and rate-limiting for 5′-flap cleavage. These changes are reported overall by fluorescence of 2-aminopurine at the 5′-flap:template junction, indicating that local DNA distortion (e.g. disruption of base stacking observed in structures), associated with positioning the 5′-flap scissile phosphodiester bond in the hFEN1 active site, controls catalysis. hFEN1 residues with distinct roles in the catalytic mechanism, including those binding metal ions (Asp-34 and Asp-181), steering the 5′-flap through the active site and binding the scissile phosphate (Lys-93 and Arg-100), and stacking against the base 5′ to the scissile phosphate (Tyr-40), all contribute to these rate-limiting conformational changes, ensuring efficient and specific cleavage of 5′-flaps.

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thread through the active site; (ii) a hydrophobic wedge that fronts Tyr-40 to pack against and help position the last 5'-flap:template base pair at the junction; and (iii) the active site comprising highly conserved acidic residues that coordinate Mg\(^{2+}\) (e.g. Asp-34 and Asp-181) for the two-metal-ion-mediated hydrolysis mechanism (17) and invariant basic residues (Lys-93 and Arg-100) that form ion pairs with the scissile phosphate to facilitate hydrolysis (Fig. 1) (12, 18).

The substrate- and product-bound enzyme structures make it clear that DNA undergoes global (bending of duplex arms) and local (ss/dsDNA junction deformation) conformational changes to achieve a catalytically competent state (Fig. 1). Evidence for corresponding conformational changes in the protein (e.g. disorder-to-order transition of the cap-helical gateway) argues for an induced-fit mechanism of interaction that probably improves substrate specificity (9, 14, 18–21). Single-molecule data indicate that the protein actively bends the DNA, which in turn drives protein ordering into a catalytically active form (15). Fluorescence-based equilibrium binding studies of hFEN1-DNA complexes formed under non-catalytic conditions have probed both global and local DNA conformational changes as well. FRET-based measurements of the duplex arm positions indicate that a bent hFEN1-DNA substrate complex is formed in the absence of metal ions or with Ca\(^{2+}\) \((K_d \sim 10–50 \text{ nM})\), suggesting that this global change occurs before engagement of the phosphodiester backbone by a catalytically competent active site (9, 15, 16, 22). In terms of local DNA conformation, changes in the exciton-coupled circular dichroic spectra (ECCD) of a pair of 2-aminopurine adenine analogs at \(-1/2\) or \(+1/1\) positions in the 5'-flap strand have been interpreted as unpairing of the last two base pairs at the 5'-flap:template junction needed to position the scissile phosphate in the active site (16, 23). Assuming that the ECCD signal of hFEN1-DNA complexes formed with Ca\(^{2+}\), which does not support catalysis, reports catalytically viable conformations, the studies indicate differential contributions of active-site residues to this process. On a single 3'-flap substrate analog missing the 5'-flap, mutations of Asp-181 (hFEN1\(^{D181A}\)), which coordinates metal ion A to facilitate nucleophile formation, and Tyr-40 (hFEN1\(^{Y40A}\)) appear to disrupt the proposed unpairing between \(+1/1\) bases in the 5'-flap strand and template, but mutations of Arg-100 (hFEN1\(^{R100A}\)) and Lys-93 (hFEN1\(^{K93A}\)) have little to no disruptive effect, respectively. On a 5'/3' double-flap substrate, hFEN1\(^{R100A}\) and hFEN1\(^{Y40A}\) partially disrupt the proposed base unpairing (16).

These equilibrium binding data were supported by the structure of a wildtype hFEN1-nicked product complex in which the \(-1\) base in the 5'-flap strand was unpaired from the template (18). A more recent structure of hFEN1\(^{R100A}\)-DNA substrate complex also has the 5'-flap strand \(+1\) and \(-1\) bases unpaired from the template as it threads through the cap-helical gateway; however, the scissile phosphate remains \(\sim 4–5\) Å away from the metal ions in the active site, suggesting that this conformation is not catalytically competent (12). More significantly, the structure of an hFEN1\(^{D86N}\)-DNA substrate complex shows the scissile phosphate coordinated to metal ion A, indicating a catalytically competent conformation, but the \(+1\) and \(-1\) bases remain paired to the template strand (Fig. 1C; PDB code 5UM9; metal ion B is absent because the Asp-86 ligand is mutated to Asn) (12). Notably, bases at the junction remain paired in recent hExo1-DNA structures as well (13). Despite the intact base pairing, both 5'-flap and template strands are distorted around the scissile phosphate, such that stacking is disrupted between the \(+1\) and \(-1\) bases and absent between the \(-1\) and \(-2\) bases in the 5'-flap strand, and the \(-2\) base stacks with the template strand instead. There is also a dramatic inversion of the phosphodiester backbone between the \(+1\) and \(+2\) nucleotides under the cap-helical gateway, such that the \(+2\) base is flipped outward (Fig. 1, B and C); this inversion, observed in hExo1-DNA structures as well, is proposed to protect the 5'-flap from premature cleavage during the threading process. In the hFEN1\(^{D86N}\)-DNA substrate structure and that of an hFEN1\(^{D233N}\)-DNA product complex (Fig. 1C; PDB code 5K97), basic residues lining the cap-helical gateway, including Lys-93 and Arg-100, appear necessary to electrostatically steer the phosphodiester backbone into a catalytically competent conformation, with the scissile phosphate coordinated by the metal ions and a nucleophilic water within striking distance in the active site. Meanwhile, Tyr-40 adopts different rotamer conformations, stacked with the \(+1\) base of the double-flap substrate or the \(-1\) base of the nicked duplex product, suggesting that this residue helps lock the junction in position before and after catalysis (12). Because the structural data imply that Lys-93, Arg-100, and Tyr-40 play important roles in the hFEN1-DNA complex achieving a catalytically competent state, the relatively mild effects of their mutations reported by the ECCD signal are somewhat surprising (16, 23).

The new structural data present novel hypotheses about changes in DNA conformation en route to catalysis, most notably that unpairing of bases at the 5'-flap:template junction is not required to achieve an active state, contrary to previous interpretations of DNA-binding data (16, 22, 23). Moreover, because the DNA binding studies of hFEN1 were conducted in equilibrium under non-catalytic conditions with Ca\(^{2+}\), the significance of the proposed conformational changes for the hFEN1 kinetic mechanism is not fully understood. To address this gap in our knowledge, we examined multiple steps of the hFEN1 reaction, from substrate binding to product release and any associated DNA isomerization, under catalytic conditions with Mg\(^{2+}\) by ensemble stopped-flow and rapid-quench experiments. We also examined active-site residue mutants of hFEN1 under these conditions to parse their contributions to the kinetic mechanism. The results show that after the bent DNA complex is formed at a diffusion-limited rate, slower Mg\(^{2+}\)-specific local changes in the 5'-flap:template junction conformation limit the rate of catalysis. These changes are reported by unquenching of 2-aminopurine fluorescence, implying that they involve changes in the extent of base stacking at the junction, as suggested by the structural data (12). In addition, active-site residues known for distinct roles in the catalytic mechanism all contribute to this rate-limiting stage of the 5'-flap cleavage mechanism.

