Specialization of an Exonuclease III family enzyme in the repair of 3' DNA lesions during base excision repair in the human pathogen Neisseria meningitidis

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ABSTRACT

We have previously demonstrated that the two Exonuclease III (Xth) family members present within the obligate human pathogen Neisseria meningitidis, NApe and NExo, are important for survival under conditions of oxidative stress. Of these, only NApe possesses AP endonuclease activity, while the primary function of NExo remained unclear. We now reveal further functional specialization at the level of 3'-PO4 processing for NExo. We demonstrate that the bi-functional meningococcal glycosylases Nth and MutM can perform strand incisions at abasic sites in addition to NApe. However, no such functional redundancy exists for the 3'-phosphatase activity of NExo, and the cytotoxicity of 3'-blocking lesions is reflected in the marked sensitivity of a mutant lacking NExo to oxidative stress, compared to strains deficient in other base excision repair enzymes. A histidine residue within NExo that is responsible for its lack of AP endonuclease activity is also important for its 3'- phosphatase activity, demonstrating an evolutionary trade off in enzyme function at the single amino acid level. This specialization of two Xth enzymes for the 3'-end processing and strand-incision reactions has not previously been observed and provides a new paradigm within the prokaryotic world for separation of these critical functions during base excision repair.

INTRODUCTION

Oxidative stress is one of the principle causes of DNA damage, and base excision repair (BER) is the main mechanism in living cells responsible for repairing the consequent DNA lesions. BER is therefore a well studied pathway in model organisms such as Escherichia coli, Saccharomyces cerevisiae and humans (1). Initially, damaged nucleobases are recognized and removed by DNA glycosylases, and the resulting apurinic/apyrimidinic (AP) sites, or incised products, are acted upon by the downstream AP endonucleases of the Exonuclease III (Xth) or Endonuclease IV (Nfo) family (2).

DNA glycosylases are responsible for recognizing and removing damaged DNA bases, and those involved in oxidative damage frequently remove multiple DNA lesions that can arise following oxidative damage (3). Glycosylases can also be bi-functional and incise the DNA backbone following removal of the base. The chemistry of these backbone incisions proceeds via an AP lyase activity in which base expulsion results from nucleophilic attack of the 1'-0 of the ribose sugar, typically by the N-terminal amine or a lysine residue in the enzyme, with subsequent ring opening and strand cleavage occurring either via β-elimination to yield a 3'-unsaturated aldehyde (3'-Ald; Figure 1) or β-δ elimination leaving a 3'-phosphate (3'-PO4; Figure 1) (4,5). These 3'-lesions cannot be repaired by a DNA polymerase and are therefore referred to as 3'-blocking lesions (2,6,7); such 3'-blocking lesions can also arise directly from oxidative damage leading to 3'-PO4 and 3'-phosphoglycolate lesions (8). Further processing of these 3'-lesions typically occurs via the Xth or Nfo AP endonucleases to yield a 3'-OH that is competent for extension by a DNA polymerase (2,8).
AP endonucleases are a critical component of BER since they also process AP sites that arise due to spontaneous base loss, or the action of monofunctional DNA glycosylases such as uracil DNA glycosylase (1,4). Incision of the DNA backbone at the abasic site is essential for further processing and repair, and AP endonucleases cleave 5’ to the AP site, leading to 3’-OH and 5’-deoxyribosephosphate (5’-dRP). This 5’ lesion must be processed by a deoxyribosephosphatase activity, and repair can then be effected by DNA polymerase and DNA ligase (1). The requirement for AP endonucleases to process both 3’- and 5’-blocking lesions in addition to AP sites means these enzymes form a key node in BER, through which all lesions must pass en route to complete repair.

