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Comparison of the Catalytic Parameters and Reaction Specificities of a Phage and an Archaeal Flap Endonuclease

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Flap endonucleases (FENs) catalyse the exonucleolytic hydrolysis of blunt-ended duplex DNA substrates and the endonucleolytic cleavage of 5′-bifurcated nucleic acids at the junction formed between single and double-stranded DNA. The specificity and catalytic parameters of FENs derived from T5 bacteriophage and *Archaeoglobus fulgidus* were studied with a range of single oligonucleotide DNA substrates. These substrates contained one or more hairpin turns and mimic duplex, 5′-overhanging duplex, pseudo-Y, nicked DNA, and flap structures. The FEN-catalysed reaction properties of nicked DNA and flap structures possessing an extrahelical 3′-nucleotide (nt) were also characterised. The phage enzyme produced multiple reaction products of differing length with all the substrates tested, except when the length of duplex DNA downstream of the reaction site was truncated. Only larger DNAs containing two duplex regions are effective substrates for the archaeal enzyme and undergo reaction at multiple sites when they lack a 3′-extrahelical nucleotide. However, a single product corresponding to reaction 1 nt into the double-stranded region occurred with *A. fulgidus* FEN when substrates possessed a 3′-extrahelical nt. Steady-state and pre-steady-state catalytic parameters reveal that the phage enzyme is rate-limited by product release with all the substrates tested. Single-turnover maximal rates of reaction are similar with most substrates. In contrast, turnover numbers for T5FEN decrease as the size of the DNA substrate is increased. Comparison of the catalytic parameters of the *A. fulgidus* FEN employing flap and double-flap substrates indicates that binding interactions with the 3′-extrahelical nucleotide stabilise the ground state FEN–DNA interaction, leading to stimulation of comparative reactions at DNA concentrations below saturation with the single flap substrate. Maximal multiple turnover rates of the archaeal enzyme with flap and double flap substrates are similar. A model is proposed to account for the varying specificities of the two enzymes with regard to cleavage patterns and substrate preferences.

Keywords: DNA repair; flap endonuclease; specificity; FEN; DNA binding

Introduction

The processes of DNA replication and repair require an enzymatic activity capable of catalysing the hydrolysis of bifurcated nucleic acids. In all kingdoms of life this activity is provided by flap endonucleases (FENs).[1] FENs are divalent metal ion dependent enzymes that catalyse the hydrolysis of

Abbreviations used: FENs, flap endonucleases; *Af*FEN, *Archaeoglobus fulgidus* FEN; *Pf*FEN, *Pyrococcus furiosus* FEN; FAM, 5′-fluorescein; MALDI-TOF, maltrix-assisted laser desorption ionisation- time of flight; MS, mass spectrometry.

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phosphate diesters with scission of the 3′-phosphorus oxygen bond to generate products possessing a 5′-phosphate and a 3′-hydroxyl group, respectively.[2] FENs catalyse both the 5′-exonucleolytic and structure-specific endonucleolytic reactions of DNA substrates.[3,4] Structure-specific activity occurs at the junction between single and double-stranded DNA in 5′-flap containing structures (Figure 1(a)). Eight X-ray structures of FENs are available, although none contain DNA bound in the active site.[5–12]

Studies of the FEN–DNA interaction have been greatly facilitated by the use of short oligonucleotide substrates, as these mimic the types of DNA structure that may be encountered *in vivo*. Similar models of the T5FEN–DNA and human FEN–DNA interaction have been generated using a combination of differing potential substrates and site-directed mutagenesis.[13,14] These models define areas of the protein that interact with regions of duplex DNA upstream and downstream of the site of endonucleolytic reaction of a flap-type substrate and an area of interaction with the 5′-single-stranded overhang. Analogous conclusions have been reached about the orientation of the substrate in the DNA–*Pyrococcus furiosus* (*Pf*) FEN interaction using chemically modified flap structures containing methyl phosphonate or 2′-*O*-methyl nucleoside substitutions in combination with mutated proteins.[15]

Broadly similar reaction specificities have been described for FENs derived from phage, bacterial, archaeal and mammalian sources on synthetic DNA flap substrates.[4,16–19] However, differences exist between the reported behaviour of FENs with smaller DNA substrates. For example T5FEN and human FEN (hFEN) have been reported to cleave duplex or hairpin DNA with a 5′-single-stranded overhang efficiently at the junction between single and double-stranded DNA,[2,20] whereas such structures have been reported to be poor substrates for some bacterial and archaeal FENs.[21] Flap substrates possessing an extrahelical 3′-nucleotide (nt) (so called "double-flap" substrates) have been reported to bring about a rate enhancement in the reactions catalysed by FENs from bacterial, archaeal, yeast and mammalian sources.[20–25] Quantitative evaluation of the rate enhancement brought about by a 3′-extrahelical nucleotide revealed a 3–15-fold enhancement in V_{max} for a series of bacterial and archaeal FENs compared to a flap substrate lacking the 3′-extrahelical nt[21] whereas a recent study using hFEN noted a fivefold decrease in K_M but similar turnover numbers for substrate with and without the extrahelical nucleotide.[20] It is therefore unclear

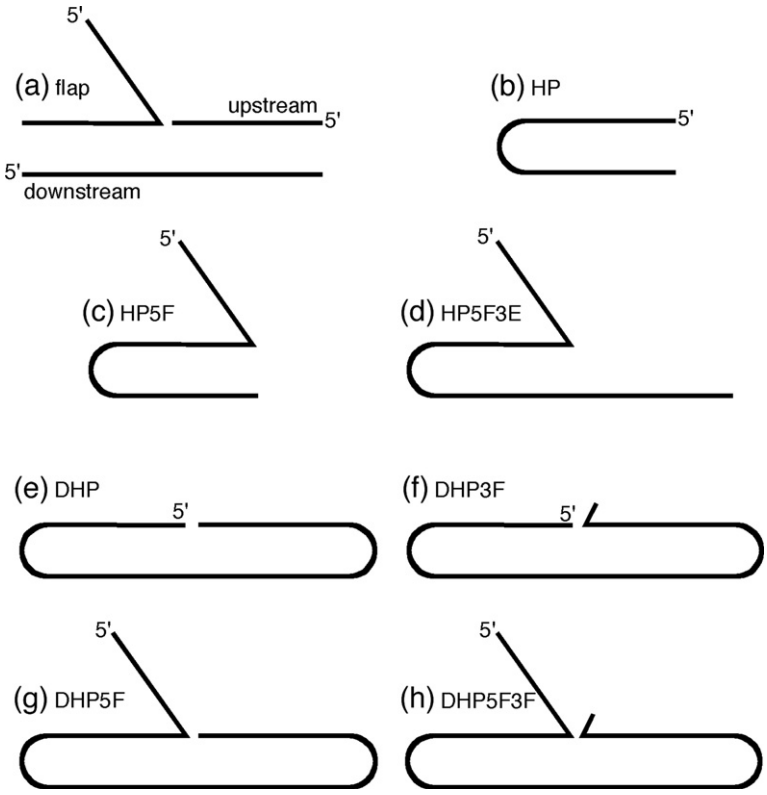

Figure 1. DNA substrates for flap endonucleases. (a) A typical flap substrate consisting of three oligonucleotides, that form duplexes downstream and upstream of the junction between single and double-stranded DNA. (b) A single oligonucleotide (hairpin) analogue of duplex DNA (HP); three hairpin substrates are used in this study of length 18 (HP(18)), 20 (HP(20)) and 22 (HP(22)) nt. (c) A single oligonucleotide analogue of a 5′-overhanging duplex (HP5F). (d) A single oligonucleotide analogue of a 5′-overhanging duplex with a 3′-extension (pseudoY structure) (HP5F3E). (e) A single oligonucleotide of nicked DNA (DHP). (f) A single oligonucleotide of nicked DNA with a 3′-extrahelical nucleotide (DHP3F). (g) A single oligonucleotide analogue of a flap substrate (DHP5F); two variants of this substrate were created with 7 nt (DHP5F(7)) and 2 nt (DHP5F(2)) 5′-single-stranded regions. (h) A single oligonucleotide analogue of a flap substrate with a 3′-extrahelical nucleotide (DHP5F3F); two variants of this substrate were created with 7 nt (DHP5F3F(7)) and 2 nt (DHP5F3F(2)) 5′-single-stranded regions. The sequences used to construct the oligonucleotides used in this study are shown in Table 1. The abbreviated nomenclature used for substrates is derived as follows: HP(no.) designates a blunted-ended duplex with a hairpin turn, with the number of nucleotides from which it is constructed in parentheses; DHP a double hairpin with two hairpin duplexes; 5F(no.) designates a substrate with a 5′-single-stranded flap with the number of single-stranded nucleotides in parentheses; 3F designates a substrate with a single 3′-extrahelical nt and 3E designates a longer single-stranded 3′-extension.

whether interactions between the enzyme and substrate with the 3′-extrahelical nt are used to stabilise the enzyme-substrate complex or to drive catalysis. In addition to rate acceleration, catalysis with double-flap substrates occurs with a marked enhancement in specificity for the reaction one nucleotide into the double-stranded region.[20–26] The molecular basis for this specificity was revealed by an X-ray structure of *Archaeoglobus fulgidus* FEN (*Af*FEN) bound to a duplex with a 3′-extrahelical nucleotide.[9] A binding pocket for the extrahelical nucleotide exists within the higher organism FENs, but the structural features that comprise this binding site appear to be absent from the smaller phage enzymes (Figure 2). To date no attempt to investigate the effects of adding a 3′-extrahelical nucleotide to the substrate of the phage enzymes has been reported.

In an attempt to understand the factors that give rise to specificity of the FEN reaction and the relationship between catalytic parameters and effectiveness or otherwise of substrate structure we have carried out a systematic study of FENs derived from a phage and archaeal source. A series of substrates of differing sizes designed to test the effects of the presence or absence of an upstream duplex, a 5′-flap of varying length, and/or a 3′-extrahelical nucleotide but where possible identical sequence, have been employed to minimise any effects due to alteration of sequence. The specificities of the two FENs were measured with the set of substrates and steady-state catalytic parameters and maximal single-turnover rates of the reactions were evaluated under as near identical conditions as possible. The patterns of reactivity can be rationalised in terms of substrate and enzyme structure and the strength of their interactions. Within this framework, some differences in the properties of FENs from alternative sources can be rationalised in terms of structural features of the enzymes, but several general features of FENs emerge.