Results

We monitored individual steps in the hFEN1-catalyzed reaction with a variety of fluorophore-labeled DNAs to investigate
the order and significance of DNA conformational changes in the kinetic mechanism. Transient kinetic measurements from ensemble stopped-flow and rapid-quench experiments yielded rate constants for multiple steps in the reaction, from substrate binding to product release, revealing rate-limiting steps that control catalysis and turnover.

**Initial rapid collision and DNA bending followed by slower cleavage and product release**

DNA binding and bending occur at a diffusion-limited rate, and engagement of the active site leads to a highly stable hFEN1-DNA\textsubscript{bent} complex—a FRET-based assay was employed first to measure global changes in DNA conformation during the reaction (9, 15, 16). A non-equilibrated double-flap DNA substrate (DNA\textsuperscript{F-T}) was constituted with a donor-acceptor dye pair by labeling the 3’-flap strand with fluorescein, 14 nt into the duplex upstream of the flap junction, and the template strand with 5- (and 6)-carboxytetramethylrhodamine (TAMRA), 13 nt into the duplex downstream of the junction (Fig. S1; 5’- and 3’-flaps are not complementary to the template in the non-equilibrated substrate). A decrease in donor fluorescence intensity and corresponding increase in acceptor fluorescence reports bending of the duplex arms upon hFEN1 binding under non-catalytic conditions (with 10 mM CaCl\textsubscript{2}; Fig. S2A), as shown previously (9, 15, 16). Control experiments performed with the same substrate labeled with either fluorescein or TAMRA alone show little to no change in fluorescence emission when mixed with hFEN1 (Fig. S2A), indicating negligible direct effect of the protein on the dyes, consistent with their locations beyond the footprint of hFEN1 (18) or Pyrococcus furiosus FEN1 on DNA (24). The kinetics of hFEN1 binding to DNA\textsuperscript{F-T} were measured first under non-catalytic conditions with 10 mM CaCl\textsubscript{2} by monitoring donor and acceptor fluorescence over time after mixing on a stopped-flow instrument. Fig. 2A shows a rapid decrease in donor fluorescence, reflecting bending of the DNA substrate. The interaction was measured next under catalytic conditions with 8 mM MgCl\textsubscript{2} (20, 25). Fig. 2B shows an initial rapid decrease in donor fluorescence (reflecting DNA bending) followed by slow recovery of the signal; Fig. S2B shows corresponding changes in acceptor fluorescence as well as a control experiment with donor-only–labeled DNA\textsubscript{F} that shows no change in signal. The rate of the first phase increases linearly with hFEN1 concentration and yields a bimolecular rate constant, \( k_{\text{on-bend}} = 2.6 \times 10^9 \text{M}^{-1} \text{s}^{-1} \) (Fig. 2C and Table 1), similar to that observed with Ca\textsuperscript{2+} (\( k_{\text{on-bend}} = 2.2 \times 10^9 \text{M}^{-1} \text{s}^{-1} \); Table 2) and close to the diffusion-controlled limit (26, 27) (note that the second, donor fluorescence recovery by hFEN1 to create a nicked product that can be sealed by DNA ligase. B, crystal structure and schematic of hFEN1 in complex with a substrate (5UM9; hFEN1\textsuperscript{D86N} mutant) (12). The enzyme makes extensive contact with duplex regions flanking the flap junction, bends DNA by \(-100^\circ\), and threads the 5’-flap (red) through a cap-helical gateway that overlooks the active site (A and B denote metal ion positions). C, close-up views of hFEN1-substrate (5UM9; hFEN1\textsuperscript{D86N}) and hFEN1-product (5K97; hFEN1\textsuperscript{D233N}) complexes show DNA distortion at the flap junction (12) and some key active-site residues: Asp-34 and Asp-86 that coordinate metal ion B, Asp-181 and Asp-233 that coordinate metal ion A, Lys-93 and Arg-100 that help position the 5’-flap, and Tyr-40 that stacks on the 1 and 2 bases in the substrate and product complexes, respectively (\( \times \) marks the absent metal ion B in the hFEN1\textsuperscript{D86N} mutant).
Slow DNA isomerization in active site controls flap cleavage

