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Human Exonuclease 1 Threads 5'-Flap Substrates Through its Helical Arch

Steven J. Shaw, L. David Finger and Jane A. Grasby*

Centre for Chemical Biology, Department of Chemistry, Krebs Institute, University of Sheffield, Sheffield, S3 7HF, UK.

Supporting Information Placeholder

ABSTRACT: Human exonuclease 1 (hEXO1) is a member of the 5'-nuclease superfamily and plays important roles in DNA repair. Alongside acting as a 5'-exonuclease on blunt, gapped, nicked and 3'-overhang DNAs, hEXO1 can also act as an endonuclease removing protruding 5'-single-stranded flaps from duplex ends. How hEXO1 and related 5'-nuclease human flap endonuclease 1 (hFEN1) are specific for discontinuous DNA substrates like 5'-flaps has been controversial. Here we report the first functional data that implies that hEXO1 threads the 5'-flap through a hole in the protein known as the helical arch, thereby excluding reactions of continuous single-strands. Conjugation of bulky 5'-streptavidin that would "block" threading through the arch drastically slowed the hEXO1 reaction. In contrast, addition of streptavidin to a pre-formed hEXO1 5'-biotin flap DNA complex trapped a portion of the substrate in a highly-reactive threaded conformation. However, another fraction behaves as if "blocked" and decayed very slowly implying there were both threaded and unthreaded forms of the substrate present. The reaction of an unmodified hEXO1-flap DNA complex did not exhibit marked biphasic kinetics, suggesting a fast re-equilibration occurs that produces more threaded substrate when some decays. The finding that a threading mechanism like that used by hFEN1 is also used by hEXO1, unifies the mode of operation for members of the 5'-nuclease superfamily that act on discontinuous substrates. As with hFEN1, intrinsic disorder of the arch region of the protein may explain how flaps can be threaded without a need for a coupled energy source.

Human exonuclease 1 (hEXO1) is a member of the divalent metal ion dependent 5'-nuclease superfamily that process a wide variety of bifurcated nucleic acid structures.[1] Playing important roles in mismatch and double-strand break repair, hEXO1 catalyses the processive exonucleolytic hydrolysis of DNA.[2] Substrates include nicked, gapped, 3'-overhang (Figure S1A) and blunt ended DNA duplexes, which are degraded on the 5'-strand producing single nucleotide products. In addition, hEXO1 also acts as an endonuclease removing 5'-single-stranded (ss) flaps from 5'-overhang or pseudo-Y (psY) DNAs (Figures S1B-C), in a manner analogous to its paralogue flap endonuclease 1 (FEN1). Indeed, this flap removal capability of hEXO1 has been suggested to initiate resection in double-strand break repair.[3]

Biochemical and more recent structural studies of FEN1 have shown that the 5'-flap portion of the substrate threads through a hole in the protein known as the helical arch (Figure 1A-C)[4-6] The helical arch of FEN1 is formed from two alpha helices (α4-α5). In turn these helices can be divided into two regions, firstly part of the "gateway" (base of α4) which provides residues to the active site and is a universal feature of the 5'-nuclease superfamily, and the helical cap (rest of α4-α5).[7] Unlike the gateway, the cap is not present in superfamily members that do not enforce a specificity for discontinuous