Results

Design of oligonucleotide substrates

A synthetic "flap substrate" typically used to assay FEN reactions is shown in Figure 1(a) and is constructed from three oligonucleotides, yielding a 5′-single-stranded flap, and two regions of duplex DNA, one upstream and one downstream of the sites of endonucleolytic reaction. A three-stranded flap substrate contains multiple possible FEN reaction sites, namely the single-stranded 5′ terminus of the flap, the double-stranded 5′ termini of the upstream and downstream duplexes and the structure-specific site at the single-stranded–double-stranded junction. 5′-Labelling of each of the three strands in turn allows all these activities to be observed with T5FEN (data not shown). Thus, even when monitoring the initial reactions of a single 5′-labelled flap strand, the complication that silent reactions potentially occur on other DNA strands exists.

The synthetic oligonucleotide substrates used in this study are illustrated in Figure 1 and the sequences are displayed in Table 1. These substrates

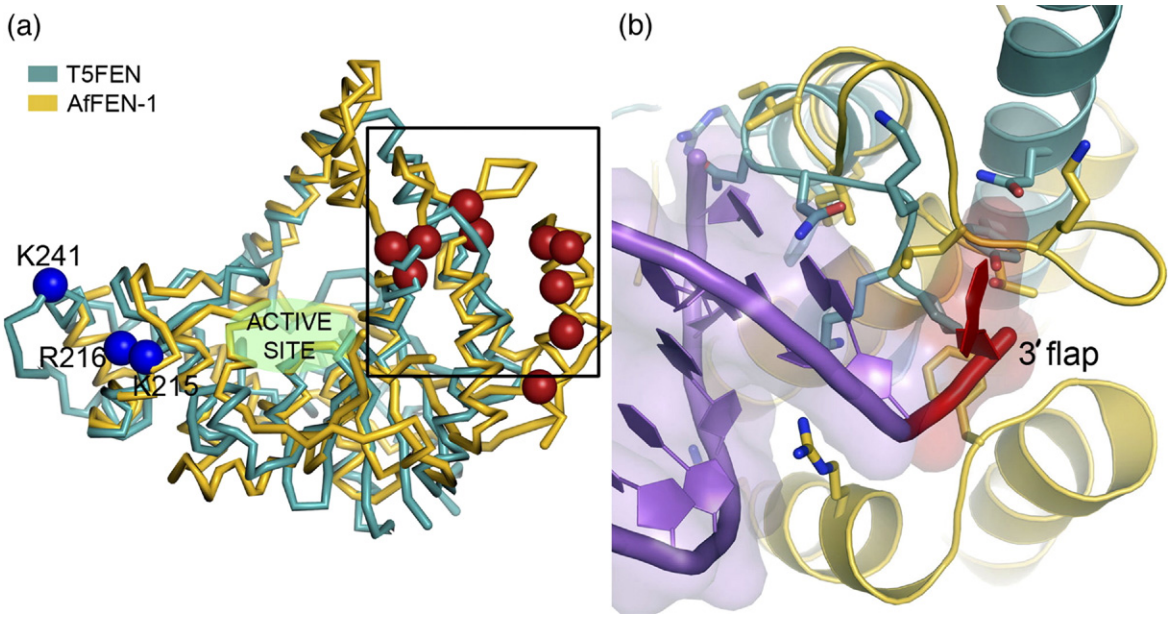

Figure 2. Structural comparisons of T5FEN and *Af*FEN proteins. (a) Structural superposition of the C-alpha atoms of T5FEN (1UT5, blue) with *Af*FEN (1RXW, yellow). Blue spheres indicate residues implicated in interaction of T5FEN with the downstream duplex (K241, K215, R216, left). Red spheres indicate residues of *Af*FEN that interact with the upstream DNA duplex (red spheres) observed crystallographically (F35, I38, L39, L47, F310, S311, R314, K317, K321, right). The boxed region highlights the key differences between T5FEN and *Af*FEN in the 3′ flap binding site (b). (b) T5FEN (blue) lacks the extended loop and helical regions of *Af*FEN (gold), which form the binding site for the 3′ flap (red) and associated upstream DNA duplex (1RXW, purple).

have been designed to probe the cleavage specificity and catalytic parameters associated with various types of FEN activity that can potentially occur. To remove the complication of unmonitored initial reactions, substrates were constructed from single oligonucleotides possessing where appropriate, either one or two hairpin turns, and thus all contain only a single 5′-fluorescently labelled terminus. Whilst FENs display no overt sequence specificity, the possibility of alternative structures as a consequence of sequence changes could give rise to changes in reaction specificity, and therefore where possible the same sequences have been conserved throughout the substrate design. The selection of substrate oligonucleotide sequence was aided by stability and structure prediction software,[27] so that no alternative folded structures were predicted to be possible. In addition, the sequences of all hairpin DNAs used in these experiments were selected to have independent predicted melting temperatures of greater than 55 °C at 50 mM NaCl (0 mM $MgCl_2$) (pH 9.3), thus ensuring that under the conditions of the reaction (25 mM potassium glycinate (pH 9.3), 10 mM $MgCl_2$, 50 mM KCl, 37 °C or 55 °C) hairpin formation was favoured. Unless otherwise indicated, a pH of 9.3 was selected for evaluation of the specificity and catalytic parameters of the FEN reactions, because both the multiple turnover number and the maximal single turnover rate of the T5FEN catalysed reaction with a magnesium ion cofactor are maximal and pH-independent at this pH value.[28,29] Substrates were synthesised using conventional methods and all contain a 5′-fluorescein (FAM) label. It has previously been demonstrated that both 5′-fluorescent and 5′-radiolabelled variants of HP5F have similar catalytic parameters and therefore the fluorophore does not interfere with the reaction.[30] Oligonucleotides were purified by reversed phase HPLC (RPHPLC). Separation of the phosphorothioate diastereoisomers of the 5′-FAM labelled psHP, designated f (fast) and s (slow) in order of their elution, was also possible using RPHPLC. However, RPHPLC resolution of the intact epimers of psHP5F was not possible and so the individual phosphorothioate diastereoisomers of this oligonucleotide were assembled from smaller separable fragments by templated ligation. Matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry was used to confirm the primary structure and homogeneity of all oligonucleotides.

Specificity of reactions with the DNA substrates

The reaction specificities of T5FEN catalysed reactions of the oligonucleotide substrates are summarised in Figure 3. The reactions of the various substrates were monitored utilising the 5′-FAM label. Reaction mixtures were separated and quantified by denaturing HPLC (dHPLC), equipped with a fluorescence detector. The dHPLC gradient and buffers used in this experiment were chosen to allow single nucleotide resolution of all possible products of length one to ten nucleotides. Oligonucleotide standards of the appropriate length were used to confirm the length of 5′-fluorescent products by co-injection. In some cases the results of these studies were verified by MALDI-TOF mass spectrometry (MS), which also allowed investigation of the nature of the unlabelled 3′-products of the reactions. The results of MALDI-TOF analysis are summarised in Materials and Methods. The data presented in Figure 3 summarise the relative amounts of reaction products observed after reaction of each substrate (100 nM) for 8 min at an enzyme concentration of 100 pM. However, in cases where accurate determination of the relative amounts of products was possible over the entire course of the reaction, the ratios of products remained constant.

T5FEN catalysed reaction of the 5′-overhanging hairpin substrate HP5F (Figure 1(c)) has been previously reported to initially produce 8mer and 21 mer products as a result of cleavage one nucleotide into the double-stranded region.[2] Although 5′-FAM-8mer is the major 5′-reaction product (69%), in this current study smaller amounts of fluorescent 7mer (29%) and 6mer (less than 2%) products were also observed in dHPLC traces (Figure 3). MALDI-TOF analysis of the reaction close to completion confirmed the presence of small amounts of 6, 7mer as well as 8mer product, but puzzlingly only detected the presence of a single phosphorylated 21mer 3′-product (data not shown). Similar MALDI-TOF

Table 1. The oligonucleotide sequences used to construct the substrates illustrated in Figure 1

Substrate	Sequence
HP(18)	FAM–d(GAACACGCTTGCGTGTTC)
HP(20)	FAM–d(GAACACCGCTTGCGGTGTTC)
HP(22)	FAM–d(GAACACACGCTTGCGTGTGTTC)
HP5F	FAM–d(CGCTGTCGAACACACGCTTGCGTGTGTTC)
HP3E	FAM–d(CGCTGTCGAACACACGCTTGCGTGTGTTCCCCAACATAGAGAGCCCC)
DHP	FAM–d(GAACACACGCTTGCGTGTGTTCCACAACATAGAGATATGTTGTG)
DHP3F	FAM–d(GAACACACGCTTGCGTGTGTTCCACAACATAGAGATATGTTGTGC)
DHP5F(7)	FAM–d(CGCTGTCGAACACACGCTTGCGTGTGTTCCATAACATAGAGATATGTTATG)
DHP5F3F(7)	FAM–d(CGCTGTCGAACACACGCTTGCGTGTGTTCCATAACATAGAGATATGTTATGC)
DHP5F(2)	FAM-d(TCTTACACACGCTGTCGTGTGTAACATAACATAGAG ATATGTTATG)
DHP5F3F(2)	FAM-d(TCTTACACACGCTGTCGTGTGTAACATAACATAGAG ATATGTTATGC)
psHP	FAM–d(GpsAACACACGCTTGCGTGTGTTC)
psHP5F	FAM–d(CGCTGTCGpsAACACACGCTTGCGTGTGTTC)
FAM-8mer	FAM–d(CGCTGTCG)