Figure 2. Kinetics of DNA substrate binding and bending by hFEN1. DNA binding and bending in the presence of Ca\(^{2+}\) (A) or Mg\(^{2+}\) or EDTA (B) was measured by change in FRET over time on mixing donor (fluorescein)- and acceptor (TAMRA)-labeled DNA (DNA-F-T) with wildtype hFEN1 on a stopped-flow instrument (final concentrations: 0.125–0.225 \(\mu\)M hFEN1, 0.025 \(\mu\)M DNA-F-T, 10 mM CaCl\(_2\), 8 mM MgCl\(_2\), or 1 mM EDTA). The data were fit to single- or double-exponential functions (n indicates data normalized to the starting point), C, an initial concentration-dependent decrease in donor fluorescence indicates DNA-F-T binding and bending at a diffusion-limited rate (\(k_{\text{on-bend}} = 2.6 \times 10^9 \text{ M}^{-1} \text{s}^{-1}\) with Mg\(^{2+}\); see Table 2 for rates with Ca\(^{2+}\) and EDTA; error bars indicate S.D.). A subsequent increase in donor fluorescence in the presence of Mg\(^{2+}\) indicates DNA-F-T unbending at a constant rate \(k_{\text{on-bend}} = 26 \pm 3 \text{ s}^{-1}\); there is no second phase with Ca\(^{2+}\) and EDTA, which suggests that DNA unbending is associated with flap cleavage. D, hFEN1 mutant interactions with DNA were measured in the presence of Mg\(^{2+}\) as in B for wildtype and yielded similar rates for DNA binding and bending (Table 1) but no subsequent recovery of donor fluorescence (except with hFEN1Y40A at a very slow rate), indicating severe defects in 5’-flap cleavage resulting from alanine substitutions of these active-site residues. E, DNA-F-T dissociation from hFEN1 was measured by mixing the complex formed in the presence of Ca\(^{2+}\) with excess unlabeled substrate as chase (final concentrations: 0.1 \(\mu\)M hFEN1, 0.025 \(\mu\)M DNA-F-T, 2 \(\mu\)M DNA chase, and 10 mM CaCl\(_2\)). With wildtype hFEN1, donor fluorescence recovers in two phases, and the data fit to a double-exponential function yield rates of 0.4 s\(^{-1}\) (35%) and 0.017 s\(^{-1}\) (65%). In contrast, DNA-F-T dissociation from the hFEN1 mutants occurs in a single phase (inset shows the hFEN1Y40A kinetic trace as an example; see Table 2 for rates), F, the recovery of donor fluorescence seen in A was followed to completion in a chase experiment in which hFEN1 was mixed first with DNA-F-T and then with excess unlabeled substrate to block labeled DNA re-binding (final concentrations: 0.17 \(\mu\)M hFEN1, 0.017 \(\mu\)M DNA-F-T, and 1.4 \(\mu\)M DNA chase). The signal increases over time in two phases at \(k_{\text{on-bend}} = 26 \pm 4 \text{ s}^{-1}\) and \(k_{\text{on-bend}} = 0.5 \pm 0.3 \text{ s}^{-1}\), indicating slow rates of DNA isomerization in active site to form the initial hFEN1-DNA\(_{\text{bent}}\) complex. This hypothesis was tested by DNA-binding experiments with hFEN1 mutants in which conserved residues Asp-181 and Asp-34, which coordinate metal ions A and B, respectively, in the active site to form the initial hFEN1-DNA\(_{\text{bent}}\) complex. The data show that DNA binding and bending by hFEN1 occur at the same fast rate in the presence of EDTA (Fig. 2B and Table 2), as noted in single-molecule measurements as well (15), indicating that metal ions need not be present in the active site to form the initial hFEN1-DNA\(_{\text{bent}}\) complex. This hypothesis was tested by DNA-binding experiments with hFEN1 mutants in which conserved residues Asp-181 and Asp-34, which coordinate metal ions A and B, respectively, in the active site to form the initial hFEN1-DNA\(_{\text{bent}}\) complex.
site, were mutated to alanine (Fig. 1) (28). Both hFEN1^{D181A}\ and hFEN1^{D34A}\ yield similar binding/bending rate constants in the presence of Mg^{2+} and Ca^{2+} as wildtype protein (Fig. 2D and Tables 1 and 2). None of the other active-site mutants tested in this study, hFEN1^{K93A}\ and hFEN1^{R100A}\ (residues that help steer the 5'-flap strand into the active site) and hFEN1^{Y40A}\ (a steric guide for the last base at the 5'-flap-template junction), exhibit significant differences in the DNA binding/bending rate either (Fig. 2D and Tables 1 and 2). In single-molecule experiments performed with some of these mutants, hFEN1^{Y40A}\ and hFEN1^{R100A}\ did show a 3- and 10-fold decrease in the binding/bending rate relative to wildtype hFEN1. The wildtype binding rate was also ~20-fold slower than that reported here, which may be due to the lower temperature of the single-molecule experiments (22 °C versus 37 °C here); therefore, small differences between wildtype and mutant rates may have been better resolved (15). Notwithstanding these differences, the results are consistent with diffusion-limited collision and bending of the DNA substrate by hFEN1, which does not require participation of the active site, including metal ions.

We also assessed the stability of the bent DNA complex by measuring the dissociation kinetics of hFEN1 and DNA^{F-T} pre-incubated with CaCl_2 and then mixed with excess unlabeled DNA chase (unlabeled double-flap DNA substrate). The recovery of donor fluorescence reports release of DNA^{F-T} in two phases from wildtype hFEN1 (Fig. 2E and Table 2), which could be indicative of two hFEN1-DNA\_bent populations with differing affinities. About 35% dissociates at \( k_{bent-off-1} = 0.4 \text{ s}^{-1} \) and ~65% at a slower \( k_{bent-off-2} = 0.02 \text{ s}^{-1} \) (similar results were obtained over a range of 1:1 or 1:7 ratios of DNA/hFEN1). The \( k_{off}/k_{on} \) ratio yields \( K_d = 0.18 \text{ nM} \) for the relatively weak complex, hFEN1-DNA\_bent-1 (note that no 5'-flap cleavage occurs under these conditions with Ca^{2+}; Fig. 3). An equilibrium titration of DNA^{F-T} with increasing concentrations of hFEN1 in the presence of Ca^{2+} yields a \( K_d \) of 0.08 nM (Fig. S2C); note that at the lowest measurable concentration of 0.5 nM DNA^{F-T}, the binding isotherms appears stoichiometric at a 1:1 DNA:hFEN1 ratio, indicating that the \( K_d \) is at least 5-fold lower than 0.5 nM. This \( K_d \) value is >250-fold tighter than reported from previous equilibrium DNA-binding studies (\( K_d \sim 20-50 \text{ nM} \)) (9, 16, 22), perhaps due to differences in experimental conditions. However, the slow \( k_{bent-off-2} \) of 0.02 s^{-1} for the relatively tight complex, hFEN1-DNA\_bent-2, is consistent with single-molecule measurements in which dissociation of hFEN1-DNA\_bent complex formed with a non-equilibrating DNA substrate in the presence of Ca^{2+} was rarely detected over 60-s acquisition times (15). Interestingly, in similar experiments performed with wildtype hFEN1 in the presence of EDTA or with active-site mutants in the presence of Ca^{2+}, the DNA dissociates in just one fast phase with \( k_{off} \) values of ~0.1 s^{-1} for hFEN1 (with EDTA), hFEN1^{D34A}, hFEN1^{D181A}, and hFEN1^{K93A} and 0.3 s^{-1} for hFEN1^{Y40A} (Fig. 2E, inset), similar to the 0.4 s^{-1} rate for the weaker hFEN1-DNA\_bent-1 complex; the \( k_{off} \) value is slightly faster at 0.7 s^{-1} for hFEN1^{R100A} (Table 2). These results indicate that metal ions and active-site residues Asp-34, Asp-181, Lys-93, and Tyr-40 are not involved in formation of the weaker hFEN1-DNA\_bent-1 complex (\( K_d = 0.18 \text{ nM} \)), whereas Arg-100 may be involved even at this early stage. Moreover, absence of the second, tighter complex observed with wildtype hFEN1 (\( k_{bent-off-2} = 0.02 \text{ s}^{-1} \)) indicates its formation requires engagement of all of the active-site residues tested here and the metal ions as well. These results are consistent with single-molecule measurements in which active-site mutants failed to form a stable bent DNA complex (15).
Rapid substrate binding and bending is followed by much slower 5′-flap cleavage—After diffusion-limited DNA binding/bending, as reported by the decrease in donor fluorescence in Fig. 2B, there is partial recovery of the signal at a much slower rate, $k_{\text{unbend}} = 26 \text{ s}^{-1}$ in the presence of $\text{Mg}^{2+}$. The rate of this second phase is independent of hFEN1 concentration (Fig. 2C), indicating that it reflects a step after DNA binding/bending. An initial hypothesis is that this step is associated with 5′-flap cleavage and perhaps involves unbending of the nicked duplex product, given that the second phase is absent when catalysis is blocked by EDTA (Fig. 2B). Moreover, the measured rate is within the 10–40 s$^{-1}$ range of cleavage rates reported previously (3, 10, 20, 22, 25). Finally, the active-site mutants, which are known to suffer severe loss of catalytic activity, also do not exhibit any recovery of donor fluorescence, except hFEN1V670A, which retains some activity and shows a corresponding slow increase in donor fluorescence (Fig. 2D) (12, 16, 22, 28). The cleavage activity of wildtype hFEN1 was measured directly under single-turnover conditions to test this hypothesis.

