BAX1 IS A NOVEL ENDONUCLEASE: IMPLICATIONS FOR ARCHAEOAL NUCLEOTIDE EXCISION REPAIR

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The helicases XPB and XPD are part of the TFIIH complex, which mediates transcription initiation as well as eukaryotic nucleotide excision repair (NER). Although there is no TFIIH complex present in archaea, most species contain both XPB and XPD, and serve as a model for their eukaryotic homologs. Recently, a novel binding partner for XPB, Bax1 (binds archaal XPB), was identified in archaea. To gain insights into its role in NER, Bax1 from Thermoplasma acidophilum was characterized. We identified Bax1 as a novel Mg2+-dependent structure-specific endonuclease recognizing DNA containing a 3’ overhang. Incision assays conducted with DNA substrates providing different lengths of the 3’ overhang indicate that Bax1 specifically incises DNA in the single-stranded region of the 3’ overhang 4 to 6 nucleotides to the ssDNA/dsDNA junction and thus is a structure-specific and not a sequence-specific endonuclease. In contrast, no incision was detected in the presence of a 5’ overhang, dsDNA or DNA containing few unpaired nucleotides forming a bubble. Several Bax1 variants were generated based on multiple sequence alignments and examined with respect to their ability to perform the incision reaction. Residues E124, D132, Y152 and E155 show a dramatic reduction in incision activity indicating a pivotal role in catalysis. Interestingly, Bax1 does not exhibit any incision activity in the presence of XPB, thus suggesting a role in NER in which the endonuclease activity is tightly regulated until the damage has been recognized and verified prior to the incision event.

Nucleotide Excision Repair (NER) is a DNA repair mechanism that is responsible for the removal of a vast diversity of bulky DNA damages (1). Failures in the NER pathway can ultimately lead to cancer as observed in the skin cancer prone disease xeroderma pigmentosum (XP). In addition, since several chemotherapeutic agents are recognized and repaired by NER, the underlying mechanism of damage recognition and repair is an important issue in cancer treatment (2).

The helicases XPB and XPD are part of the TFIIH complex, which mediates eukaryotic NER as well as transcription initiation. Mutations in either protein lead to severe diseases like XP, Cockayne syndrome (CS) or trichothiodystrophy (TTD). Although there is no TFIIH complex present in archaea, most species contain both XPB and XPD, and serve as a model for their eukaryotic homologs (3). It is important to note that the structure from T. acidophilum XPD, which was recently solved, could explain the effect of several point mutations leading to the phenotype of patients suffering from XP, TTD or XP/CS (4-6).

In contrast to the active helicase XPD, XPB exerts only limited helicase activity (7,8). However, its ATPase activity was shown to be crucial for TFIIH to unwind DNA (8). Thus it is assumed, that XPD, the major helicase in TFIIH, is responsible for further strand opening during the process of NER. Taken together, these data suggest that XPB is
recruited to the DNA lesion to locally unwind the DNA and thereby stimulate XPD. XPD further opens the DNA enabling other proteins of the TFIIH complex to bind and proceed with the removal of damaged DNA (9).

Very recently, a novel binding partner for XPB was identified, which was named Bax1 due to its interaction with XPB (binds archael XPB) (7). Richards et al. proposed a physical and/or functional interaction of XPB and Bax1 because of the close association in gene organization. Although they could verify a physical interaction by using analytical gel filtration and affinity chromatography, the interaction of XPB and Bax1 did not affect XPBs activity in ATPase, DNA binding or helicase assays (7). Thus the role of Bax1 with respect to the NER pathway remained unclear. Database analysis, however, predicted Bax1 to possess a restriction endonuclease-like DUF790 domain (7,10).

We sought to identify a potential endonucleolytic role of Bax1 in NER. We showed that complex formation of Bax1 and XPB does not only occur between S. solfataricus/acidolaricus proteins (7) but also between T. acidophilum proteins. Moreover, we discovered that Bax1 has a robust endonuclease activity in vitro and we identified suitable substrates for its incision activity. Bax1 incises DNA substrates containing a 3’ overhang, whereas no incision was observed when double-stranded DNA (dsDNA) or DNA substrates including a 5’ overhang were used. Incision by Bax1 is strictly limited to the scissile single-stranded DNA (ssDNA) of the 3’ overhang, and Bax1 cuts at a defined distance (4 to 6 nucleotides (nts)) from the dsDNA/ ssDNA junction. These findings suggest that Bax1 is an endonuclease which is specific to sequences with 3’ overhangs. Analysis of twelve Bax1 mutants reveals three different levels of incision activity indicating different roles for the mutated amino acids. Some of the point mutants do not impair incision activity compared to wildtype (WT) Bax1, whereas other mutants seem to affect either DNA binding or catalysis.

**EXPERIMENTAL PROCEDURES**

**Cloning** - The bax1 gene (Ta1017) was amplified by PCR from T. acidophilum genomic DNA (11). For expression in E. coli the PCR product was cloned into the pETM-11 vector (EMBL, Hamburg) using the restriction sites NcoI and XhoI, thus providing an N-terminal TEV-cleavable hexahistidine tag.