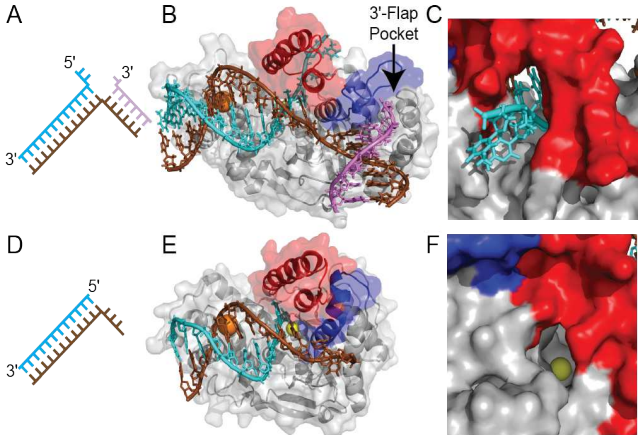

Figure 1. Similarities between 5'-nuclease superfamily members hEXO1-352 and hFEN1-336 in complex with DNA. (A) Double flap and (D) 3'-overhang substrate DNAs crystallized with (B) hFEN1-336 (5UM9) and (E) hEXO1-352 (3QE9). The helical arch (α4-α5) is shown as red and α2-α3 is shown in blue. (C) The exit site of the 5'-flap through the back of arch is shown in close up for hFEN1, with the potential exit site of a flap from hEXO1 also illustrated (F).

DNAs like GEN1 and XPG, but is a feature exclusive to FEN1 and EXO1 (Figure 1D-F). Yet, current thinking on EXO1 mechanism differs from that of FEN1 and suggests that the cap region of the protein acts as a substrate clamp, rejecting the threading mechanism for substrate specificity.[8]

To understand the mechanism of flap removal by hEXO1, we initiated a comparative study with hFEN1. We used a version of the hEXO1 protein truncated at residue 352 (termed hEXO1-352), which is sufficient for robust nuclease activity but lacks the disordered C-terminal tail that is the site of protein-protein interaction *in vivo*.[8] hEXO1-352 was crystallized with DNA previously and the structure closely resembled that of hFEN1 except that it lacks the 3'-flap binding pocket of FEN1 (Figure 1B,E).[7-8] The 3'-flap pocket of FEN1 locks the DNA substrate into a particular orientation so that reaction of a double flap substrate only occurs at one site, one nucleotide into double-stranded (ds) DNA.[9] One nucleotide into ds DNA was also the preferred reaction site of hEXO1-352 on a 3'-overhang DNA substrate (Figure 2A and S1. All experimental details are available in supporting information). In contrast, ss flap removal by hEXO1-352 from a psY substrate produced two major products that corresponded to reaction one nucleotide into the ss flap and at the ss-ds junction with minimal reaction within the ds DNA (Figure 2B and S1). Nevertheless, because the ds DNA products of EXO1 action are also substrates until they reach a minimal duplex length, eventual reaction within the dsDNA will occur.

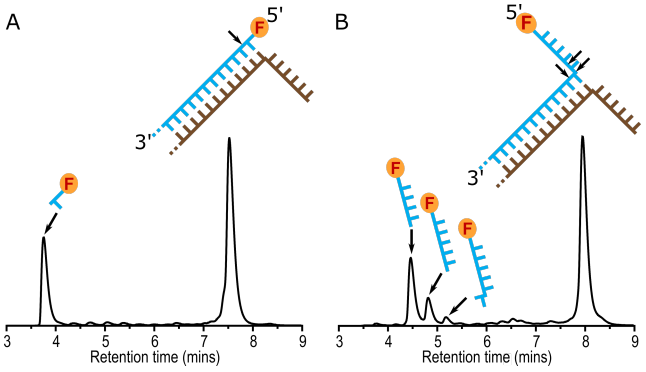

Figure 2. Products of hEXO1-352-catalyzed reactions as determined by HPLC. Chromatograms of hEXO1-352 reaction on (A) 3'-overhang and (B) psY substrates bearing 5' fluorescein labels produces (A) a single nucleotide product and (B) three ss products corresponding to reaction one nucleotide into the duplex, the ss-ds junction and one nucleotide into the flap (6, 5 & 4 nucleotides, respectively). Schematic representations of fluorescein-labelled products and substrates are shown.