In a single-mixing experiment, DNA labeled with fluorescein (FAM) at the 5′-end of the 5′-flap strand (DNA$^{39}$) was mixed with a 10-fold excess of hFEN1 in a quench-flow instrument, and the reaction was stopped with EDTA at various times. The FAM-labeled substrate and 5′-flap product were resolved by denaturing gel electrophoresis, and the fraction of product formed plotted versus time yielded a cleavage rate, $k_{\text{sto}} = 24 \text{ s}^{-1}$ (Fig. 3; a control experiment shows no cleavage in the presence of $\text{Ca}^{2+}$, even at 200 s). For comparison, the experiment was also performed with an equilibrating DNA substrate (5′- and 3′-flaps with a common 1 nt complementary to template at junction) and yielded a similar cleavage rate of 18 s$^{-1}$ (Fig. S3, A and B), consistent with a previous report (25). The match between the rates of cleavage shown in Fig. 3 and the second phase of the FRET-based experiment shown in Fig. 2B confirms rapid unbending of the nicked DNA product after 5′-flap cleavage.

Yet slower release of partially unbent nicked duplex product limits steady-state turnover—Two aspects of the DNA unbending phase observed in Fig. 2B raise further questions. One is that the recovery of donor fluorescence plateaus at a level lower than that of free DNA (Fig. 2B, time zero). The experiment was performed under single-turnover conditions with excess hFEN1 (0.13–0.23 $\mu\text{M}$) at concentrations higher than the reported $K_i$ for a nicked duplex based on product inhibition experiments (0.075 $\mu\text{M}$) (10). Therefore, it is likely that after releasing the nicked duplex product, hFEN1 rebinds and bends it, resulting in higher FRET at equilibrium (lower donor fluorescence). The other aspect is that the product inhibition experiments also indicate that release of nicked DNA from hFEN1 limits catalytic turnover (the 5′-flap is not an effective competitive inhibitor, indicating low affinity) (10, 15). However, the 26 s$^{-1}$ nicked DNA unbending rate observed here is about 10-fold faster than the reported $k_{\text{cat}}$ of ~3 s$^{-1}$ (10, 20, 25). This discrepancy suggests that nicked DNA unbending occurs before its release from hFEN1 or that another slower step after release of this product limits catalytic turnover. We resolved these two possibilities by a double-mixing experiment designed to directly measure the rate of product release from hFEN1. The fluorescein/TAMRA-labeled DNA$^{\text{F-T}}$ was mixed first with hFEN1 for 20 ms on the stopped flow, which is just enough time to complete substrate binding and bending (Fig. 2B), followed by the addition of buffer or excess unlabeled substrate as chase to block rebinding of the nicked DNA product. As shown in Fig. 2F, when the preformed hFEN1-DNA$_{\text{bent}}$ complex is mixed with buffer, donor fluorescence increases at 19 s$^{-1}$ (due to 5′-flap cleavage and DNA unbending) and plateaus before full recovery, as expected from the results in Fig. 2B. However, when the hFEN1-DNA$_{\text{bent}}$ complex is mixed with unlabeled chase, donor fluorescence increases in two phases, first at 26 s$^{-1}$ and then ~15-fold slower at 1.6 s$^{-1}$ (Fig. 2F), a rate that perfectly matches the $k_{\text{cat}}$ of 1.4 s$^{-1}$ measured in steady-state cleavage experiments (Table 1). Thus, the chase experiment reveals a two-step process of product release: first, rapid isomerization of the nicked DNA product after cleavage (perhaps partial relaxation of the bent state along with release of the 5′-flap product), followed by slower dissociation from hFEN1 (and full unbending) to complete the catalytic turnover. Recent single-molecule measurements also indicate a similar two-step product release mechanism (29). This conformational change and slow release process could facilitate coordinated handoff of the nicked duplex from hFEN1 to DNA ligase.

Slow local DNA distortion at the 5′-flap:template junction controls the rate of catalysis— Fluorescence of 2-aminopurine bases flanking the scissile phosphate reports hFEN1 endonuclease and exonuclease activities—The rates of DNA substrate binding/bending and cleavage differ over an order of magnitude (e.g. DNA binding/bending occurs at ~500 s$^{-1}$ with 0.2 $\mu\text{M}$ hFEN1 in the reaction, whereas 5′-flap cleavage occurs at 24 s$^{-1}$ at 37°C (Table 1)). Therefore, we asked what events might be rate-limiting for catalysis after formation of the initial hFEN1-DNA$_{\text{bent}}$ complex. Because key active-site residues engage with the substrate after initial collision and DNA bending (Fig. 2D and Table 2), isomerization of the complex to position the scissile phosphate in the active site could be slow and rate-limiting. Recent crystal structures of hFEN1, hExo1, and T5Fen bound to their DNA substrates with the scissile phosphate close to or in the active site show significant deformation of both the 5′-flap and template strands at the junction, including disruption of base stacking (12–14). Moreover, ECDX spectra of 2-aminopurine (2-AP) bases at the 5′-flap:template junction also report local distortion in hFEN1-DNA complexes formed under non-catalytic conditions with $\text{Ca}^{2+}$, which has been interpreted as base unpairing (16, 23).