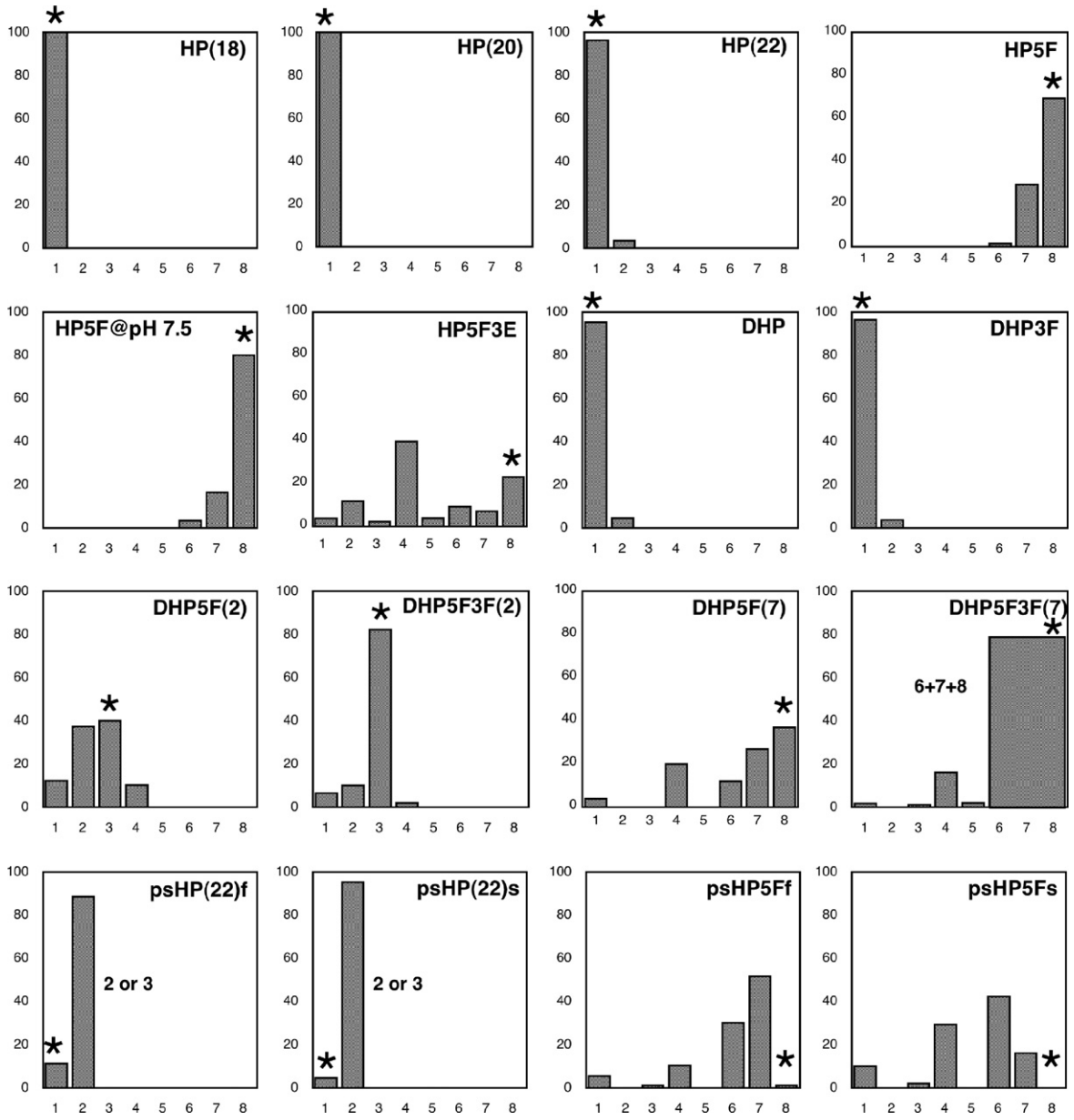

Figure 3. The relative amounts of differing length products produced by T5FEN-catalysed hydrolysis of the substrates used in this study. Enzyme reactions (100 pM T5FEN, 100 nM substrate, 25 mM potassium glycinate (pH 9.3), 10 mM $MgCl_2$, 50 mM KCl, 0.01 mg/ml BSA, 37 °C) were allowed to progress for 8 min. Each set of data is the average of at least three experiments. The length of product is shown on the *x* axis, as a percentage of total product on the *y* axis. An asterisk indicates the site of reaction 1 nt into the double-stranded region. For DHP5F3F(7), it was impossible to quantify the individual amounts of 6, 7 and 8mer due to peak overlap. In the case of psHPf and psHPs we do not possess the relevant phosphorothioate-containing product FAM-d(GpsA) and FAM-d(GpsAA) standards and are therefore unable to assign whether the major product of these reactions is a 2mer or 3mer. A Table of the data used to construct this Figure is shown in Table 1S (Supplementary Data).

results were obtained regardless of whether HP5F was 5′-phosphorylated or was a 5′-FAM derivative, indicating that the presence of the fluorophore had not changed the reaction specificity. If the 6mer and 7mer oligomers observed by MALDI-TOF MS originated from reaction of intact HP5F it would be expected that the presence of the corresponding phosphorylated 22mer and 23mer products would be detected. This raised the possibility that the smaller 6mer and 7mer products could be the consequence of further reaction of the 8mer product. However, when independently synthesised 5′-FAM-8mer was incubated under identical conditions used for enzymatic reaction of HP5F (100 pM FEN, 100 nM FAM-8mer, 25 mM potassium glycinate (pH 9.3), 10 mM $MgCl_2$, 50 mM KCl, 0.01 mg/ml BSA, 37 °C) for 24 h, there was no reaction. Small (<2.5%) traces of 1mer product were observed from this reaction after 72 h. We therefore concluded that oligomers of 6 and 7 nt in length are products of

reaction of intact HP5F, but that the corresponding phosphorylated 23mer and 22mer 3′-products must be substrates for T5FEN and react within the time scale of complete reaction of intact HP5F generating the phosphorylated 21mer that is the only 3′-product detected by MS analysis. As has been previously noted for the reactions of hFEN with flap substrates, reducing the pH of the T5FEN-catalysed reaction of HP5F enhanced the preference for reaction 1 nt into the double-stranded region.[26]

To study the double-stranded exonucleolytic activity of T5FEN and verify the conclusions regarding the origin of reaction products of the 5′-overhanging hairpin substrate, we also investigated the reaction of a blunt ended 22mer hairpin, HP(22) (Figure 1(b)), of identical sequence to the hairpin component of HP5F. By analogy to the reaction of the 5′-overhanging hairpin, this proved to be an efficient substrate for T5FEN and the major product of the reaction was 5′-FAM-dG corresponding to the same major reaction site of HP5F, one nucleotide into the double-stranded region. Minor traces of 5′-FAM-d(GA) were also observed during product analyses by dHPLC (less than 4%) (Figure 3). As with reaction of HP5F, the major 3′-product of reaction is phosphorylated 21mer, which is resistant to further enzyme-catalysed reaction under the same conditions and timescale that produce reaction of HP(22). Two shorter hairpin substrates HP(20) and HP(18) produced only a single 5′-FAM-dG product (Figure 3). A single oligonucleotide nicked DNA substrate was created by addition of a second hairpin duplex to the 3′ terminus of HP to create DHP (Figure 1(e)). This did not alter the substrate specificity of the reaction and identical products were observed to those seen with HP(22), namely a major 1mer product with trace amounts of 2mer (Figure 3).

Extending the 3′ terminus of HP5F, produces HP5F3E (Figure 1(d)), a single oligonucleotide analogue of a pseudo-Y structure. Surprisingly the initial products of T5FEN-catalysed hydrolysis of HP5F3E, were eight different 5′-FAM-oligomers ranging in length from 1–8 nt (Figure 3). The major products of this reaction were 8, 7, 6, and 4mers, the generation of 8mer corresponding to reaction 1 nt into the double-stranded region, whereas all other reaction sites were in the single-stranded 5′-flap region. An analogous lack of specificity has been observed with some pseudo-Y substrates and hFEN.[13] The single oligonucleotide analogue of a three-stranded flap structure, DHP5F (Figure 1(g)), has two hairpin turns and contains the same sequence as HP5F with the addition of an upstream duplex identical to that of DHP. The T5FEN-catalysed reaction of DHP5F(7) is less specific than the reaction of the 5′-overhanging hairpin substrate HP5F producing 1, 4, 6, 7 and 8mer. Addition of a 3′-extrahelical nucleotide to flap structures, has been reported to enhance the specificity of reaction of flap endonucleases from several sources, producing a site-specific reaction 1 nt into the double-stranded region.[21,23–25] However the double-flap substrate DHP5F3F(7) (Figure 1(h)), that contains a 3′ extrahelical nucleotide, possesses further reaction sites when compared to the 5′-overhanging double-hairpin structure (DHP5F(7)) without a 3′-flap (Figure 3). Analogous results were obtained with flap and double-flap substrates with a shortened 5′-single-stranded region (DHP5F(2) and DHP5F3F(2)), except that in these cases reaction was also observed 2 nt into the double-stranded region. These substrates produced 1, 2, 3 and 4mer products, where the 3mer product results from cleavage 1 nt into the double-stranded region. Similarly, addition of an extrahelical 3′-nucleotide to the nicked DNA substrate (DHP3F, Figure 1(f)), did not produce any change in the cleavage specificity of this reaction, resulting in the same 5′-FAM-dG major product in analogy to HP and DHP, but modest amounts of 5′-FAM-d(GA) were also observed (Figure 3).