The Xth family of AP endonucleases are remarkably conserved across nature at the sequence and structural level (2,9–11). The E. coli ExoIII and human hAP1 enzymes form a four layered α/β sandwich structure with a binding pocket for the abasic deoxyribose that can accommodate the flipped out ribose in the enzyme-substrate complex (12,13). Both enzymes exhibit specific AP endonuclease cleavage in a Mg2+-dependent manner (2). In addition, ExoIII has significant 3’–5’ exonuclease activity (14), although this is limited in hAP1 (15,16). Furthermore, both enzymes are able to process the 3’-Ald group (2,16,17) and ExoIII was initially described as a 3’-phosphatase (18), although this activity is significantly reduced in hAP1 (15). The Xth family of enzymes thus exhibit a wide range of biochemical activities and functions in living cells, although the exact complement and efficiencies of these reactions differ between specific enzymes (2).

We have previously demonstrated that, of the two Xth family enzymes in Neisseria meningitidis, only NApE possesses AP endonuclease activity (10). Although NExo exhibits a high degree of structural conservation with ExoIII and hAP1, it is the first example of this class of enzymes that is not a functional AP endonuclease. Despite this, both NApE and NExo make significant independent contributions to the ability of the meningococcus to withstand oxidative killing and to successful systemic bloodstream infection (10). The function of NExo is therefore a key unresolved issue in understanding the precise roles of the Xth family enzymes in meningococcal BER, as all its other activities (i.e. processing of 3’-Ald and 3’–5’ exonuclease) are also efficiently performed by NApE. Here, we sought to define the role of these Xth family enzymes, and the mechanisms employed by the meningococcus for processing AP sites and 3’-blocking lesions during BER.

MATERIALS AND METHODS

Construction of Neisserial strains

For the cloning of selected N. meningitidis genome fragments, DNA from strain MC58 (serogroup B) was used as a template in PCRs. Reactions were performed with Pfu polymerase (Promega) and appropriate primers. Neisseria meningitidis VPTL2 (mutY) was kindly provided by Dr Pelicic (Imperial College London). Details of all strains and plasmids are provided in Supplementary Table S1. To delete nth, mutM, mutT, nexo, ung and tag, the following strategy was employed. DNA fragments of between 650 and 1200bp flanking the target gene were amplified by PCR with oligonucleotide primers; (primers detailed in Supplementary Table S2). The products were digested with appropriate restriction enzymes and ligated into pUC19 (19). Finally, antibiotic resistance genes were amplified from pDG1728 (for spc) (20) or pGCC4 (erm) (21) and inserted between the upstream and downstream fragments, generating the series of pDel plasmids (Supplementary Table S1), which were used to knock-out the target BER genes by a double-crossover recombination event. Transformation of N. meningitidis MC58 resulted in replacing the target BER gene by an antibiotic marker due to exchanging of the complementary regions between the pDel plasmids and the chromosome. Transformants were analysed by PCR and western blotting to confirm that allelic replacement had occurred and that the protein was no longer expressed.

Bacterial survival assays under oxidative stress

Strains were grown on plates overnight, harvested into PBS and the bacterial suspensions adjusted to 10⁹ colony-forming units (CFU)/ml. Subsequently, 100 μl of the bacterial suspension was incubated with 0.4 mM H₂O₂ (Sigma) for 30 min at room temperature. The same number of bacteria was incubated in PBS alone under the same conditions. After half an hour, serial dilutions were plated onto BHI medium. The number of CFU were counted the next day and the survival of each strain was expressed as the proportion of surviving bacteria after incubation with the oxidative agent(s) to the number recovered after incubation in PBS only. Assays were performed in triplicate on at least three independent occasions. Statistical significance was determined using a one-tail t-test.

Expression and purification of recombinant proteins

The gene for mutM (NMB1295) was amplified from MC58 using oligonucleotides MutMpET1 and
MutMpET2 (Supplementary Table S1) and the product inserted into pET-21a(+) using the NdeI and HindIII restriction sites in the primers. Nth (NMBO533) was amplified using oligonucleotides Nthl and Nth2 (Supplementary Table S1) then ligated into pProEX-HTb (Invitrogen) using BamHI and HindIII. The terminating codon was added at the end of the nth gene using site-directed mutagenesis and oligonucleotides NthStop1 and NthStop2 (Supplementary Table S1). The gene using site-directed mutagenesis and oligonucleotides pProEX-HTb (Invitrogen) using BamHI and HindIII. (Supplementary Table S1) then ligated into amplified using oligonucleotides Nth1 and Nth2

Substrate 50-PO4 was generated by enzymatic digestion of 50-U; see phosphatase assay for details.