**Site directed Mutagenesis** - Bax1 mutants were generated using a modified version of the QuickChange™ Site-Directed Mutagenesis protocol (Stratagene) (12). A single-primer extension reaction was performed for eight cycles to avoid dimerization of the two complementary mutagenesis primers. 20 additional cycles were carried out after combining the two corresponding single-primer reactions. The PCR product was digested with DpnI for 1 h at 37 °C and chemically transformed into DH5α competent cells. All mutations were confirmed by resequencing the entire gene.

**Protein expression and purification** - Bax1 WT (46 kDa) as well as Bax1 mutant proteins were expressed in BL21-CodonPlus® (DE3)-RIL cells (Stratagene) using the auto-inducing medium ZYM-5052 (13) supplemented with 34 µg/ml chloramphenicol and 50 µg/ml kanamycin. The cells were grown at 37 °C and 200 rpm to an OD_{600} of 0.4. Protein expression was accomplished at 15 °C over night. Cells were harvested by centrifugation and pellets were stored at -20 °C.

Cells were resuspended in 50 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, pH 7.5, and 1 µl DNAsel and lysed using a cell disruptor system (Constant Systems). The lysate was clarified from insoluble fractions by centrifugation at 75,000 g and 4 °C for 30 min. The supernatant was subsequently loaded onto a 1 ml Ni-MAC® cartridge (Merck) pre-equilibrated with lysis buffer. Protein was eluted applying a linear gradient to 500 mM imidazole in lysis buffer and collected in 1 ml fractions. Fractions
containing pure protein were identified by SDS-PAGE (14), pooled and further purified by size exclusion chromatography employing a HiLoad™ 26/60 Superdex™ 200 column (or alternatively HiLoad™ 16/60 Superdex™ 200, GE Healthcare). Isocratic elution of the protein was carried out in 20 mM Tris-HCl, 500 mM NaCl, pH 7.5. Fractions containing pure protein were identified by SDS-PAGE (14), pooled and concentrated to 50 mg/ml. XPB (52 kDa) was expressed as described for Bax1 and co-purified with Bax1. Protein concentrations were determined spectrophotometrically employing molar extinction coefficients of ε280 = 49,900 M⁻¹ cm⁻¹ and ε280 = 58,800 M⁻¹ cm⁻¹ for Bax1 and XPB, respectively.

**Analytical size exclusion chromatography and analytical ultracentrifugation** - To investigate protein-protein interactions analytical size exclusion chromatography was performed using a Superdex™ 200 10/300 GL column (GE Healthcare). Protein was eluted isocratically with 20 mM Tris-HCl, 500 mM NaCl, pH 7.5 and collected in 0.5 ml fractions which were subsequently analyzed by SDS-PAGE (14). Purified Bax1 (46 kDa) and the XPB/Bax1 complex (98 kDa) were subjected to sedimentation velocity analytical ultracentrifugation. Sedimentation velocity (SV) experiments were conducted in a Beckman Optima XL-I analytical ultracentrifuge (Beckman Coulter) using an eight-hole An-50 Ti rotor at 40,000 rpm and 20 °C, with 400 µl samples in standard double-sector charcoal-filled Epon centerpieces equipped with sapphire windows. Data were collected in continuous mode at a step-size off 0.003 cm without averaging using absorption optical detection at a wavelength of 280 nm. Data were analyzed using the software SEDFIT to determine continuous distributions for solutions to the Lamm equation c(s), as previously described (15). Analysis was performed with regularization at confidence levels of 0.68 and floating frictional ratio (FLF), time-independent noise, baseline, and meniscus position to RMSD values of < 0.007.

**Incision assays** - Incision assays were performed through incubation of protein and DNA substrates for 30 min at 45 °C in a total volume of 10 µl containing 20 mM MES, pH 6.5, 150 mM NaCl, 10 mM DTT, 1 mg/ml BSA and 10 mM MgCl2. 2x urea sample buffer (Invitrogen) was added and samples were heated to 90 °C for 10 min prior to electrophoresis at 300 V on a 15 % urea polyacrylamide gel. Gels were exposed to a phosphorimager screen over night, visualized by a Personal Molecular Imager (PMI) System (BioRad) and quantified employing the software Quantity One (BioRad).