Reactions involving both variants of DHP5F3F and DHP5F were also carried out using FEN from *A. fulgidus* (*Af*) (Figure 4). These were carried out under similar conditions to those employed for the T5 enzyme except that DTT was added to the reaction mixtures and the experiments were carried out at 55 °C. The concentration of substrate and enzyme were varied from the T5 case to produce reaction on a similar timescale. In contrast to T5FEN, *Af*FEN produced a specific reaction with both variants of DHP5F3F hydrolysing the phosphate diester 1 nt into the double-stranded region. The finding that *Af*FEN performs a specific reaction on both DHP5F3F substrates implies that these oligomers are correctly folded in the proposed double-hairpin form, even at 55 °C. Nicked DNA (DHP and DHP3F) was also cleaved by the enzyme, with a specific reaction observed with the presence of a 3′-extrahelical nucleotide. Like T5FEN, DHP and DHP5F(2), substrates without a 3′-extrahelical nucleotide and with either no or a shortened 5′-flap, produced a product corresponding to reaction 2 nt into the double-stranded region in addition to reaction 1 nt into this region and in the single-stranded flap when present. All other substrates required the presence of very elevated concentrations of enzyme and prolonged incubation to detect any evidence of reaction, which was generally non-specific. The results observed here are in line with those reported previously for a range of thermophilic FENs.[21]

Substrates where a chiral phosphorothioate linkage replaces the scissile phosphate diester could potentially be employed to monitor the stereochemical course of the FEN-catalysed reaction, revealing whether the FEN reaction proceeds by direct attack of water (or hydroxide) or involves covalent catalysis. However, based on previous precedents with metalloendonucleases such as EcoRV and Vsr endonucleases,[31,32] it is likely that replacement of the scissile bond with a phosphorothioate would be both refractory to reaction and impair substrate binding. In line with these expectations, replacement of the phosphate diester that is the major site of reaction of unmodified HP(22) with a phosphorothioate linkage of either stereochemistry (psHPf and psHPs, Figure 3) results in a change in the major

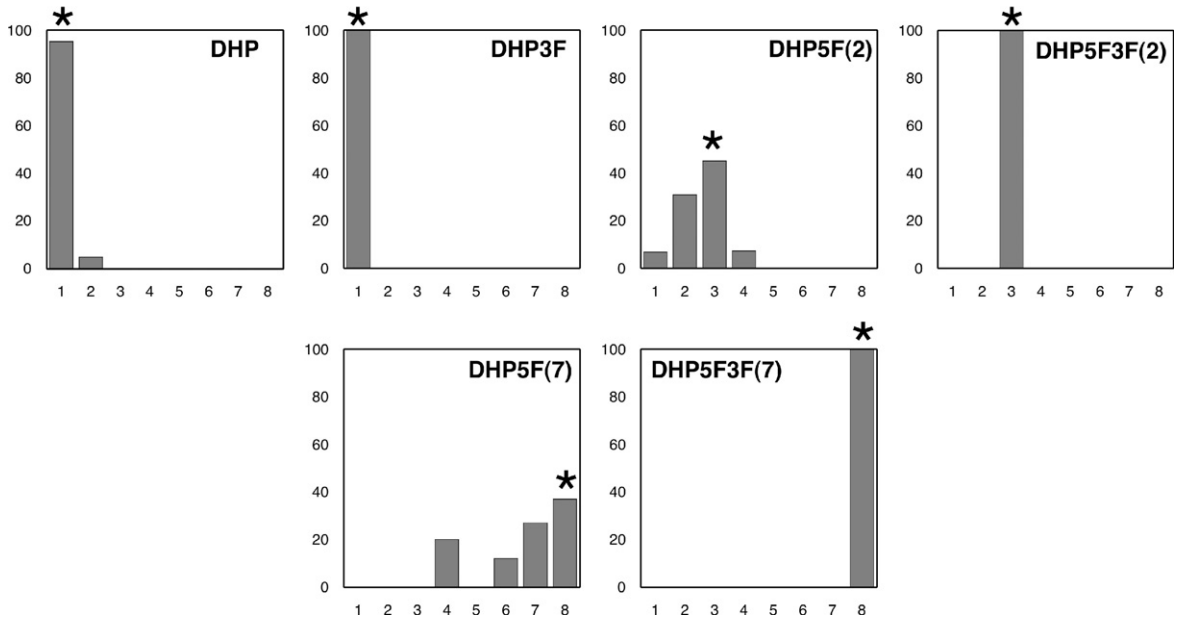

Figure 4. The relative amounts (%) of differing length products produced by *Af*FEN-catalysed hydrolysis of the substrates used in this study. Enzyme reactions (8 μM substrate and 0.1 μM *Af*FEN, 25 mM potassium glycinate (pH 9.3), 10 mM $MgCl_2$, 50 mM KCl, 0.01 mg/ml BSA, 1 mM DTT, 55 °C) were allowed to progress for 8 min. Each set of data is the average of at least three experiments. The length of product is shown on the *x* axis as a percentage of total product on the *y* axis. An asterisk indicates the site of reaction 1 nt into the double-stranded region. Smaller substrates (HP5F3E, HP5F and HP (22)) did not undergo any reaction under these conditions on this timescale. A Table of the data used to construct this Figure is shown in Table 2S (Supplementary Data).

reaction site of T5FEN compared to the corresponding unmodified oligomer. Similarly, replacement of the phosphate diester 1 nt into the double-stranded region in HP5F (psHP5Ff and psHP5Ff) with a phosphorothioate linkage of either stereochemistry, resulted in reactions within the single-stranded region of the molecule (Figure 3).

Kinetic characterisation of the substrates

Variation in catalytic parameters of T5FEN with the length of the downstream duplex

To determine the minimal substrate requirements for the T5FEN-catalysed reaction the steady-state catalytic parameters of three blunt ended duplex hairpin substrates (Figure 1(b)) of varying length (HP(18), HP(20) and HP(22)) were determined and are shown in Table 2. The use of fluorescent substrates for determination of the catalytic parameters of the FEN reaction has been discussed in detail elsewhere.[30] The formation of multiple products was dealt with by measuring the total amounts of all products relative to substrate at various time intervals to generate the initial rates of reaction. Analysis of the catalytic parameters of hairpin substrates of varying length, suggests that the 22mer serves as an effective minimal substrate. Decreasing the length of the duplex region by 1 bp (HP(20)), has a negligible effect on catalytic efficiency. However, decreasing the length of the substrate by a further base-pair (HP(18)) results in

Table 2. The catalytic parameters of T5FEN-catalysed reactions

Substrate	k_{cat} (min^{-1})	K_M (nM)	k_{cat}/K_M (nM^{-1} min^{-1})	k_c (min^{-1})	k_c/k_{cat}
HP(18)	226±23	(45.6±10.6)×10^3	5.0×10^{-3}	ND	ND
HP(20)	678±69	483±150	1.40	1106±123	1.6
HP(22)	237±6	109±10	2.2	1466±271	6.2
HP5F	79±2	31±3	2.5	329±29	4.2
HP5F3E	154±5	13±2	11.6	840±190	5.5
DHP	70±2	32±4	2.2	1518±168	21.8
DHP3F	68±3	39±7	1.7	881±52	13.1
DHP5F(7)	44±2	7±3	6.9	881±121	20.1
DHP5F3F(7)	40±1	7±2	5.8	1195±52	29.9

Full experimental details are contained in Materials and Methods. Steady-state parameters were determined by measuring the initial rates of reaction of substrates at concentrations around their respective K_Ms. Maximal single-turnover rate constants (k_c) were measured using concentrations of enzyme at least 8×K_M and at a substrate concentration of 1 nM. In the case of HP(18) the high K_M precluded an evaluation of k_c. Each set of data is the average of at least three experiments.

a very dramatic decrease in catalytic efficiency as a consequence of a 100-fold increase in K_M.

As it has been previously demonstrated that the T5FEN-catalysed steady-state multiple-turnover reaction of HP5F is rate-limited by product release,[29,30] maximal single turnover rates were also determined where possible using quenched flow apparatus. Maximal single turnover rates were measured using enzyme concentrations that were at least eightfold higher than K_M. The amount of enzyme was then increased until constant maximal rate was observed. The maximal single turnover rates of HP(22) and HP(20) are identical within experimental error. Small differences in the multiple turnover catalytic parameters of HP(22) and HP(20) are likely to reflect very small changes in the stability of the respective enzyme-substrate and enzyme-product complexes. Together these data suggest that a minimal hairpin substrate for T5FEN must be 20 or 22 nt in length (assumed eight or nine base-pairs) and that interactions between enzyme and substrate must take place with the terminal eighth base-pair of these substrates. Similar conclusions about potential interactions of *Pf*FEN and its downstream DNA substrate have been reported.[15]

Comparison of the catalytic parameters of a range of T5FEN substrates

The kinetic parameters of the T5FEN-catalysed reactions of a range of substrates of varying structure are shown in Table 2. All these substrates possess the downstream duplex HP(22) sequence. An example of the data used to construct Table 2 is shown in Figure 5(a). Several general trends in the steady-state catalytic parameters are evident. When compared to the minimal substrate HP(22), addition of either a 5′ single-stranded flap and/or a 3′-upstream duplex lowers the Michaelis constant. Increasing the size of the substrates also lowers the turnover number of the enzyme. A noteworthy consequence of these effects is that several substrates have similar catalytic efficiencies. Thus under conditions where blunt-ended duplexes, 5′-overhanging duplexes, nicked DNAs and flap constructs with a 3′-extrahelical nucleotide (HP5F, HP, DHP, DHP3F and DHP3F5F(7)) substrates are present at physiologically relevant concentrations below K_M, all will react with similar efficiency.

The measured maximal single-turnover rates vary from 300–1500 min^{-1} (5–25 s^{-1}) and are towards the higher end of values observed for other enzyme-catalysed phosphate diester hydrolysis reactions and represent a rate acceleration of approximately 10^{16} compared to the uncatalysed hydrolysis of phosphate diesters.[33] With the exception of HP5F the maximal single-turnover rates vary only twofold with respect to one another and are in the region of 800–1500 min^{-1}. This suggests that, with the exception of HP5F, T5FEN catalyses the hydrolysis of all phosphate diesters presented in the active site at equal rate regardless of substrate structure. However the maximal single-turnover rate of HP5F

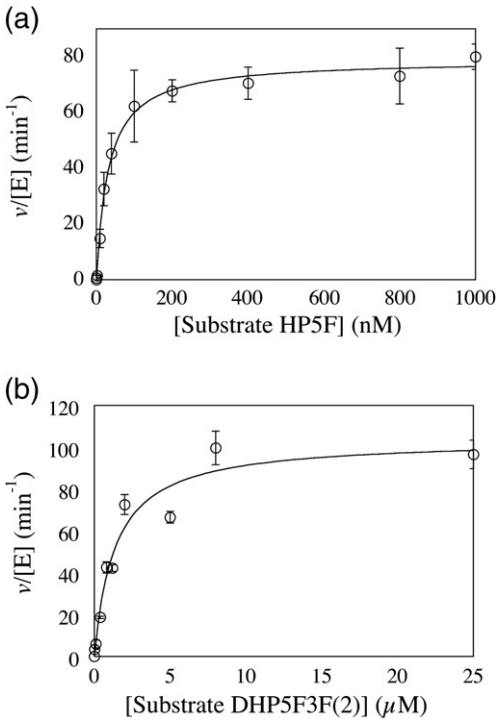

Figure 5. Examples of multiple turnover kinetic data used to construct Tables 2 and 3. Normalised initial rates of reaction (v/[E]) were determined in triplicate at substrate concentrations around the respective K_Ms. Values of v/[E] were averaged and the standard error is shown as error bars. Non-linear regression fitting to the Michaelis-Menten equation (equation (1), Materials and Methods) was used to generate the catalytic parameters and their associated standard errors. (a) T5 FEN-catalysed hydrolysis of HP5F substrate yields catalytic parameters $K_M = 31(\pm 3)$ nM, $k_{cat} = 79(\pm 2)$ min^{-1}. The R factor for this curve fit is 0.99. (b) *Af*FEN-catalysed hydrolysis of DHP5F3F(2) substrate yields catalytic parameters $K_M = 1.4(\pm 0.3)$ μM, $k_{cat} = 104(\pm 8)$ min^{-1}. The R factor for this curve fit is 0.98.