THF is a tetrahydrofuran analogue; * is a methyl phosphonate linkage; U is deoxyuracil.

| Name         | Sequence                                                                 |
|--------------|---------------------------------------------------------------------------|
| Uracil hairpin (U-hp) | 5′-(HEX) GAC TAA IUA TGA TGA CGG TTA CGC AGT CAT TGT TAG TCT*+T*+T-*+3′     |
| AP·G         | 5′-(HEX) GCT ATG GAC TAA (THF) AA TGA TGA CGT G-3′                        |
| C·G          | 5′-(HEX) CGA TAC CGT ATG TT ACT GAC GCA C-5′                               |
| 50-U         | 5′-(HEX) GCT ATG GAC TAA CCA TGA TCG CGT G-3′                             |
| 50-PO4*      | 5′-(HEX) GCT GTA TGA CAT CTA ACT ATU ATC GCG CTC ATC TGC TGC CTC GCC ACC GT-3′ |
| 20-PO4       | 5′-(HEX) GCT GTA TGA CAT CTA ACT (PO4) ATU ATC GCG CTC ATC TGC TGC CTC GCC ACC GT-3′ |
| 20-PO4       | 5′-(HEX) GCT GTA TGA CAT CTA ACT (PO4) ATU ATC GCG CTC ATC TGC TGC CTC GCC ACC GT-3′ |
| 20-OH        | 5′-(HEX) GCT GTA TGA CAT CTA ACT AT-3′                                    |
| ss20-OH      | 5′-(HEX) GCT GTA TGA CAT CTA ACT AT-3′                                    |
| ss20-PO4     | 5′-(HEX) GCT GTA TGA CAT CTA ACT (PO4) AT-3′                              |

THF is a tetrahydrofuran analogue; * is a methyl phosphonate linkage; U is deoxyuracil.

*Substrate 50-PO4 was generated by enzymatic digestion of 50-U; see phosphatase assay for details.
Lyase activity was monitored with native abasic substrates generated by treating uracil containing oligonucleotides U-hp and 50-U with 5nM hUDG (23) for 2h to generate AP-hp and 50-AP respectively. The hairpin oligonucleotide was subsequently purified by gel filtration on a Superdex 75 column and concentrated with an Amicon ultra 3 k filter (Millipore); 50-AP oligonucleotides were used without further purification to avoid degradation. Steady-state reactions were initiated by mixing enzymes with substrates at a minimum ratio of 1:100 in reaction buffer (50 mM Tris pH 7.5, 125 mM NaCl, 1 mM EDTA, 1 mM β-ME and 0.1 mg/ml BSA) at 25°C; substrate concentrations were varied from 5 to 400 nM. Reactions were stopped at set time points in an equal volume of quench solution (0.01% xylene cyanol, 0.01% bromophenol blue, 40 mM EDTA in formamide), and separated by 20% denaturing PAGE. Gels were scanned on a fluorescence scanner FLA-5000 (Fujifilm), and quantified using Phoretix™1D software. Initial velocities were determined by straight line fitting to the linear portion of the data. The initial velocities were corrected for enzyme concentration and the resulting reaction rates used to determine $k_{cat}$ and $K_m$. Fitting was performed using Grafit 6 (Erithacus software).

Phosphatase and exonuclease assays

Double stranded 50-PO$_4$ was generated by treating 2μM of annealed 50-U oligonucleotide with 50 nM hUDG (23) and 25 nM meningococcal MutM for 3h. Digestion was verified by 20% denaturing PAGE and the 50-PO$_4$ substrate was used without further purification to avoid dissociation of the strands. 20-PO$_4$ and 20-OH were generated by annealing ss20-PO$_4$ or ss20-OH with an equimolar amount of complementary DNA. Reactions were initiated by mixing the enzyme with the substrate solution in exonuclease reaction buffer (50 mM Tris pH 7.5, 50 mM NaCl, 10 mM MgCl$_2$, 0.5 mM EDTA, 1 mM DTT and 0.1 mg/ml BSA) at 25°C, and quenched at specified time points as above, heated to 95°C for 5 min, cooled on ice, then separated on 20% denaturing PAGE and analysed as described above.