To test the mode of interaction of hEXO1 with 5'-flapped DNAs, we used a 5'-biotinylated substrate that could form a very stable interaction with the 211.2 kDa streptavidin tetramer. The substrate was formed from a single 5'-fluorescent and biotinylated oligonucleotide that adopted a psY shape, abbreviated Bio-psY (Figure 3A and S1). As all discontinuous 5'-ends can potentially be sites of reaction with hEXO1, this unimolecular substrate construction ensured only one binding orientation on hEXO1.

Three different types of complexes were constructed with Bio-psY (Figure 3A) and hEXO1 (Figure 3B, for materials and methods see supporting information). In the first complex, termed "pre-mixed", the biotinylated substrate and hEXO1 were pre-incubated together in the presence of the catalytically inert cofactor Ca^{2+} ions. Excess streptavidin was added to some of this pre-mixed complex. If the 5'-ss DNA flap was threaded underneath the helical cap of hEXO1 the streptavidin would trap the DNA on the enzyme; therefore, this complex was referred to as "trapped". In the final complex excess streptavidin was added to Bio-psY prior to its interaction with the enzyme in the presence of Ca^{2+} ions. The bulky streptavidin is large enough to prevent the 5'-ss flap from threading through the helical arch; thus, this mixture is referred to as "blocked". In contrast, if the arch instead acts as a clamp, then the 5'-streptavidin should not interfere with reaction.

To investigate which of the complexes was capable of fast hEXO1-catalyzed hydrolysis and therefore which complex was positioned correctly for reaction, catalysis was initiated by addition of excess Mg^{2+} ions. Reactions were monitored on the ms timescale using quenched flow rapid handling apparatus followed by analysis of samples using denaturing HPLC with a fluorescence detector (Figure 3C). The pre-mixed complex decayed at a rate of 0.43 s^{-1} (Table 1, note that the 1 mM catalytically inert Ca^{2+} ions added to form the complex are inhibitory to some extent in the presence of 8 mM Mg^{2+} ions). In contrast, the streptavidin "blocked" mixture decayed at a rate that was ~ 240 times slower. Because prior streptavidin conjugation would not be expected to impede a clamping mechanism, this implies that a threaded structure is required for reaction on a biologically-relevant timescale. The results closely mirror the properties of hFEN1 with similar 5'-streptavidin substrates.[5]

The most interesting behavior was observed with the hEXO1 streptavidin "trapped" substrate complex, which exhibited distinctly biphasic kinetics when reaction was initiated. A portion (~20 %) of this complex decayed at a rate of 2.05 s^{-1}, comparable to the "pre-mixed" complex (Figure 3C and Table 1). The other fraction decayed much more slowly with a rate that was similar to that of the "blocked" complex. These results have some resemblance to, but some differences with, an earlier streptavidin "trapping" experiment with hFEN1, where close to 100% of the substrate decayed with a very fast rate constant.[5]

We interpreted the biphasic behavior exhibited by the hEXO1 "trapped" mixture as arising from one portion of the substrate being constrained by streptavidin in a