(329 min^{-1}) is modestly slower than that measured for all the other substrates. This small decrease may be the consequence of subtle changes in the positioning of the scissile bond within the active site that are the result of the differing structures of the substrates.

For all the substrates tested here, maximal single turnover rates (k_c) are faster than the multiple turnover number of the enzyme-catalysed reaction (k_{cat}) and product release is therefore wholly or partially rate-limiting under multiple turnover conditions (Table 2). As the rates of product release are slower than the chemical step of the T5FEN-catalysed reaction, the steady-state catalytic parameters k_{cat} and K_M are not true microscopic equilibrium and rate constants and therefore some caution is required in interpreting these values. In cases where $k_c >> k_{cat}$, the multiple turnover rate of the enzyme is a measure of the rate at which products are released. The ratio k_c/k_{cat} is a measure of the extent to which reaction is rate-limited by product release. These values are greater for the

larger substrates possessing an upstream double-stranded region, which produce larger 3′-products. This reflects the greater stability of enzyme-product complexes that result from substrates that possess an upstream double-stranded region.

For reactions that are rate-limited by steps other than the chemical reaction under steady-state conditions, K_M values define the concentration of substrate required to convert half the enzyme into the form(s) preceding the rate-limiting step(s).[34] Thus for the FEN-catalysed reaction K_M reflects the dissociation constant of ES and EP complexes and their rate of interconversion. For all substrates, with the exception of HP5F, the rate of interconversion of ES and EP (k_c) is constant within the limits of experimental error. Thus K_M values reflect the stabilities of ES and EP, and as might be expected, decrease with increasing size and therefore potential for interactions with the protein. Similarly, the decrease in turnover number observed with larger substrates reflects the greater stabilities of their EP complexes.

Catalytic parameters of Af*FEN*

The kinetic parameters of the *Af*FEN-catalysed reactions of double hairpin variants of flap substrates are shown in Table 3. Reactions were monitored at 55 °C to ensure that substrates remained base-paired under assay conditions, although reactions of *Af*FEN proceed faster at more elevated temperatures.[17] All other reaction conditions were identical to the phage enzyme except that 1 mM DTT was included in the buffer. *Af*FEN-catalysed hydrolysis of the double-flap substrate DHP5F3F(2) yields a turnover number of 104(±8) min^{-1} (Figure 5(b)). A previous determination of the catalytic parameters of *Af*FEN, also at 55 °C, produced drastically lower values of maximal rates of reaction.[17] However, this study did not include a reducing agent in the reaction buffer and we observed similar much lower rates of reaction when this was not present. Others have reported the stimulation of archaeal FENs when non-ionic detergents have been included in the buffer[21] and a recent publication included both detergents and DTT in the hFEN reaction mixture.[20]

Table 3. The catalytic parameters of *Af*FEN-catalysed reactions of DHP5F(2) and DHP5F3F(2)

Substrate	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M (μM^{-1} min^{-1})	$\Delta\Delta G$ ($kJmol^{-1}$)
DHP5F(2)	>50	>50	1.1±0.04	11.5
DHP5F3F(2)	104±8	1.4±0.3	74.3	–

Full experimental details are contained in Materials and Methods. Steady-state parameters were determined by measuring the initial rates of reaction of substrates at concentrations around their respective K_Ms. In the case of DHP5F(2), the reaction did not achieve saturation at [S]=75 μM but k_{cat}/K_M could be determined from a plot of v/[E] *versus* [S] when the concentration of [S]<K_M (Figure 1S, Supplementary Data). Each set of data is the average of at least three experiments. $\Delta\Delta G$, the energetic penalty incurred upon removal of the 3′-extrahelical nucleotide, was calculated as described (equation (2), Materials and Methods).[48]

The value of K_M observed with the corresponding single flap substrate lacking the 3′-extrahelical nucleotide (DHP5F(2)) is dramatically increased with respect to the double-flap substrate (Table 3). Whilst we were unable to determine the saturating maximal multiple turnover rate of reaction with this substrate, the normalised rate observed at 75 μM DHP5F(2) (51(±3) min^{-1}) is only half the k_{cat} value of DHP5F3F(2), suggesting that a k_{cat} value for DHP5F(2) will be close to or exceed that of the substrate possessing the 3′-extrahelical nucleotide (Figure 1S, Supplementary Data). Due to the high concentration of enzyme that would be required to achieve maximal rate under single-turnover conditions we were unable to determine whether the reactions of *Af*FEN are rate-limited by product release, although this may not be the case at elevated temperatures. However, the large differences in catalytic efficiency observed with the flap and double-flap substrates seem likely to originate from differing stabilities of ES complex, rather than stimulation of the chemical rate of reaction by binding of the 3′-extra nucleotide. The "specificity" of the thermophilic archaeal and bacterial proteins for flap structures, over smaller substrates lacking the upstream duplex, is also likely to originate from a lack of ability to form an ES complex.

Lack of knowledge of the rate-limiting step in the *Af*FEN reaction, coupled with a rate-limiting product release with the phage enzyme, and the suboptimal temperature at which the *Af*FEN reaction had to be monitored, also complicates direct comparisons of the catalytic parameters of the phage (Table 2) and archaeal (Table 3) enzymes. However the maximal multiple turnover rate of reaction observed here in the presence of reducing agent is in line with k_{cat} values reported for the phage enzyme, although about tenfold less than the maximal single-turnover rates. The Michaelis constant measured for the *Af*FEN reaction, even with double-flap substrate DHP5F3F(2) is much greater than any measured for the phage enzymes, although those of the T5FEN are lowered to some extent by the existence of a stable EP complex. The difference in Michaelis constants for the T5 and *Af* enzymes with flap substrates lacking the 3′-extra nucleotide is close to three orders of magnitude.

Discussion

An intriguing feature of the reaction of FENs is the capacity to act on a number of different substrates and catalyse both structure-specific endonucleolytic reactions of bifurcated nucleic acid structures and the 5′-exonucleolytic degradation of duplex and nicked DNA substrates. Additionally some flap endonucleases possess the ability to 5′-exonucleolytically degrade single-stranded nucleic acids under forcing conditions and the 5′-single-stranded termini of bifurcated substrates under comparable

conditions to those that give the endonucleolytic reactions.[19,35,36] Moreover, any explanation of the substrate specificity of FENs must also account for the contrasting behaviour of archaeal FENs that favour the reactions of larger flap-type substrates in contrast to smaller duplexes and hairpins.[21] Furthermore, in the context of these multiple activities, the ability of bacterial, yeast, archaeal and mammalian FENs to produce a specific reaction with a double-flap substrate deserves consideration.[21,23–25]

To understand and rationalise the apparent multiple activities of some FENs we studied the reaction of two FENs with various substrates. Differences in observed Michealis constants but similar turnover numbers for the phage and archaeal enzymes reported here suggest that the variation in the catalytic efficiency of FENs derived from differing sources with differing substrates most likely arises from their DNA binding properties. Thus the phage enzyme, in line with mammalian FENs, readily accepts blunt ended duplexes and hairpins as its minimal substrate, whereas the archaeal FENs, require much larger DNA substrates to produce a stable ES complex. It therefore seems likely that the large rate enhancements brought about by addition of an upstream duplex, previously reported to be 60,000-fold for *Af* and *Pf*FENs,[21] originate from stability of ES complex rather than any stimulation in the rate of phosphate diester hydrolysis as a consequence of forming additional interactions with the substrate. Similarly, the addition of a 3′-extrahelical nucleotide, that results in a specific reaction and is reported to stimulate the activity of bacterial, archaeal, yeast and mammalian FENs does so because the FEN–DNA interaction is improved leading to a greater rate of reaction at lower concentrations of substrate, rather than an acceleration of the rate constant.

In contrast with the results obtained here where the *Af*FEN reaction occurred only at one site with double flap substrates (DHP5F3F(7) and DHP5F3F (2)), reactions of T5FEN occurred at multiple sites with these substrates. In the case of *Af*FEN crystallographic studies have revealed that interactions are formed between the protein and the phosphate diester and 3′-hydroxyl group of this extrahelical 3′ nucleotide (Figure 2(b)).[9] This 3′-extrahelical nucleotide binding site is a feature of the FENs of higher organisms, as similar specificities have been observed with mammalian FENs and mutation of the residues that contact the extrahelical nucleotide relax this specificity.[24] Interactions with the 3′-extrahelical nucleotide presumably favour only a single binding mode of the substrate, by stabilising it in preference to all others. In the case of the substrate used here, removal of the 3′-extrahelical nucleotide incurs an energetic penalty of 11.5 kJmol^{-1}, consistent with the nature of the interactions observed in the X-ray structure. The regions of the *Af* protein that interact with the 3′-extrahelical nucleotide are absent in the smaller bacteriophage FEN (Figure 2), explaining the failure to observe a specific reaction with double flap substrates and the phage enzyme.