Nuclease assays with bacterial cell lysates

The bacteria-free extracts were obtained by spinning down 3 ml of O/N liquid culture and re-suspending the pellet in the mixture containing: 0.2 ml of BugBuster lysis buffer (Merck), 0.12 ml lysozyme (20 mg/ml) and protease inhibitor (Roche complete EDTA free) and gently agitating for 30 min at room temp. Once the solution was transparent, 0.8 ml of storage buffer [50 mM Tris pH 7.5, 500 mM NaCl, 1 mM EDTA and 50% (w/v) glycerol] was added and the sample spun down for 1 min (13 000 rpm) to remove the cell debris. Samples were stored at −20°C. The collected supernatant was used for activity assay with 20-PO$_4$ and 20-OH substrates. Reactions were carried out in exonuclease reaction buffer at 25°C, 20 μl of lysate corresponding to 1.5 × 10$^8$ CFU was mixed with 100 nM substrate in a 200 μl reaction for 60 min.

RESULTS

Contribution of BER enzymes to survival of N. meningitidis under oxidative stress

Initially we determined the contribution of all BER enzymes to survival of the meningococcus following exposure to oxidative stress. Strains were constructed with insertion mutations in genes encoding known or predicted BER enzymes, identified through biochemical or bioinformatics analyses (24,25). The level of survival of the mutants against the wild-type strain MC58 was compared after incubation in 0.4 mM H$_2$O$_2$ for 30 min before plating. Mutants deficient in the Nth and MutM enzymes, which are predicted to act against oxidative DNA lesions, do not exhibit any enhanced sensitivity as compared to those deficient in enzymes that act against the non-oxidative lesions 3-methyladenine (Tag) or uracil (Ung). However, it should be noted that oxidative conditions may increase rates of uracil deamination, and human UNG has been shown to have activity against minor oxidative lesions of cytosine (26,27). Data for NApe and NExo have here been measured with new strain deletions (Supplementary Table S1) and confirm our previous observations (10). These data emphasize the contribution of NApe and NExo to resistance against oxidative stress. Loss of NExo has the highest impact on bacterial survival with only 24% of the mutant surviving compared with the wild-type strain under the conditions tested (P < 0.001, Figure 2). This demonstrates that NExo has a critical role in bacterial survival during oxidative lesion repair, and must have a key function(s) within the meningococcus, distinct from the 3’-Ald activity that it shares with NApe (10).

Processing of abasic sites in N. meningitidis

Of the two Xth family members in N. meningitidis, only NApe has AP endonuclease activity (10). This single enzyme specialization is somewhat surprising given the overlapping activities frequently observed in other organisms, such as between ExoIII and EndoIV in E. coli (2). To fully evaluate the pathways for abasic site processing in the bacterium, we therefore investigated the two putative bi-functional DNA glycosylases, MutM and Nth identified in the genome (24). Escherichia coli MutM was initially named Fpg because formamidopyrimidine (FAPY) was its first characterized substrate (28,29). However, as the E. coli and meningococcal enzymes both recognize other DNA lesions including 8-oxoG (25,30), we refer to this enzyme as MutM (31). Nothing is known about the activity of the putative meningococcal Nth (encoded by NMB0533), which shares 72% amino acid identity with the E. coli enzyme that recognizes a broad range of oxidized pyrimidines (32). Potential AP lyase activity in these two enzymes means that they may act directly on abasic sites produced either by the action of mono-functional glycosylases or by direct depurination/ depyrimidination following oxidative damage (33,34). Thus they may provide an alternative pathway for the processing of abasic sites, independent of NApe.
To investigate this possibility, we examined the binding and lyase activities of the Neisserial MutM and Nth enzymes on AP sites and non-specific DNA. The meningococcal genes encoding MutM and Nth were cloned and expressed in E. coli, and the recombinant proteins purified to homogeneity (see ‘Materials and Methods’ section). Binding to AP sites was examined using double-stranded oligonucleotides containing an abasic furanose analogue, which cannot be cleaved by lyase activity (AP·G; Table 1), and a non-specific DNA (C·G; Table 1). The oligonucleotide was labelled at its 5'-end with HEX and binding monitored by fluorescence anisotropy and intensity. With MutM, there was no clear discrimination between the abasic DNA and a non-specific DNA. However, with Nth, a significant difference was observed with good binding to abasic DNA and only a relatively weak affinity to the non-specific oligonucleotide (Figure 3). Further to this, we also determined that there was no preference for binding to the abasic site, for either enzyme, when it was paired with either a G or an A (Supplementary Figure S1).