To investigate whether these conformational changes in DNA limit catalysis, we utilized 2-AP fluorescence as a reporter of 5′-flap:template junction conformation during the cleavage reaction. Two DNA substrates comprising the same sequence as the non-equilibrating cleavage assay substrate were prepared with 2-AP incorporated in the 5′-flap strand at the +1 position (5′ to the scissile phosphate; DNA$^\text{A}$) or at both the +1 and −1 positions (5′ and 3′ to the scissile phosphate; DNA$^\text{AA}$). In single-mixing experiments performed under catalytic conditions with 8 mM MgCl$_2$, hFEN1 was mixed with DNA in the stopped flow, and changes in 2-AP fluorescence were monitored over...
time. Fig. 4A shows that with DNAAA (2-AP at +1 and −1), 2-AP fluorescence increases rapidly at a rate of 25 s⁻¹, which is the same as the 24 s⁻¹ rate of 5'−flap cleavage (Fig. 3 and Table 1) and suggests that these two events are coupled (note that no change in 2-AP fluorescence is detectable when Ca²⁺ is substituted for Mg²⁺). The reaction monitored over a longer time
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shows an additional, slower increase in 2-AP fluorescence at a rate of 0.01 s⁻¹ (Fig. 4B; note that even on this time scale, there is no change in 2-AP fluorescence with Ca²⁺). This second phase is absent when the experiment is performed with the DNA³⁻ substrate, which does not contain 2-AP at the −1 position (Fig. S4). Therefore, we hypothesized that the secondary slow increase in signal arises from release of the −1 2-AP into solution by 5’−3’ exonucleolytic activity of hFEN1 that cuts into the nicked duplex after endonucleolytic cleavage of the 5’-flap. This hypothesis was tested by directly measuring hFEN1 exonuclease activity using a DNA substrate labeled with FAM at the 3’-end of the 5’-flap strand (DNA³⁻). In a single-mixing experiment, DNA³⁻ was mixed with 10-fold excess of hFEN1 in a quench-flow instrument, and the reaction was stopped with EDTA at various times. The FAM-labeled substrate and 3’-end-labeled products resulting first from removal of the 15-nt 5’-flap (18 nt) and subsequently from cleavage of the nicked product (<18 nt) were resolved by denaturing gel electrophoresis, and the fraction of <18-nt products plotted versus time yielded an exonuclease rate, kₐₒₓ = 0.016 s⁻¹ (Fig. 4B). This rate is comparable with the 0.01 s⁻¹ fluorescence increase reported by 2-AP at the −1 position of the 5’-flap strand, confirming that this signal reflects hFEN1 exonuclease activity. Interestingly, no exonuclease activity is detected with hFEN1¹⁴⁰A (Fig. 4C), implying a role for this residue in readying the nicked product for further cleavage.

DNA distortion while positioning the scissile phosphate in the active site limits catalysis—Next we attempted to determine the underlying cause of the initial 25 s⁻¹ increase in 2-AP fluorescence upon mixing DNAAA with hFEN1, which appears coupled to 5’-flap cleavage (Figs. 3 and 4A). One possibility is that the signal arises as the 5’-flap containing the +1 2-AP is cleaved and released rapidly into solution (i.e. after cleavage). Another possibility is that the signal arises from conformational changes at the junction leading to catalysis (i.e. before cleavage). We turned to hFEN1 active-site mutants (Fig. 1) to resolve these options. The rationale was that one or more of these mutants may have differential effects on isomerization of the hFEN1-DNA₃⁻ complex versus catalysis, and any resulting differences between the 2-AP fluorescence change and cleavage rates would identify the event reported by 2-AP and its order in the reaction. First, single-mixing experiments were performed under catalytic conditions with 8 mM MgCl₂ by mixing hFEN1 mutants with DNAAA in the stopped flow and monitoring the increase in 2-AP fluorescence over time. The results from this assay show that all mutants are severely impacted, but to varying extents (Fig. 4C and Table 1). Mutation of Tyr-40 to alanine slows down the change in 2-AP fluorescence from 25 s⁻¹ with wildtype hFEN1 to 0.3 s⁻¹ when hFEN1¹⁴⁰A is mixed with DNA³⁻ (the signal amplitude is larger possibly due to loss of the quenching effect of Tyr-40 base-stacking with 2-AP at the junction) (23). Mutation of the Asp-34 residue (hFEN1¹³⁴⁰A) has a similar effect, lowering the rate to 0.1 s⁻¹ (in this case, the signal amplitude is similar to that of wildtype). Mutations of Lys-93 (hFEN1¹⁹⁶⁰A) and Arg-100 (hFEN1¹⁰⁰⁰A) have an even more drastic effect, reducing the rate to 0.01 s⁻¹ and lower, respectively, and finally, mutation of Asp-181 (hFEN1¹¹⁸¹A) abolishes the 2-AP signal.

We also measured the corresponding single turnover cleavage rates of these mutants as described for wildtype hFEN1 earlier (Fig. 3). The 5’-flap strand FAM (5’-end)-labeled DNA³⁻ was mixed with each hFEN1 mutant in a quench-flow instrument and the reaction was stopped with EDTA at various times. The FAM-labeled substrate and 5’-flap product were resolved by denaturing gel electrophoresis, and the fraction of product formed plotted versus time yielded the cleavage rate (Fig. 4D). hFEN1¹⁴⁰A exhibits a cleavage rate of 0.25 s⁻¹, which matches the 0.3 s⁻¹ rate of the 2-AP signal change (Fig. 4C and Table 1) and indicates that the two events remain coupled in this mutant, without resolving which one occurs first. Analogous results were obtained with the hFEN1¹⁹⁶⁰A, hFEN1¹⁰⁰⁰A, and hFEN1¹¹⁸¹A mutants, which have progressively lower cleavage rates (Fig. 4D) that match the low rates of the 2-AP signal change (Fig. 4C). In the case of hFEN1¹³⁴⁰A, however, the cleavage activity is completely abolished (Fig. 4D), although this mutant can undergo an increase in 2-AP fluorescence at a rate of 0.1 s⁻¹ that is comparable with hFEN1¹⁴⁰A (Fig. 4C). Thus, the D34A mutation uncouples the two events measured by the 2-AP and 5’-flap cleavage assays and reveals that the 2-AP signal reports an event before catalysis. The unquenching of 2-AP fluorescence indicates that it involves changes in DNA conformation at the junction, including disruption of base stacking at the +1 and/or −1 positions in the 5’-flap strand. Clearly, Asp-34, which coordinates metal ion B, participates in this DNA distortion preceding catalysis, because its mutation to alanine slows the process by ~250-fold (from 25 s⁻¹ for wildtype hFEN1 to 0.1 s⁻¹ for hFEN1¹³⁴⁰A); however, the residual rate indicates that its contribution is less than Lys-93, Arg-100, and Asp-181/metal ion A, perhaps because these amino acids are located closer to the scissile phosphate and therefore play a greater role in positioning the bond for cleavage (Fig. 1) (12). Asp-34 is, however, absolutely essential for catalysis because metal ion B stabilizes the pentavalent phosphorus transition state and the enzyme–product complex during the reaction (17, 30); therefore, its mutation shuts down catalysis. Based on these results, we can conclude that in wildtype hFEN1, conforma-
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Scheme 1. Kinetic model of the hFEN1-catalyzed reaction. Key steps in the 5'-flap cleavage reaction are depicted along with rate constants that globally fit all of the kinetic data (see Fig. S5) (33). hFEN1 binds and bends its DNA substrate rapidly at a diffusion-limited rate to form an initial complex that achieves higher affinity through additional interactions with the active site (inferred from binding/dissociation rates with Mg^{2+} or Ca^{2+}). This complex undergoes slower changes in local DNA conformation, reported by disruption of base stacking at the 5'-flap:template junction as it is positioned for catalysis through the cap-helical gateway with the help of multiple active-site residues (see hFEN1D86N; Fig. 1). This rate-determining isomerization step is followed by rapid cleavage and partial unbending of the nicked DNA product (and 5'-flap release, not measured in this study). The nicked product is released at a much slower rate that limits steady-state turnover, which is unsurprising, given extensive hFEN1 interactions with the duplex portions of the product and key contacts in the active site, such as Tyr-40, which may help stabilize DNA in the correct conformation for handoff to DNA ligase (see hFEN1D233N; Fig. 1) (12, 18). *, nicked duplex binding rate estimated from reported k_i of 75 nm (10).