Models of the interaction of FENs with their DNA substrates suggest three binding sites for structural elements of their flap DNA substrates. One region of the protein interacts with duplex DNA downstream of the scissile bond, a second region interacts with 3′-upstream DNA and a third region is responsible for contacts with the single-stranded region of bifurcated structures (Figure 2(a)). In T5FEN the downstream DNA duplex is contacted by Lys241, Lys215 and Arg216 (blue spheres Figure 2(a)).[14] Similarly located residues have been implicated in downstream substrate binding in hFEN.[13] The upstream binding residues of *Af*FEN have been visualised crystallographically (red spheres Figure 2(a)).[9] In accord with these proposals are the finding that T5FEN R216A and K215A mutant proteins have significantly impaired catalytic efficiency compared to the wild-type enzyme when HP5F is employed as a substrate, defining the downstream binding surface and confirming that HP5F occupies this site.[14] The analogues of simple duplex substrates used in this study, HP, presumably also bind in this site, indicating that only interaction with this portion of the protein is strictly necessary for catalysis. Whilst reaction of HP5F is formerly designated as an endonucleolytic activity, whereas reaction of HP (22) is a 5′-exonucleolytic reaction, here we show that these reactions share the common feature that a preferential reaction occurs 1 nt into the double-stranded region of the substrate, although both of these substrates have additional minor reaction sites. Thus the structure-specific endonucleolytic activity of 5′-overhanging duplex and hairpin substrates and the exonucleolytic behaviour of FENs on duplex substrates can be explained by occupation of the same downstream duplex binding site. In contrast the nicked DNA substrates DHP and DHP3F, must occupy both the duplex binding regions, as must all the other substrates used in this study.

The 5′-single-stranded DNA interaction site of FENs involves the region of the protein that contains the helical arch or clamp. Basic residues at the base of the helical arch of T5 and DNA polymerase I FENs have been implicated in contacting the DNA substrate.[25,28,37] The active site of FENs lies at the base of the helical arch or clamp (Figure 2(a)). Earlier models of the T5FEN–DNA interaction threaded single-stranded DNA through the arch.[5,14] However the recent observation that hFEN can catalyse structure-specific cleavage of bifurcated DNA structures that have an oligodeoxynucleotide hybridised to their single-stranded regions and therefore no free 5′ terminus,[20] and that T5FEN can cleave closed circular DNA substrates,[36,38,39] suggests that threading cannot be operational in these substrates. It would therefore appear more likely that 5′-single-stranded DNA passes in front of the helical arch, or is clamped by the helical arch folding over the DNA. Another possibility is that the arch could fold backwards, or become disordered, allowing DNA to pass over. The observation of small amounts of reaction of HP(22) and DHP, 2 nt into the double-stranded region, a reaction site not observed with

any of the substrates possessing a 7 nt 5′-flap, suggests that placing DNA, even if it is base-paired, in the flap binding site may be beneficial to interaction, especially as with these substrates this must occur with the penalty of shortening the amount of duplex present in the downstream binding site. T5FEN reaction further into the duplex region also occurs with HP(22) when binding and reaction is inhibited at the site 1 nt into the double stranded region by inclusion of a phosphorothioate linkage. Beneficial interactions in both the T5FEN and *Af*FEN flap-binding site may also extend further as reaction 2 nt into the double-stranded region is also observed with substrates with a shorter 2 nt 5′-overhang (DHP5F(2)).

Flap endonucleases have an absolute requirement for a divalent metal ion cofactor. The maximal single turnover rate of the T5 enzyme is proportional to the concentration of metal-bound hydroxide, or its ionic equivalent, at pH values below the pK_a of metal-bound water within the FEN active site.[29] It is also likely that metal ion(s) play the role of Lewis acid catalysis or leaving group activation in the FEN-catalysed reaction, requiring the direct association of metal ion(s) to one of the non-bridging oxygen atoms or the leaving group oxygen of the scissile bond.[29] Thus phosphate diester hydrolysis must involve intimate contact between one or more metal ions and the cleaved phosphate diester. Most X-ray structures of flap endonucleases contain two divalent metal ions that are liganded by conserved active site carboxylates, located at the base of a helical arch or clamp.[20–25] The separation of the metal ions differs in the various structures but generally exceeds the less than 4 Å separation demanded by nucleases that adopt a "two metal ion mechanism".[5,7–11] Most studies support the view that metal one, closest to the helical arch or clamp, is absolutely required for reaction.[25,40–42] Thus models of the catalytically active FEN–substrate interaction require the scissile phosphate diester bond to be located adjacent to metal site one.

Multiple initial reaction sites are observed with all the DNA substrates utilised in this study with T5FEN and with all *Af*FEN substrates lacking the 3′-extrahelical nucleotide. Furthermore all of these reaction products appear to originate from a reaction of the intact substrate used in these experiments as a subsequent reaction of any 5′-FAM labelled products of the FEN-catalysed reaction can be ruled out as a single-stranded 8mer is not a cleaved by either FEN under the reaction conditions used. To account for the varying specificities observed, we propose that each of the DNA substrates is capable of subtly different modes of interaction with FEN within the confines of occupying the global substrate-binding areas identified in previous models. The interaction modes place different phosphate diester linkages that become the scissile bond in intimate contact with metal one. The cleavage specificity of T5FEN with HP5F could be explained by a small change in position of the bound duplex region within the downstream binding site. The most favoured interaction mode of this substrate must be one that places metal one in contact with the phosphate diester 1 nt into the double-stranded region, as the greatest amount of reaction occurs at this site. Other binding modes that place the two 5′-adjacent phosphates of the single-stranded flap in intimate contact with metal one result from placing the duplex region of the substrate further away from the active site. Both of these less favoured binding modes, would still allow duplex DNA to interact with Lys241, Lys215 and Arg216 in T5FEN.

Adding a further increment of potential binding energy in the form of either a single-stranded 3′-extension (HP5F3E) or an upstream duplex (DHP5F and DHP5F3F) changes the specificity of the observed reactions of the substrate with T5FEN, leading to further reactions within the single-stranded region. A similar lack of specificity is observed with *Af*FEN and the flap substrates lacking a 3′-extrahelical nucleotide. This presumably arises because interactions with the upstream binding site stabilise additional binding modes where the downstream duplex is moved further from the active site under the reaction conditions. The flexibility of the 3′-single-stranded extension in HP5F3E may allow changes in the position of the downstream DNA to be accommodated whilst still realising interactions with the upstream binding site. Despite the greater rigidity of the upstream duplex in the flap substrates compared to a 3′-single strand, we suppose it can adjust position by stabilising various interactions with the downstream DNA within its binding site, perhaps by changing the angle subtended by the two duplex regions.

Results obtained here with flap (DHP5F(7) and DHP5F3F(7)) and pseudo-Y (HP5F3E) substrates contrast with results obtained for larger two or three-stranded variants of these type of substrates with T5FEN.[14,42] Enzymatic hydrolyses of these larger substrates generate initial endonucleolytic products that result from reactions around the region of the bifurcation and smaller exonucleolytic products of 3 and 5 in length, but no products of intermediate length. The length of the 5′-single-stranded flaps used in these larger substrates is typically in the region of 20 nt. The difference between endonucleolytic and exonucleolytic reactions is not obvious in the substrates used in this study that have smaller single-stranded regions of 7 nt. The exonucleolytic reactions observed with substrates with longer single-stranded regions may involve a looping of the 5′-single-stranded DNA, to position the favoured reaction sites located towards the 5′ terminus in the enzyme active site. A looping of the single-stranded DNA portion of bifurcated substrates has previously been suggested as a pre-requisite for threading through the helical arch.[25] However, such a looping could still occur to position the exonucleolytic reaction sites over metal site one without the need to invoke threading. An alternative binding mode that positions the exonucleolytic scissile phosphate diesters close to metal site one without looping must eventually result in no inter-

action between the downstream double-stranded DNA and the enzyme. Indeed such a looping could also be the origin of the smaller products observed in this study. Further work is required to clarify the reasons for the preferred reaction sites in substrates with larger single-stranded regions.

An important finding of this work is that for all the substrates tested with T5FEN, maximal single-turnover rates are greater than those measured in the steady state, indicating that product release is the slowest step in the T5FEN reaction pathway for all substrates. A consequence of this is that simple interpretation of steady-state catalytic parameters, which assume a Michaelis-Menten-type mechanism, are inappropriate. As a result of a rate-limiting product release, the measured Michaelis constant is lower than the true dissociation constant of the ternary (protein-metal ions-DNA) complex and similarly the measured turnover number can severely underestimate the rate of the chemical reaction. Furthermore, analysis of altered enzymes and variation in reaction conditions that rely solely on the determination of steady-state parameters may well produce misleading results. For example, a tenfold decrease in the rate of the chemical reaction of any of the larger substrates containing an upstream duplex, would produce a negligible change in the observed value of k_{cat}, if the rate of product release remained the same.

At least one other FEN reaction, that of the 5′-nuclease domain of DNA polymerase I, is rate-limited by product release with flap type substrates.[25] It seems likely that this may be a common feature of FEN reactions, especially those that take place at 37 °C. Mammalian FENs possess binding sites for other proteins involved in DNA replication and repair, such as the Werner and Bloom syndrome helicases that stimulate the multiple turnover rates of the enzymes.[43,44] One possibility is that the binding of these proteins may stimulate release of the FEN reaction products. The FENs of archaea, yeast and mammals contain interaction sites for the sliding clamp proliferating cell nuclear antigen (PCNA), but these are not present in the phage enzymes.[9,11,45–47] Addition of PCNA to reactions catalysed by these FENs has been demonstrated to stimulate activity. One possibility is that PCNA interaction stabilises ES complexes of archaeal proteins, although further experiments are required to clarify whether this is the case.

Materials and Methods

T5FEN and *Af*FEN were purified to homogeneity as described.[17,35]

Synthesis, purification and characterisation of 5′-FAM-labelled substrates

The fluorescent oligonucleotide substrates were synthesised using an ABI model 394 DNA/RNA synthesiser using 5′-fluorescein (FAM) reagent (Glen Research *via* Cambio) and standard reagents and conditions for other base additions. Following deprotection, the oligonucleotides were purified by RP HPLC (Alltech Alphabond C18 column; buffer A, 100 mM triethylammonium acetate (TEAAc) (pH 6.5), 5% (v/v) acetonitrile; buffer B, 100 mM TEAAc (pH 6.5), 65% acetonitrile) and then desalted using NAP-10 columns.