The lyase activity of Nth and MutM with a natural AP site was also examined and differences in gel migration clearly shows that the two enzymes yield products with different chemistry. Comparison with previous characterization of elimination products (33) demonstrates that Nth proceeds via β-elimination, generating a 3'-Ald, while MutM proceeds via β-δ elimination to yield a 3'-PO4 (Figure 4A). Examination of the reaction profiles shows that the initial rates for both enzymes are very similar (Figure 4B and C). A steady state analysis of the enzymes demonstrated that they both had similar $k_{cat}$ and $K_m$ values (Figure 4D) but the reaction with MutM clearly does not proceed to completion and accumulation of product wanes as the reaction proceeds (Figure 4C and D). This can be explained by product inhibition; as the product accumulates it remains bound by the enzyme, reducing the availability of the enzyme to process substrates. Since steady-state rates are determined by the initial reaction velocity, product inhibition does not affect the determination of the $k_{cat}$ and $K_m$ of MutM.

3'-phosphatase and exonuclease activities of Xth family enzymes

Nicks and gaps in DNA that do not have 3'-OH groups are potentially highly cytotoxic to the meningococcus.
since they cannot be repaired by DNA polymerase and ligase. They thus present blocking lesions that hinder further downstream processing. We have previously demonstrated that both NApe and NExo can very efficiently remove 3'-Ald lesions (10). However, examination of the bi-functional DNA glycosylases present in N. meningitidis reveals that both 3'-PO4 groups as well as 3'-Ald lesions are produced (Figure 4A). Since no enzyme has been identified that is responsible for the removal of 3'-PO4 blocking lesions in the meningococcus, we investigated both NApe and NExo in relation to this activity. In addition, we performed the same assays on the well characterized ExoIII and HAP1 enzymes to provide a comparison of activities across the Xth family of enzymes.

Enzymes were incubated with a double stranded 50 bp oligonucleotide (50-PO4; Table 1), which contained a 3'-PO4 at an internal gap site generated by enzymatically by digestion of a uracil containing oligonucleotide with Ung and MutM. Oligonucleotides with a 3'-PO4 migrate faster on PAGE than oligonucleotides of the same length with a 3'-OH, enabling monitoring of 3'-phosphatase activity (Figure 5A). Reaction aliquots were quenched at specific time points and resolved by denaturing PAGE; this was repeated with different concentrations of enzyme to observe the dynamic range of activities (Figure 5A–C). Even at the lowest enzyme concentration, it can be seen that NExo is the most efficient enzyme at removing the 3'-PO4 to yield the 20-OH product, and is more active than ExoIII, for which this activity is well established (18,36). In comparison, neither NApe nor hAP1 show any 3'-phosphatase activity at this level of enzyme. As the concentration of enzyme was increased to 10 nM, the exonuclease activity of NExo and ExoIII can be seen to further degrade the DNA, and this occurs fastest for ExoIII. Even at the highest concentration of enzyme, the 3'-PO4 is completely refractory to cleavage by NApe, although a small amount of 3'-phosphatase activity can be observed by hAP1 (Figure 5C).
Analysis of the 3′–5′ exonuclease activities of the enzymes reveals that ExoIII has the highest cleavage activity, NApe and NExo possess comparable activity, while hAP1 is clearly the slowest (Figure 5D).