**Discussion**

We measured a series of transient events during the hFEN1-catalyzed 5'-flap cleavage reaction under the same experimental conditions to address outstanding questions about the kinetic mechanism of this critical DNA metabolic enzyme. Our findings are broadly supportive of recent structural and single-molecule studies of 5'-structure-specific nucleases, including FEN1, indicating that a multistep process involving protein and DNA conformational changes is employed by these enzymes to achieve substrate specificity and catalytic efficiency (12–15, 21, 29, 31, 32). This study provides new and complementary ensemble kinetic information by identifying rate-limiting steps in the reaction mechanism that control catalysis and steady-state turnover. All of the kinetic data obtained for wildtype hFEN1 were fit simultaneously to a minimal model of the reaction using KinTek Explorer (Scheme 1) (33). The measured rates were used as initial estimates for global fitting, and the best fits are shown with corresponding data in Fig. S5. The steps in the model are described below with transient events related to available structural data.

**Stepwise progression of hFEN1-DNA toward a catalytically active complex**

The results show that hFEN1 initiates the cleavage reaction by binding and bending a 5'/3' double-flap DNA substrate at a diffusion-limited rate on the order of 10^9 M^-1 s^-1. This initial interaction occurs at the same rate with Mg^{2+} or Ca^{2+} or in the presence of EDTA (Tables 1 and 2) (15) and even when some of the active-site residues are mutated to alanine (Asp-34, Lys-93, Asp-181, and Tyr-40). Dissociation rates of the DNA substrate bound to hFEN1 in the presence of Ca^{2+} indicate two populations (hFEN1-DNA_{bent-1} and hFEN1-DNA_{bent-2}), one less stable than the other (k_{bent off-1} = 0.4 s^-1 and k_{bent off-2} = 0.02 s^-1). Only the weaker complex is formed without metal ions or with the active-site mutants (Table 2). Prior evidence from single-molecule experiments indicates that DNA binding/bending is not affected if the 3'-flap is absent or if 5'-flap threading is blocked, and the resulting complexes are transient (15). We interpret these data to mean that an initial, weak complex (hFEN1-DNA_{bent-1}) forms before 3'-flap binding and 5'-flap threading, while the cap-helical gateway is still disordered, perhaps simply by dint of the major interaction interface between hFEN1 and the duplex arms flanking the junction. This interpretation is consistent with an early reaction intermediate from a recent series of hExo1-DNA crystal structures, which shows that the mobile arch (analogous to the hFEN1 helical gateway) is open and the active site does not contain the 5'-flap:template junction or metal ions ([Mg^2+]) (13). Interestingly, we observed that mutation of Arg-100 further weakens hFEN1-DNA_{bent-1} by 2–4-fold (Table 2), a subtle feature of the interaction that was noted recently in single-molecule experiments as well (15). In addition to its role in stabilizing the charge on the scissile phosphate oxygens during catalysis, Arg-100 is part of a set of basic residues that help electrostatically steer the 5'-flap through the helical gateway (Fig. 1) (12, 18); thus, early contact with this residue in the weak complex could facilitate transition to a tighter complex, such as hFEN1-DNA_{bent-2} (Table 2), involving coordinated binding of the 3'-flap, threading of the 5'-flap, and ordering of the helical gateway, previously reported as critical to formation of a stable bent complex (15); note that mutating other 5'-flap steering residues also weakens the affinity slightly (12). Another early intermediate hExo1-DNA structure, in which the 5'-flap is only partially threaded through the mobile arch, offers a snapshot of this transition process ([Mg^2+]) (13). Note that Arg-100 is not essential at this stage, because the crystal structure of hFEN1R100A shows a fully threaded and inverted 5'-flap (12); however, slower transition to the hFEN1-DNA_{bent-2} complex by this mutant may contribute to the dramatic reduction in its cleavage rate (Table 1).

Once formed, the tight complex rarely dissociates and is always cleaved (k_{bent off-2} = 0.02 s^-1; Table 2) (15). All of the active-site residues tested here as well as metal ions are involved at this stage, because the interaction remains weak with wild-type hFEN1 in the presence of EDTA or with the mutants, as noted above (Table 2) (15). Thus, the major elements of substrate recognition appear to be in place within the hFEN1-DNA_{bent-2} complex, and the 5'-flap:template junction is engaged with the active site. Nonetheless, the stage is not yet completely set for catalysis. Ca^{2+} ions support formation of the hFEN1-DNA_{bent-2} complex but not 5'-flap cleavage (Table 2).
and Fig. 3). This intermediate state may be reflected in the hFEN1R100A structure, in which the 5'-flap:template junction is in the active site but the scissile phosphate is still 4–5 Å away from the metal ions, Tyr-40 is not stacked fully against the +1 base, and the junction conformation differs from that in hFEN1D386N, which is deemed to be in a more active state with the phosphate coordinated to metal ion A and Tyr-40 holding the junction in position (Fig. 1) (12). Yet other intermediate structures of hExol-DNA also show the junction bound to the active site but the scissile phosphate not yet coordinated to the metal ions (rII and rIII) (13). These subtle differences indicate the need for additional conformational transitions before the complex is locked into a catalytically competent state.