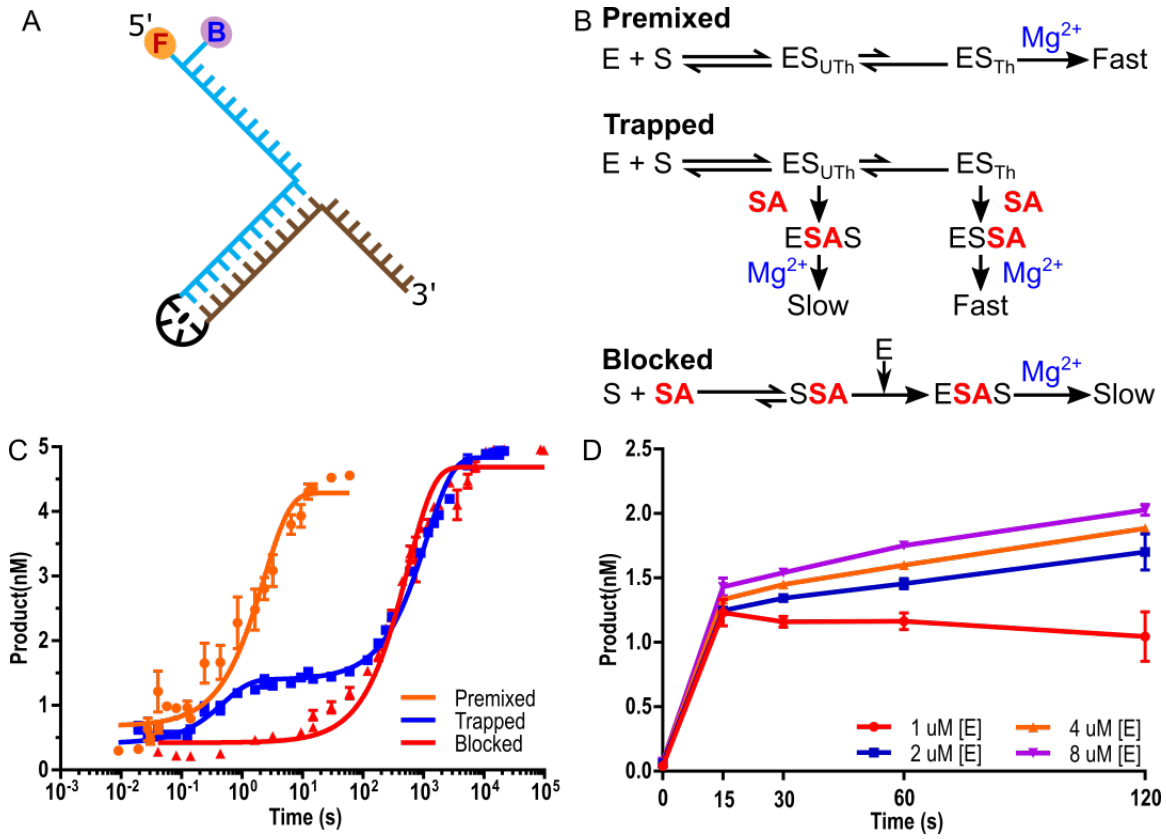

Figure 3. Streptavidin trapping and blocking experiments of EXO1 substrates reveals role for threading in the hEXO1-352-catalyzed endonucleolytic reaction. (A) Illustration of the unimolecular pseudo-Y (Bio-psY) substrate with a 5' fluorescein [F] label and a serinol-biotin TEG [B] used in the trapping experiments. (B) Reaction scheme for the formation of "pre-mixed", "trapped" and "blocked" complex involving enzyme hEXO1 [E], Bio-psY [S] and streptavidin [SA]; UTh = un-threaded and Th = threaded. (C) Decay of "pre-mixed" and streptavidin "trapped" and "blocked" complexes of Bio-psY with hEXO1 upon addition of Mg^{2+} ions. Rates constants from fits to single ("pre-mixed" and "blocked") or double exponential ("trapped") decays are shown in the Table 1. (D) Lack of variation in the proportion of fast decaying species with concentrations of hEXO1 (1-8 μM) in a streptavidin trapping experiment.

position that is capable of fast reaction, with the 5'-ss flap threaded through the helical arch. The other fraction of the substrate had not adopted a threaded state and when streptavidin was added, it became "blocked".

An important question was whether the substrate was saturated with hEXO1 in our experiment, because this could have been what prevented all the substrate from adopting a threaded conformation and being capable of fast reaction. To test this, the concentration of hEXO1 was increased and the amount of fast decaying species that arose in the streptavidin "trapping" experiment was determined (Figure 3D). Despite changing the concentration of enzyme, the amount of trapped fast decaying substrate remained constant under the concentrations of protein tested. This implied that the partition between threaded (fast decaying) and unthreaded (slow decaying) portions in the trapping experiment reflects an on-enzyme equilibrium.