Moreover, this demonstrates that the inability of NApe and hAP1 to cleave the 3′-PO_4 substrate is unrelated to their exonuclease activities and highlights the blocking capacity of the 3′-PO_4 lesion, since it cannot be

**Figure 5.** Comparative activity of Xth family endonucleases. Comparison of the 3′-phosphatase and 3′–5′ exonuclease activities of NExo, NApe, *E. coli* ExoIII and human hAP1. The substrates 50-PO_4 (100 nM; A–C) and 20-OH (200 nM; D) were incubated with different concentrations of enzyme as indicated under standard phosphatase assay conditions. Samples were taken at the time points indicated and the reactions quenched before separation by 20% denaturing PAGE.
NExo is used as the substrate to measure removal of a 3'-blocking PO4 from an internal AP site. NExo (1 nM) was incubated with 50-PO4 (50 nM) under standard phosphatase assay conditions and aliquots removed at the indicated time points, quenched and resolved by denaturing 20% PAGE. (B) A number of reactions were performed with varying substrate concentrations using substrate 20-PO4 (Table 1); quantified data are shown with the best fit to the Michaelis–Menten equation with $k_{cat} = 0.49 \pm 0.02/\text{s}$ and $K_m = 28 \pm 6\text{nM}$.

bypassed through the exonuclease activity of the Xth enzymes.

**Processing of 3'-PO4 lesions by NExo**

The above data indicated that NExo is able to process 3'-PO4 lesions. We therefore characterized the kinetics of NExo against this specific lesion. To avoid enzyme pre-treatment synthetic 20-PO4 substrate (Table 1) was used for Michaelis–Menten kinetics (both 20-PO4 and 50-PO4 substrates were processed with same rate; data not shown). It was notable that NExo exhibited highly efficient 3'-phosphatase activity, with a $k_{cat}$ of 0.49/s (±0.02) and $K_m$ of 28 nM (±6) (Figure 6B). Therefore, NExo is a highly efficient phosphatase that recognizes and removes blocking 3'-PO4 lesion at internal gap sites and recessed 3'-termini.

**NExo is the only 3'-phosphatase in N. meningitidis**

Examination of the genome of MC58 did not reveal any other enzyme homologies, such as poly-nucleotide kinase (PNK), that may be associated with 3'-phosphatase activities. To determine whether NExo is the only enzyme with 3'-phosphatase activity, we assayed cell extracts from MC58, MC58Δnexo, MC58Δnape and MC58Δnexo/Δnape with the 20-PO4 substrate (Figure 7). Extracts from the wild type meningococcus can clearly be seen to remove the 3'-phosphate with similar efficiency to 1 nM purified NExo, followed by rapid degradation through exonuclease activity. The NExo containing mutant extract (MC58Δnape) matched the activity of the purified NExo enzyme with efficient 3'-phosphate processing, but reduced exonuclease activity. No 3'-phosphatase activity was observed with the MC58Δnexo or MC58Δnexo/Δnape double mutant. Analysis of the 3'-5' exonuclease activity with 20-OH substrate demonstrated that NApE and NExo account for all of the observed exonuclease activity in the cell extract. While both enzymes contribute to this, it is evident that NApE is responsible for the majority of exonuclease activity (Figure 7).

**A key amino acid in NExo dictates its specificity**

We have previously demonstrated that the activity of NExo is modulated by a His residue, which is a Ser or Gly in active AP endonucleases such as ExoII and hAP1, respectively (10). This amino acid resides in the position that forms an abasic ribose binding pocket in the homologous human enzyme, hAP1 (10). The His residue thus sterically blocks recognition of the abasic site and modification to either Ser or Gly confers AP endonuclease activity to NExo (10). We therefore investigated whether this residue has an impact on 3'-PO4 lesion processing by NExo. NExo His167Ser and His167Gly were both incubated with 20-PO4 DNA containing a 3'-PO4 lesion. Processing of the lesion was still observed with both mutants (Figure 8A–C). However, when the rate of 3'-phosphatase activity was determined it could be seen that both NExo His167Ser and His167Gly had ~10-fold lower activity than wild type (Figure 8C). Therefore, His167 is a critical residue for efficient 3'-PO4 lesion processing by NExo.