**Final, fine adjustment of the 5'-flap:template junction in the active site licenses catalysis**

Monitoring changes in fluorescence of 2-AP at +1 and −1 positions in the 5'-flap strand as the reaction proceeds through cleavage revealed for the first time that this adjustment is rate-limiting for catalysis. Distortion of DNA around the 5'-flap:template junction, as observed in the hFEN1D386N structure, shifts the scissile phosphate into the active site, and while base pairing remains intact at the +1 and −1 positions, base stacking is disrupted, which would result in unquenching of 2-AP fluorescence (Fig. 1) (12). Additional intermediate hExol-DNA structures considered to be more catalytically competent show the scissile phosphate finally engaged with metal ions. His-36 (analogous to Tyr-40 in hFEN1) stacked against the last base at the junction, and basic residues Lys-85 and Arg-92 (analogous to Lys-93 and Arg-100 in hFEN1) in contact with the phosphate oxygens. Notably, these structures also indicate disruption of base stacking at the junction (rIV–rVIII) (13). Our findings indicate that in wildtype hFEN1, transition to this catalytically active state occurs at an overall rate of ~25 s⁻¹ and licenses cleavage (Fig. 4). In contrast, hFEN1R100A exhibits minimal unquenching of 2-AP fluorescence and correspondingly low cleavage activity, consistent with structural data indicating that this mutant cannot achieve the final active state (12). Lys-93 serves a similar function as Arg-100, helping steer the 5'-flap through the gateway and contacting the scissile phosphate oxygens; accordingly, hFEN1K93A also exhibits little 2-AP unquenching and catalytic activity. hFEN1D381A cannot adopt a catalytically active state either, while hFEN1D394A can, albeit at a ~250-fold slower rate than wildtype hFEN1 (kₐ₈ = 0.1 s⁻¹ for hFEN1D394A versus 25 s⁻¹ for wildtype; Fig. 4). Although both metal ions are essential for catalysis, this difference implies a more prominent role for metal ion A (coordinated by Asp-181 and located 5.2 Å from the phosphate) than metal ion B (coordinated by Asp-34 and located 7.9 Å from the phosphate) in correct positioning of the phosphodiester bond in the active site; indeed, the structure of hFEN1D386N shows that this mutant can adopt an active conformation despite the loss of ion B (Fig. 1) (12).

The hFEN1Y40A mutant retains catalytic activity, but both the transition to active state reported by 2-AP and the 5'-flap cleavage rate are ~80-fold slower than wildtype (k₅₈ = 0.3 s⁻¹, k₅₈ = 0.25 s⁻¹; Fig. 4). Single-molecule measurements of hFEN1Y40A show multiple bending/unbending events after substrate binding, before a successful bending event leads to 5'-flap removal, indicating that unproductive substrate bending events contribute to slowing hFEN1Y40A cleavage activity. However, even in the case of the rare productive bending events, a subsequent step limits catalysis (15). The match between the k₈₈ and k₅₈ rates of hFEN1Y40A reported in this study implicates local distortion of the 5'-flap:template junction as this step. Accordingly, if substrate bending does not lead to FEN1 and DNA locked in a catalytically competent conformation that allows subsequent junction distortion/scissile phosphate positioning coupled to catalysis, the equilibrium shifts in favor of DNA unbending. In this particular case, the transition to active state is slowed by loss of Tyr-40 stacking against the +1 base, which may allow the junction and 5'-flap to slip within the active site, but one could imagine similar consequences when hFEN1 binds non-cognate DNA as well. Together, these results suggest that hFEN1 employs a kinetic proofreading mechanism, whereby correct reaction intermediates that can be locked in an active state are cleaved rapidly, whereas incorrect reaction intermediates are rejected.

**Stepwise release may influence further processing of the nicked product**

Following cleavage, the single-stranded 5'-flap is released readily by hFEN1, as indicated by its inability to competitively inhibit the reaction (10, 15). We observed fast, partial unbending of the nicked duplex product while bound to hFEN1 (k₈₈₉₈ = 26 s⁻¹), indicating relaxation of the DNA to a less bent conformation upon loss of interactions between the enzyme and the 5'-flap. This product remains bound to the enzyme and is released at a ~15-fold slower rate than cleavage (k₈₉₉₈ = 1.5 s⁻¹ versus k₈₈₉₈ = 25 s⁻¹; Fig. 2 and Table 1). We recently observed these two steps of nicked product release by single-molecule imaging as well (29). Such a prolonged dwell time could enable direct handoff of the nicked duplex from hFEN1 to DNA ligase, thus minimizing release of a genome-disturbing intermediate. It has been proposed previously that the ligase I DNA-binding domain can interact with an hFEN1-DNA complex, with the only clash occurring at the cap region topping the helical gateway (18). Movement/disordering of this structure coupled with loss of the 5'-flap and partial duplex unbending, even as hFEN1 maintains contact with the duplex arms, could facilitate coordination of flap cleavage with ligase binding and completion of Okazaki fragment maturation. Movement of the analogous mobile arch in hExol after cleavage is consistent with this hypothesis (13); in this case, slow release of the product favors cleavage of the next nucleotide by hExol.

In summary, our kinetic study of hFEN1 supports a multistep process of DNA substrate binding and selection, whereby the enzyme-substrate complex transitions from initial weakly bound species toward tight recognition intermediates. The initial species are likely to have high conformational entropy, as proposed previously (13, 34), with most interactions occurring distant from the active site, whereas the tight recognition intermediates have lower conformational entropy as the enzyme progressively engages with the site of cleavage on the DNA, which in turn enables specific contacts between the active site and the scissile bond that license catalysis. After cleavage, the enzyme-product also undergoes transitions that involve initial disengagement of local contacts before the loss of distant con-
tacts leads to product release, thus allowing time for nicked product handoff to DNA ligase in the case of hFEN1 and processive cleavage activity in the case of hExo1. The kinetics nicely complement structural data available for these enzymes, indicating that this stepwise reaction mechanism is employed by the 5′-nuclease superfAMILY.

**Experimental procedures**

**DNA and protein**

Fluorophore-modified DNAs were purchased from Integrated DNA Technologies (IDT) and purified by urea gel electrophoresis (sequences provided in Fig. S1). Non-equilibrated (5′- and 3′-flaps non-complementary to template) and equilibrated (5′- and 3′-flaps with a common 1 nt complementary to template at junction) flap substrates for cleavage assays were prepared by first mixing 5′-flap and template strands in annealing buffer (30 mM HEPES-KOH, pH 7.4, 40 mM KCl, and 8 mM MgCl₂), heating the mixture to 65 °C for 10 min, and cooling slowly to 25 °C. The 3′-flap strand was added next, followed by heating at 37 °C for 1 h and cooling slowly to 25 °C. The ratio was 1:1.25:2.5 for the 5′- flap/template/3′- flap strands. Annealing efficiency was tested by 12% non-denaturing PAGE, and the DNAs were visualized on a Typhoon imager (GE Healthcare).

Substrates for FRET and 2-AP assays were prepared by mixing the labeled/unlabeled strands in a 1:1.1 ratio in annealing buffer (50 mM HEPES-KOH, pH 7.4, and 100 mM KCl), heating the mixture to 80 °C for 5 min, and cooling slowly to 25 °C. Wild-type human FEN1 was overexpressed in E. coli BL21 (DE3) cells and purified as described previously (35). hFEN1 mutants were prepared using the QuikChange protocol (Agilent Technologies) using the primers listed in Fig. S1 and were purified using the same protocol as wildtype protein.