Phosphorothioate-containing oligomers (5′-FAM-GsAACACACGCTTGCGTGTGTTC (psHP) and 5′-pGsAACACACGCTTGCGTGTGTTC) were synthesised using Beaucage sulfurising reagent (Glen Research via Cambio). Chemical phosphorylating reagent (Glen Research *via* Cambio) was used for 5′-phosphorylation of the 3′-phosphorothioate fragment of psHP5F. The fast and slow isomers of oligonucleotides containing phosphorothioate linkages were purified by RP HPLC (Waters μBondapak C18; buffer A, 100 mM triethylammonium acetate (TEAAc) pH 6.5, 5% (v/v) acetonitrile; buffer B, 100 mM TEAAc (pH 6.5), 65% acetonitrile). For resolution of diastereoisomers of psHP $t=0$ min 0% B, $t=30$ min 100%B; retention times psHP fast 13.22 min; ps HP slow 13.78 min. For resolution of diastereoisomers of 5′-pGsAACACACGCTTGCGTGTGTTC $t=0$ min 1% B, $t=5$ min 1% B; $t=35$ min 40% B; retention times fast 20.36 min; slow 21.60 min. psHP5F substrates were then assembled by templated ligation of a 5′ fragment (5′-FAM-CGCTGCT-3′) and a 5′-phosphorylated 3′ fragment (5′-pGsAACACACGCTTGCGTGTGTTC-3′) annealed to a template oligonucleotide (5′-ACACGCAAGCGTGTGTT-CGACAGCG-3′). The oligonucleotides were reacted in the ratio of 3 OD units 3′ fragment to 4 OD units template to 9 OD units 5′ fragment in a reaction (125 μl) containing T4 DNA ligase (2600 units; NE Biolabs) in 50 mM Tris-HCl (pH 7.5 at 25 °C), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 μg/ml BSA for 72 h at 16 °C. The products were separated from the unligated oligonucleotides by 20% (w/v) PAGE, followed by gel elution into deionised water. Following gel elution the oligonucleotide was desalted using a NAP-25 column.

All oligonucleotides were characterised by MALDI-TOF mass spectrometry and molecular weights were in good agreement with their calculated values.

Determination of the reaction specificity and steady-state parameters of FEN-catalysed reactions

T5FEN

Reaction mixtures containing appropriate concentrations of FAM labelled oligonucleotides in 25 mM potassium glycinate (pH 9.3), 50 mM KCl, 0.01 mg/ml BSA, 10 mM $MgCl_2$ were heated to 95 °C for 1 min and cooled to 37 °C to pre-fold the substrate. Enzyme stock was diluted in 125 mM potassium glycinate (pH 9.3), 250 mM KCl, 0.05 mg/ml BSA, 50 mM $MgCl_2$, and kept on ice until used. Reactions were initiated by addition of enzyme. Final concentrations of 10–1000 nM substrate (100 nM–160 μM for HP(18) and 25–3600 nM for HP(20)) were used. Enzyme concentrations ranging from 3–200 pM were employed depending on the substrate under study. In the case of HP(18), where the K_M was drastically raised, concentrations ranging from 4–100 nM enzyme were used. Aliquots were removed at eight time intervals and quenched with an equal volume of 20 mM EDTA. dHPLC (Wave® fragment analysis system with a fluorescence detector; Transgenomic, Warrington, UK) was used to separate and quantify the fluorescent product from the labelled substrate. A DNA Sep® column was used with the following buffers: buffer A, 2.5 mM tetrabutyl

ammonium bromide, 1 mM EDTA, 0.1% acetonitrile; buffer B, 2.5 mM tetrabutyl ammonium bromide, 1 mM EDTA, 70% acetonitrile; gradient, $t=0$ min, 5% B; $t=9$ min, 50% B; $t=13.5$ min, 100% B; retention times of products, fluorescent 8mer product 9.5 min, fluorescent 7mer product 9.1 min, fluorescent 6mer product 8.7 min, fluorescent 5mer product 8.0 min, fluorescent 4mer product 7.5 min, fluorescent 3mer product 6.8 min, fluorescent 2mer product 6.2 min, fluorescent 1mer product 5.6 min. Initial rates of reaction at the various substrate concentrations were determined and the kinetic parameters were calculated by non-linear regression fitting of the data to the Michaelis-Menten equation:

$$\frac{v}{[E]} = \frac{k_{cat}[S]}{(K_M = [S])} \quad (1)$$

All curve fitting was carried out using Kaleidagraph software (Synergy Software, Reading, PA).

A comparison of the reaction of specificity of T5FEN with the varying substrates was carried out at 100 nM substrate and 100 pM enzyme.

Af*FEN*

Experiments with *Af*FEN were carried out essentially as described for the phage enzyme except that reactions were carried out at 55 °C and DTT (final concentration 1 mM) was added after substrate denaturation and refolding. The specificity of the reaction was studied at concentrations of 8 μM substrate and 0.1 μM enzyme, 25 mM potassium glycinate (pH 9.3), 50 mM KCl, 0.01 mg/ml BSA, 10 mM $MgCl_2$, 1 mM DTT at 55 °C. Kinetic parameters were assessed using final substrate concentrations 0.05–75 μM and enzyme of 0.1–10 nM. The energetic penalty incurred by removal of the 3′ extrahelical nt ($\Delta\Delta G$) was calculated using equation (2):

$$\Delta\Delta G = -RT\ln\frac{(k_{cat}/K_M)_{DHP5F3F(2)}}{(k_{cat}/K_M)_{DHP5F(2)}} \quad (2)$$

Single turnover rapid quench experiments

Rapid quench experiments were carried out at 37 °C using a RQF-63 device from Hi-Tech Ltd (Salisbury, UK). An 80 μl aliquot of enzyme in reaction buffer (25 mM potassium glycinate (pH 9.3), 10 mM $MgCl_2$, 50 mM KCl, 0.01 mg/ml BSA) was mixed with an equal volume of oligonucleotide substrate in reaction buffer. Enzyme was used at final concentrations of at least 8 x K_M and then increased until a constant maximal rate of reaction was observed. Oligonucleotide substrates were used at a final concentration of 1 nM. After a controlled time delay of 9.1 ms to 6.4 s, 80 μl of quench (1.5 M sodium hydroxide, 20 mM EDTA) was added. Solutions of enzyme, substrate and quench were held in a thermostatted bath within the instrument during the reactions. The quenched reaction mixtures were recovered from the sample recovery loop and were analysed using denaturing HPLC as described previously for the steady-state analyses. The first-order rate of the reaction was determined by plotting the appearance of product against time and by non-linear regression fitting to the equation:

$$P_t = P\infty(1 - \exp^{-k_c t}) \quad (3)$$

where P_t is the amount of product at time t, P_8 is the amount of product at time 8, and k_c is the maximal rate of reaction under single turnover conditions.

MALDI-TOF mass spectral analysis of cleavage products

Substrate (HP5F, 100 μM) was incubated at 37 °C in a solution of 25 mM potassium glycinate (pH 9.3), 50 mM KCl, 10 mM $MgCl_2$. T5FEN was then added to a concentration of 80 nM to initiate the reaction. Aliquots of reaction mixture were taken at intervals from 5 to 60 min, quenched by the addition of equal volume of 20 mM EDTA. Salts were removed using Dowex beads (NH_4^+ form) and the sample was mixed with matrix (50 mg/ml 3-hydroxypicolinic acid 3:1 H_2O:CH_3CN). Samples were then submitted to MALDI-TOF mass spectrometry in positive ion mode on a Bruker Reflex III instrument to confirm their identities.

Reaction of HP5F: HP5F calc. 9398.24 found 9401; product 21mer calc. 6477.19 found 6480; product FAM-8mer calc. 2939.07, found 2942; product FAM-7mer calc. 2609.86, found 2612; product FAM-6mer calc. 2320.68, found 2323. No peaks were observed corresponding to 22mer or 23mer products.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2007.04.063

References

1. Liu, Y., Kao, H. I. & Bambara, R. A. (2004). Flap endonuclease 1: a central component of DNA metabolism. *Annu. Rev. Biochem.* **73**, 589–615.
2. Pickering, T. J., Garforth, S. J., Thorpe, S. J., Sayers, J. R. & Grasby, J. A. (1999). A single cleavage assay for T5 5′–>3′ exonuclease: determination of the catalytic parameters for wild-type and mutant proteins. *Nucl. Acids Res.* **27**, 730–735.
3. Lieber, M. R. (1997). The FEN-1 family of structure-specific nucleases in eukaryotic DNA replication, recombination and repair. *Bioessays*, **19**, 233–240.
4. Lyamichev, V., Brow, M. A. D. & Dahlberg, J. E. (1993). Structure-specific endonucleolytic cleavage of nucleic acids by eubacterial DNA polymerases. *Science*, **260**, 778–783.
5. Ceska, T. A., Sayers, J. R., Stier, G. & Suck, D. (1996). A helical arch allowing single-stranded DNA to thread through T5 5′-exonuclease. *Nature*, **382**, 90–93.
6. Kim, Y., Eom, S. H., Wang, J. M., Lee, D. S., Suh, S. W. & Steitz, T. A. (1995). Crystal structure of *Thermus aquaticus* DNA polymerase. *Nature*, **376**, 612–616.