**DISCUSSION**

The Neisserial exonuclease, NExo, is critical to the survival of the meningococcus under conditions of oxidative stress, since its removal has the most marked impact...
The presence of only a single AP endonuclease, NApe, within the bacterium is unusual when compared to the *E. coli* and *S. cerevisiae* paradigms, which both have functional ExoIII and Nfo enzymes, both of which possess AP endonuclease activity (2). We determined that AP site processing can also be performed by the bi-functional DNA glycosylases Nth and MutM (Figure 3). Further to this, we characterized the 3'-lesions generated by Nth and MutM, which must be processed by the next stage of the BER pathway. Nth proceeds via β-elimination to produce a 3'-Ald, while MutM processes the lesion further, consistent with β-δ elimination to produce a 3'-PO₄ (Figure 4A). These chemistries are consistent with the reactions observed for this class of enzymes from other organisms (33,34,37,38).

It is therefore evident that both 3'-Ald and 3'-PO₄ lesions can arise either by the glycosylases-lyase action of Nth and MutM, or indeed by direct incision at abasic sites by these enzymes. Additionally, these lesions can form slowly by spontaneous β and subsequently δ-elimination or through hydrolysis of relatively unstable abasic sites that occur by spontaneous depurination and/or depyrimidination (39). Since both lesions present a block to repair by DNA polymerase, it is important to consider how they can be processed in the meningococcus.

We have here demonstrated that only NExo is able to process 3'-PO₄ lesions, and that this activity for NExo is more efficient than that of the well characterized *E. coli* ExoIII enzyme (Figure 5). In contrast, NApe is completely unable to cleave 3'-PO₄ lesions, a unique observation for this class of enzyme (Figure 5). When taken together with our previous observation that NApe is a highly efficient AP endonuclease, whereas NExo is completely resistant to processing of AP sites (10), we see extreme specializations for this family of enzymes within the meningococcus. Their activities are thus complementary and the balance of Xth family enzymes present in *N. meningitidis* represents a highly specialized solution to the processing of intermediates in BER.

A subpathway of BER in humans has previously been proposed that is independent of hAP1: the Nei-like enzymes, NEIL1 and 2 are bi-functional DNA glycosylases that incise the DNA backbone leaving 3'-PO₄ termini, which may then be processed by the 3'-phosphatase activity of polynucleotide kinase (PNK) (17). It has also been demonstrated that PNK is essential for rapid repair of single strand breaks under oxidative stress (40). *Escherichia coli* ExoIII can efficiently process all 3'-lesions (Figure 5), but the very slow 3'-PO₄ processing observed with hAP1 (Figure 5) supports the proposal that hAP1 is not sufficiently active against 3'-phosphates to support this activity *in vivo* (17). The 3'-phosphatase activity of NExo is of particular relevance to *N. meningitidis*, since assays with cell extracts have confirmed that it is the only 3'-phosphatase activity in the meningococcus (Figure 7). Our data indicate that NExo serves an analogous role to PNK: steady-state reactions demonstrate that the kinetic constants for removal of 3'-PO₄ by NExo ($K_m = 28\text{ nM}$ and $k_{cat} = 0.49/s$; Figure 6) are remarkably similar to values obtained for PNK ($K_m = 16\text{ nM}$ and $k_{cat} = 0.46/s$) (17). Therefore,

Figure 8. His167 is a key amino acid determining NExo activity. (A–C) Comparison of the phosphatase activity of NExo, NExo His167Gly and NExo His167Ser were performed by incubating 3nM enzyme with 150 nM substrate 20-PO₄ under phosphatase assay conditions. Aliquots were removed at specified times, quenched and separated by 20% denaturing PAGE; representative lanes are shown for clarity. (D) Analysis of the full reaction profile is shown with fits to straight line plots to determine the initial velocity for NExo (solid circles, solid line; $k = 0.15/s$), NExo His167Gly (open triangles, dashed line; $k = 0.022/s$) and NExo His167Ser (closed triangles, dotted line; $k = 0.023/s$).