**Equilibrium and stopped-flow FRET assays for DNA binding, bending, cleavage, and product release**

DNA binding by hFEN1 was detected by FRET between fluorescein (donor) and TAMRA (acceptor) dyes on a non-equilibrated double-labeled substrate, DNA-F-T (0.01 μM), in the absence and presence of protein (0.2 μM) in 50 mM HEPES-KOH, pH 7.4, 100 mM KCl, 10 mM CaCl₂, 0.1 mg/ml BSA, and 1 mM DTT at 37 °C (λ_ex = 490 nm; λ_em = 515–650 nm); control experiments were performed similarly with donor (DNA-F) or acceptor (DNA-T)-labeled substrates. The DNA binding affinity was measured by titrating DNA-F-T and DNA-F (0.5 μM) with hFEN1 (0–20 nm) in 30 mM HEPES-KOH, pH 7.4, 40 mM KCl, and 8 mM MgCl₂ at 37 °C. FRET efficiency was calculated (E = 1 - I_D/A), where I_D and A are fluorescence intensities of DNA-F and DNA-A, respectively, and normalized mean E values and error bars representing S.D. obtained from two independent experiments were plotted versus hFEN1 concentration. The K_d was calculated by fitting the data with a quadratic equation, [F-D] = [(K_d + [D] + [F])] - [(K_d + [D] + [F])]² - 4[D][F][]³/ (2[D]), where F-D is the fraction of hFEN1-DNA complex, and D and F are total molar concentrations of DNA and hFEN1, respectively.

The kinetics of DNA binding/bending were measured by a single-mixing scheme in which wildtype or mutant hFEN1 was mixed with DNA-F-T in 30 mM HEPES-KOH, pH 7.4, 40 mM KCl, and 1 mM EDTA or 8 mM MgCl₂ or 10 mM CaCl₂ at 37 °C on a KinTek SF-2001 stopped-flow instrument, and changes in fluorescein (λ_em = 510–530 nm) and TAMRA (λ_em > 570 nm) fluorescence were monitored over time (final concentrations: 0.125–0.225 μM hFEN1 and 0.025 μM DNA-F-T). All reported data are from 2–3 independent experiments. For each experiment, the signal from 3–5 traces was averaged, normalized to initial value, and fit to a single- or double-exponential function to determine the binding, bending, and unbending rates; the S.E. of the regression is provided as a measure of goodness of fit. The kinetics of DNA substrate dissociation were measured by a single-mixing scheme in which wildtype or mutant hFEN1 was preincubated with DNA-F-T (in buffer containing 1 mM EDTA or 10 mM CaCl₂) and mixed with buffer or unlabeled DNA substrate as chase (final concentrations: 0.03–0.175 μM hFEN1, 0.025 μM DNA-F-T, and 2 μM DNA chase). As noted above, the signal from 3–5 traces was averaged for each experiment, normalized to initial value, and fit to a single- or double-exponential function to determine the substrate dissociation rate. The kinetics of DNA unbending and nicked product release were measured by a double-mixing scheme in which hFEN1 was first mixed with DNA-F-T in buffer with 8 mM MgCl₂ and with buffer or unlabeled DNA substrate as chase (final concentrations: 0.17 μM hFEN1, 0.017 μM DNA-F-T, and 1.4 μM DNA chase). As noted above, the signal from 3–5 traces was averaged for each experiment, normalized to initial value, and fit to a single or double exponential function to determine the unbending and product release rates.

**Rapid quench and steady-state assays for 5′-flap cleavage and exonuclease activity**

Single turnover experiments to measure 5′-flap cleavage were performed by mixing 15 μl of wildtype or mutant hFEN1 with 15 μl of a non-equilibrated 5′-flap strand FAM (5′-end)-labeled DNA substrate (DNA-F-EQ) in 30 mM HEPES-KOH, pH 7.4, 40 mM KCl, and 8 mM MgCl₂ at 37 °C on a KinTek RQF-3 rapid quench-flow instrument. The reaction was quenched at varying times with 75 μl of 200 mM EDTA (final concentrations: 2.5 μM hFEN1 and 0.25 μM DNA-F-EQ); control experiments were performed similarly in buffer with 10 mM CaCl₂ instead of MgCl₂. The quenched samples were placed on ice under cover until analysis by denaturing PAGE. A 25-μl aliquot of each sample was mixed with an equal volume of denaturing dye (2% w/v) for 40 min at 12 W. The FAM-labeled substrate and 5′-flap product were quantified on a Typhoon imager (λ_ex = 488 nm). The fraction of 5′-flap formed was determined from two or more independent experiments, and the mean values and error bars representing S.D. were plotted versus time. The data were fit to a single-exponential function to obtain the cleavage rate, k_cat; the S.E. of the regression is provided as a measure of goodness of fit. Similar experiments were performed with an equilibrated DNA substrate, DNA-F-EQ (1-nt overlap between 5′ and 3′ flaps at the junction).
The 5′–3′ exonuclease rate was also measured on a rapid quench-flow by mixing hFEN1 with non-equilibrated 5′-flap strand FAM (3′ end)-labeled DNA (DNAAA), quenching the reaction at varying times with EDTA, and analyzing the products by denaturing PAGE as described above (final concentrations: 2.5 μM hFEN1 and 0.25 μM DNAAA). The fraction of 3′-FAM end-labeled cleavage products (shorter than the 18-nt strand remaining after 5′-flap removal) was determined from two or more independent experiments, plotted versus time, and fit to a single exponential function to determine the exonuclease rate kcat. Steady-state 5′-flap cleavage rates were measured by mixing 1 nM hFEN1 with 800 nM DNAAA (10× Kcat) (20) in 30 mM HEPES-KOH, pH 7.4, 40 mM KCl, and 8 mM MgCl2, or 10 mM KCl and 0.1 mg/ml BSA at 37 °C, and quenching 15-μl aliquots of the reaction with 4 μl of 100 mM EDTA at varying times to determine initial velocity (<20% product). The samples were analyzed by denaturing PAGE as described above, and the plot of 5′-flap product versus time fit to a linear equation yielded kcat (slope/[hFEN1]).

**Stopped-flow 2-AP assays for DNA isomerization and flap cleavage**

The kinetics of DNA distortion at the 5′-flap:template junction were measured by changes in fluorescence of 2-aminopurine bases positioned at −1 and +1 positions in the 5′-flap strand of a non-equilibrated DNA substrate (DNAAA). Wild-type or mutant hFEN1 was mixed with DNAAA in 30 mM HEPES-KOH, pH 7.4, 40 mM KCl, and 8 mM MgCl2, or 10 mM CaCl2 at 37 °C on a stopped-flow instrument, and changes in 2-AP fluorescence were monitored over time (final concentrations: 2.5 μM hFEN1 and 0.5 μM DNAAA). All reported data are from 2–3 independent experiments. For each experiment, the signal from 3–5 traces was averaged, corrected for low DNA background signal, normalized to initial value, and fit to a single- or double-exponential function to determine the DNA isomerization/cleavage and exonuclease rates; the S.E. of the regression is provided as a measure of goodness of fit. Similar experiments were performed with a non-equilibrated DNA substrate labeled with 2-AP at only the +1 position in the 5′-flap strand (DNAA).

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