7. Mueser, T. C., Nossal, N. G. & Hyde, C. C. (1996). Structure of bacteriophage T4 RNase H, a 5′ to 3′ RNA-DNA and DNA-DNA exonuclease with sequence similarity to the Rad2 family of eukaryotic proteins. *Cell*, **85**, 1101–1112.
8. Hosfield, D. J., Mol, C. D., Shen, B. H. & Tainer, J. A. (1998). Structure of the DNA repair and replication endonuclease and exonuclease FEN-1: coupling DNA and PCNA binding to FEN-1 activity. *Cell*, **95**, 135–146.
9. Chapados, B. R., Hosfield, D. J., Han, S., Qiu, J. Z., Yelent, B., Shen, B. H. & Tainer, J. A. (2004). Structural basis for FEN_1 substrate specificity and PCNA-mediated activation in DNA replication and repair. *Cell*, **116**, 39–50.
10. Hwang, K. Y., Baek, K., Kim, H. Y. & Cho, Y. (1998). The crystal structure of flap endonuclease I from *Methanococcus jannaschii*. *Nature Struct. Biol.* **5**, 707–713.
11. Sakurai, S., Kitano, K., Yamaguchi, H., Hamada, K., Okada, K., Fukuda, K. *et al.* (2005). Structural basis for recruitment of human flap endonuclease 1 to PCNA. *EMBO J.* **24**, 683–693.
12. Matsui, E., Musti, K. V., Abe, J., Yamasaki, K., Matsui, I. & Harata, K. (2002). Molecular structure and novel DNA binding sites located in loops of flap endonuclease-1 from *Pyrococcus horikoshii*. *J. Biol. Chem.* **277**, 37840–37847.
13. Qiu, J. Z., Liu, R., Chapados, B. R., Sherman, M., Tainer, J. A. & Shen, B. H. (2004). Interaction interface of human flap endonuclease_1 with its DNA substrates. *J. Biol. Chem.* **279**, 24394–24402.
14. Dervan, J. J., Feng, M., Patel, D., Grasby, J. A., Artymiuk, P. J., Ceska, T. A. & Sayers, J. R. (2002). Interactions of mutant and wild-type flap endonucleases with oligonucleotide substrates suggest an alternative model for DNA binding. *Proc. Natl Acad. Sci. USA*, **99**, 8542–8547.
15. Allawi, H. T., Kaiser, M. W., Onufriev, A. V., Ma, W. P., Brogaard, A. E., Case, D. A. *et al.* (2003). Modeling of flap endonuclease interactions with DNA substrate. *J. Mol. Biol.* **328**, 537–554.
16. Harrington, J. J. & Lieber, M. R. (1994). The characterization of a mammalian DNA structure-specific endonuclease. *EMBO J.* **13**, 1235–1246.
17. Hosfield, D. J., Frank, G., Weng, Y. H., Tainer, J. A. & Shen, B. (1998). Newly discovered archaebacterial flap endonucleases show a structure-specific mechanism for DNA substrate binding and catalysis resembling human flap endonuclease-1. *J. Biol. Chem.* **273**, 27154–27161.
18. Bhagwat, M., Hobbs, L. J. & Nossal, N. G. (1997). The 5′-exonuclease activity of bacteriophage T4 RNase H is stimulated by the T4 gene 32 single-stranded DNA-binding protein, but its flap endonuclease is inhibited. *J. Biol. Chem.* **272**, 28523–28530.
19. Garforth, S. J. & Sayers, J. R. (1997). Structure-specific DNA binding by bacteriophage T5 5′–>3′ exonuclease. *Nucl. Acids Res.* **25**, 3801–3807.
20. Liu, R., Qiu, J. Z., Finger, L. D., Zheng, L. & Shen, B. H. (2006). The DNA-protein interaction modes of FEN-1 with gap substrates and their implication in preventing duplication mutations. *Nucl. Acids Res.* **34**, 1772–1784.
21. Kaiser, M. W., Lyamicheva, N., Ma, W. P., Miller, C., Neri, B., Fors, L. & Lyamichev, V. I. (1999). A comparison of eubacterial and archaeal structure-specific 5′-exonucleases. *J. Biol. Chem.* **274**, 21387–21394.
22. Lyamichev, V. I., Brow, M. A., Varvel, V. E. & Dahlberg, J. E. (1999). Comparison of the 5′ nuclease activities of Taq DNA polymerase and its isolated nuclease domain. *Proc. Natl Acad. Sci. USA*, **96**, 6143–6148.
23. Kao, K. I., Henricksen, L. A., Liu, Y. & Bambara, R. A. (2002). Cleavage specificity of *Saccharomyces cerevisiae* flap endonuclease 1 suggests a double-flap structure as the cellular substrate. *J. Biol. Chem.* **277**, 14379–14389.
24. Friedrich-Heineken, E. & Hubscher, U. (2004). The Fen1 extrahelical 3′-flap pocket is conserved from archaea to human and regulates DNA substrate specificity. *Nucl. Acids Res.* **32**, 2520–2528.
25. Xu, Y., Potapova, O., Leschziner, A. E., Grindley, N. D. F. & Joyce, C. M. (2001). Contacts between the 5′ nuclease of DNA polymerase I and its substrate DNA. *J. Biol. Chem.* **276**, 30167–30177.
26. Qiu, J. Z., Bimston, D. N., Partikian, A. & Shen, B. H. (2002). Arginine residues 47 and 70 of human flap endonuclease-1 are involved in DNA substrate interactions and cleavage site determination. *J. Biol. Chem.* **277**, 24659–24666.
27. Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucl. Acids Res.* **31**, 3406–3415.
28. Pickering, T. J., Garforth, S. J., Sayers, J. R. & Grasby, J. A. (1999). Variation in the steady state kinetic parameters of wild-type and mutant T5 5′–3′ exonuclease with pH. Protonation of Lys_83 is critical for DNA binding. *J. Biol. Chem.* **274**, 17711–17717.
29. Tock, M. R., Frary, E., Sayers, J. R. & Grasby, J. A. (2003). Dynamic evidence for metal ion catalysis in the reaction mediated by a flap endonuclease. *EMBO J.* **22**, 995–1004.
30. Patel, D. V., Tock, M. R., Frary, E., Feng, M., Pickering, T. J., Grasby, J. A. & Sayers, J. R. (2002). A conserved tyrosine residue aids ternary complex formation but not catalysis, in phage T5 flap endonuclease. *J. Mol. Biol.* **320**, 1025–1035.
31. Elliott, S. L., Brazier, J., Cosstick, R. & Connolly, B. A. (2005). Mechanism of the *Escherichia coli* DNA T: G-mismatch endonuclease (Vsr protein) thiophosphate-containing probed with oligodeoxynucleotides. *J. Mol. Biol.* **353**, 692–703.
32. Thorogood, H., Grasby, J. A. & Connolly, B. A. (1996). Influence of the phosphate backbone on the recognition and hydrolysis of DNA by the EcoRV restriction endonuclease—a study using oligodeoxynucleotide phosphorothioates. *J. Biol. Chem.* **271**, 8855–8862.
33. Schroeder, G. K., Lad, C., Wyman, P., Williams, N. H. & Wolfenden, R. (2006). The time required for water attack at the phosphorus atom of simple phosphodiesters and of DNA. *Proc. Natl Acad. Sci. USA*, **103**, 4052–4055.
34. Gutfreund, F. (1995). *Kinetics for the Life Sciences, Cambridge University Press, Cambridge, UK.*
35. Sayers, J. R. & Eckstein, F. (1990). Properties of overexpressed phage T5 D15 exonuclease-similarities with *Escherichia coli* DNA polymerase I 5′–3′ exonuclease. *J. Biol. Chem.* **265**, 18311–18317.
36. Sayers, J. R. & Eckstein, F. (1991). A single-strand specific endonuclease activity copurifies with overexpressed T5 D15 exonuclease. *Nucl. Acids Res.* **19**, 4127–4132.
37. Garforth, S., Ceska, T., Suck, D. & Sayers, J. (1999). Mutagenesis of conserved lysine residues in T5 5′–3′ exonuclease suggests separate mechanisms of endo- and exonucleolytic cleavage. *Proc. Natl Acad. Sci. USA*, **96**, 38–43.
38. Sayers, J. R., Evans, D. & J.B., T. (1996). Identification

and eradication of a denatured DNA isolated during alkaline lysis-based plasmid purification procedures. *Anal. Biochem.* **241**, 186–189.
39. Kiss-Toth, E., Dower, S. K. & Sayers, J. R. (2001). A method for enhancing the transfection efficiency of minipreps obtained from plasmid cDNA libraries. *Anal. Biochem.* **288**, 230–232.
40. Xu, Y., Derbyshire, V., Ng, K., Sun, X. C., Grindley, N. D. F. & Joyce, C. M. (1997). Biochemical and mutational studies of the 5′–3′ exonuclease of DNA polymerase I of *Escherichia coli*. *J. Mol. Biol.* **268**, 284–302.
41. Shen, B. H., Nolan, J. P., Sklar, L. A. & Park, M. S. (1997). Functional analysis of point mutations in human flap endonuclease-1 active site. *Nucl. Acids Res.* **25**, 3332–3338.
42. Feng, M., Patel, D., Dervan, J. J., Ceska, T., Suck, D., Haq, I. & Sayers, J. R. (2004). Roles of divalent metal ions in flap endonuclease-substrate interactions. *Nature Struct. Mol. Biol.* **11**, 450–456.
43. Sharma, S., Sommers, J. A., Wu, L., Bohr, V. A., Hickson, I. D. & Brosh, R. M. (2004). Stimulation of flap endonuclease-1 by the Bloom's syndrome protein. *J. Biol. Chem.* **279**, 9847–9856.
44. Brosh, R. M., Driscoll, H. C., Dianov, G. L. & Sommers, J. A. (2002). Biochemical characterization of the WRN-FEN-1 functional interaction. *Biochemistry*, **41**, 12204–12216.
45. Wu, X. T., Li, J., Li, X. Y., Hsieh, C. L., Burgers, P. M. J. & Lieber, M. R. (1996). Processing of branched DNA intermediates by a complex of human FEN-1 and PCNA. *Nucl. Acids Res.* **24**, 2036–2043.
46. Gomes, X. V. & Burgers, P. M. J. (2000). Two modes of FEN1 binding to PCNA regulated by DNA. *EMBO J.* **19**, 3811–3821.
47. Tom, S., Henricksen, L. A. & Bambara, R. A. (2000). Mechanism whereby proliferating cell nuclear antigen stiumlates flap endonulcease 1. *J. Biol. Chem.* **275**, 10498–10505.
48. Fersht, A. R. (1985). *Enzyme Structure and Mechanism*, 2nd edit., W.H. Freeman and Company, New York.

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