Table 1. Single Turnover Rates of the Various Bio-psY Complexes with hEXO1-352

State	k_{ST}, s^{-1}	Amplitude
Premixed	0.43 ± 0.05	100
Trapped		
Fast	2.05 ± 0.89	22.3
Slow	0.0009 ± 0.00004	77.7
Blocked	0.0018 ± 0.00010	100

The picture that emerges from our experiments is that EXO1, like FEN1, uses a threading mechanism and that, when present, passing the 5'- ss flaps of substrates underneath the helical cap is a pre-requisite for fast reaction. However, unlike FEN1 which processes its optimal double flap substrates at maximum diffusion controlled rates,[7] hEXO1 is not evolutionarily optimized to thread 5'-flap substrates. Thus, even when flap substrates were fully saturated with enzyme an on-enzyme partition between threaded and unthreaded substrate favored the unthreaded non-catalytically proficient state. Nevertheless, the "pre-mixed" sample does not decay with the markedly fast (threaded) and slow (unthreaded) behavior of the "trapped" mixture. This appears to rule out the possibility that unmodified reactions of hEXO1 typically occur in both threaded and non-threaded states (assuming the non-threaded rate of reaction of an unmodified

substrate is similar to that of a "blocked" one). The lack of a biphasic pre-mixed profile would be expected if re-equilibration producing more threaded material occurs when some reacts, so long as the rate of re-equilibration is faster than, or similar to, the rate of reaction. Accordingly, our data are consistent with a model where all flapped substrates typically react from a threaded state.

The threading mechanism was initially (sensibly) rejected for EXO1 based on the perceived difficulties of passing DNA through a small hole in a protein without a coupled energy source.[8] A possible reconciliation between this and the data reported here may be provided by proposals for the mode of action of other 5'-nucleases where regions of intrinsic disorder are suggested to be essential for enzyme function.[5, 7, 10]. Passing DNA through a disordered arch region, which could then form secondary structure to permit reaction, would be expected to have a much less significant entropic barrier. Indeed, crystallographic B-factors for backbone amide nitrogens in structures of hEXO1 bound to DNA show large variations (Figure S2).[8] Residues in the arch and between α2-α3 (Figure 1) possess very large (100-250) B-factors, implying a high degree of motion and lending weight to the plausibility of our disorder-thread-order proposal. Differences in the partition between threaded and unthreaded state observed for hEXO1 compared to hFEN1 may reflect the tendency of the arch regions of the respective proteins to form secondary structure, although this requires further investigation.

The unified 5'-nuclease superfamily threading mechanism has important implications for EXO1 and FEN1 function during DNA replication and repair. The need to thread the 5'- portion of substrates through a hole in the protein confines reactions to discontinuous DNA ends that possess free 5'-termini. However, in contrast reactions of continuous DNAs, such as the unbroken strand in a gapped DNA substrate are prevented on a biological timescale by the threading mechanism. Thus, threading, likely together with intrinsic disorder, plays a vital role in 5'-nuclease reaction specificity.

ASSOCIATED CONTENT

Supporting Information. Full details of materials and methods and Table S1 Oligonucleotide Sequences and Constructs, Figure S1 DNA constructs used and Figure S2 Plot of B-factors for hEXO1-352 are available as supporting information. The Supporting Information is available free of charge on the ACS Publications website as a PDF file.

AUTHOR INFORMATION

Corresponding Author

Jane A Grasby, Centre for Chemical Biology, Department of Chemistry, Krebs Institute, University of Sheffield, Sheffield, S3 7HF, UK j.a.grasby@sheffield.ac.uk

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

During the review of this manuscript, a structure of hEXO1 with a threaded flap substrate was published.[11]

ABBREVIATIONS

ds, double-stranded; hEXO1, human exonuclease 1; hFEN1, human flap endonuclease 1; ss, single-stranded; psY, pseudo Y (DNA structure).

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