on bacterial survival compared with any other single component of the bacterium’s BER system (Figure 1). Our previous studies have also highlighted the additive effect of deleting both NApe and NExo, indicating specialist roles for the enzymes (10). However, it was not evident what the distinct functions of these enzymes were within the bacterium. This is a key consideration since the Xth family of enzymes operate at a critical node in BER in the processing of AP sites and 3'-lesions (2,9,17), and we therefore investigated these activities within *N. meningitidis*. 
NExo and PNK are members of unrelated protein families, yet strikingly share almost identical activity against 3'-PO₄ lesions.

The importance of the 3'-phosphatase activity of NExo can be seen from the observed sensitivity of the MC58Δnexo mutant under oxidative stress as compared to all other strains lacking single BER enzymes (Figure 1). 3'-PO₄ lesions are a blocking lesion that cannot be extended by DNA polymerase and since data from cell extracts demonstrate that NExo is the only enzyme in the meningococcus capable of processing this lesion, we conclude that 3'-phosphates are highly toxic to the cell and the loss of this activity largely accounts for the sensitivity of MC58Δnexo to oxidative stress. It is also possible that NExo processes other lesions generated by direct strand cleavage from oxidative damage, which can also lead to 3'-blocking lesions such as 3'-glycollate (2) and which may contribute to the phenotypic sensitivity of MC58Δnexo.

The effect of this can be fully appreciated in the meningococcus, which not only has enzymes with a high degree of specialization, but also lacks an inducible SOS response that may otherwise mask the phenotype (24).

The data that we have presented here demonstrate that N. meningitidis possesses two Xth family enzymes that have evolved highly specific activities. The evolution of NExo to become a specialist 3'-end processing enzyme is evident from the critical His¹⁶⁷ residue. We have previously shown that His¹⁶⁷Ser and His¹⁶⁷Gly modifications restore a significant degree of AP endonuclease activity to NExo (10). Here, we have shown that the same mutations reduce the efficiency of the 3'-phosphatase activity, demonstrating the evolutionary trade-off in activities that centres on this key amino acid (Figure 8A). Although the structure of NExo provides insights into the mechanism by which His¹⁶⁷ impairs AP endonuclease activity (10), it is not evident how the same residue contributes to its phosphatase activity. It is also notable that a mutation in the active site of HAP1 can increase its 3'-phosphatase and 3'-phosphodiesterase activities at the expense of AP site cleavage (41). There is thus a plasticity in this class of enzymes for the spectrum of activities that they possess, and in the case of both the Neisserial NExo and human HAP1 enzymes, broadening the range of activity reduces the efficiency of the specialized functions. This therefore points to the efficient 3'-phosphatase activity being a desirable evolutionary trait in this human pathogen, which has to survive persistent oxidative stress by the innate immune system during infection.

In conclusion, we have here defined the distinct roles for the two Xth family enzymes present in N. meningitidis: NExo is a specialized 3'-end processing enzyme, with sole activity against 3'-PO₄ lesions, while NApe is a specialized AP endonuclease. Although Nth and MutM may provide alternative pathways for strand incision, no such functional redundancy exists for 3'-PO₄ processing. These data demonstrate the importance of NExo in this pathogen, since it is required for efficient defence against oxidative stress, one of the primary responses of the host innate immune system to infection. This high degree of specialization between a pairing of Xth enzymes within an organism has not previously been observed and it is more frequent to have functional redundancy at this critical node in BER, even between enzymes of a different family, such as ExoIII and EndoIV in E. coli (2). Our previous bioinformatic study (10) suggests that this specialist enzyme pairing is widely dispersed within the prokaryotic world and it may present a new paradigm for separation of these two critical functions at the enzyme level.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online: Supplementary Tables 1–4 and Supplementary Figure 1.

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