**RESULTS**

**T. acidophilum Bax1 binds to XPB**

Bax1 has been identified as a novel binding partner of the XPB protein in *S. solfataricus* (7). Several archaeal genomes, among them *T. acidophilum*, comprise the genes coding for XPB and Bax1 in close proximity. To verify complex formation of the two *T. acidophilum* proteins, Bax1 was subjected to both analytical ultracentrifugation and analytical size exclusion chromatography first.
individually and subsequently together with XPB (Figure 1). Peak fractions from analytical size exclusion chromatography were analyzed by SDS-PAGE to confirm their protein content. The peak including both proteins is clearly shifted compared to the peak containing Bax1 only, indicating stable complex formation in solution (105 kDa). The elution volume of the complex peak suggests a 1:1 stoichiometry of XPB and Bax1. The sedimentation curves derived from analytical ultracentrifugation support this finding. Bax1 displays a monomer-dimer equilibrium with sedimentation coefficients of approximately 3 S for the monomer and 5 S for the Bax1 dimer. The purified XPB/Bax1 complex shows a sedimentation coefficient of 5 S. Optimized fits to the data were obtained with frictional ratios of 1.65 and 1.35 for Bax1 and the XPB/Bax1 complex, respectively. The different frictional ratios indicate different geometries for the particles in the two samples, while the similar sedimentation coefficients confirm similar sizes for the Bax1 dimer and the XPB/Bax1 complex, consistent with a heterodimeric XPB/Bax1 complex and hence a 1:1 stoichiometry.

**Bax1 is a novel structure specific endonuclease**

Despite the fact that Bax1 binds to XPB, Bax1 had no known function to date. Database research, however, revealed that Bax1 encloses a DUF790 domain which includes a restriction endonuclease-like fold (10). Therefore, incision assays were conducted and a specific incision product was formed in a concentration dependent manner (Figure 2A). Moreover, Bax1 only incises DNA substrates comprising a free 3’ overhang, whereas it does not cut dsDNA, DNA substrates forming a bubble with a maximum of 16 unpaired bases or DNA substrates including a 5’ overhang (Supplemental Data, Figure S1). Incision assays employing different DNA substrates with respect to the length of the 3’ overhang indicate that Bax1 cuts 4 to 6 nts to the ssDNA/dsDNA junction in the scissile 3’ overhang. This finding indicates that Bax1 is a structure-specific endonuclease (Figure 2B/C).

To verify that the observed incision activity is not due to contamination but is initiated by Bax1, the protein was purified as described above including size exclusion chromatography as a final purification step. The peak fractions were analyzed by SDS-PAGE with respect to their protein content and were then subjected to incision assays (Figure 3). Comparing the resulting incision product to the employed amount of Bax1, a strong correlation can be observed confirming that the endonuclease activity is specific to Bax1.

Moreover, incision assays were performed in the presence of XPB. Surprisingly they reveal that XPB inhibits the endonuclease activity of Bax1 as the XPB/Bax1 complex is not able to form an incision product at comparable concentrations (Figure 4). To further confirm that the nuclease activity is intrinsic to Bax1 we created a series of site-directed mutants, described below.

**Bax1 mutants show impaired nuclease activity revealing essential residues for DNA incision**

Bax1 mutants were generated to further scrutinize its endonuclease activity. Since there is no structural information available for Bax1, multiple sequence alignments of homologous proteins from diverse archaeal organisms were performed and revealed a patch of highly conserved residues (Figure 5A). Moreover, similarities with the restriction endonuclease *Tsp*RI were identified in a BLAST search (Figure 5B). Although the overall sequence identity of Bax1 and *Tsp*RI is only 14 %, a small patch of 14 amino acids shares 60 % identical residues. Interestingly, the two patches identified by multiple sequence alignment and BLAST search overlap, indicating a crucial role for the conserved sites with regard to DNA binding or catalysis. To confirm the importance of individual residues, site directed mutagenesis was performed. After successful expression and purification, the mutants were first analyzed by CD spectroscopy. The CD spectra are almost identical for Bax1 WT and all Bax1
mutant proteins, indicating that the point mutations do not affect the overall structure of the protein (Supplemental Data, Figure S2).

Quantification of the incision assays revealed three different groups of mutants (Figure 5C): The first group is only slightly impaired in the incision assays as it exerts more than 70% of WT Bax1 activity, i.e. F116A, E125A and N153A. The second group shows impaired incision activity in a range of 25 to 35% of WT activity, i.e. E124A/E125A, Y128A, D130A, E134A and L137E. Mutants of the third group display a dramatically reduced activity of less than 20% of the WT activity, i.e. E124A, D132A, Y152A and N153A/E155A. These different levels of incision activity strongly indicate varying roles for the mutated amino acids either in DNA binding or catalysis.

Mg$^{2+}$ is crucial for Bax1 incision activity

Several endonucleases require divalent cations. The prokaryotic endonuclease involved in NER UvrC, requires one divalent cation for its N-terminal active site and most likely two divalent cations for the C-terminal endonuclease site (16,17). To investigate whether divalent cations are also essential for the incision activity of Bax1, assays were conducted in the presence of different divalent cations: Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$ or the chelating reagent EDTA (Figure 6). A specific incision product can only be observed in the presence of Mg$^{2+}$, whereas an unspecified smear can be detected when Mn$^{2+}$ is added. Both Ca$^{2+}$ and EDTA completely abolish Bax1 incision activity.

**DISCUSSION**

Here, we present the first characterization of an endonuclease predicted to possess a DUF790 domain. The DUF790 family, which comprises hypothetical proteins from archaea and cyanobacteria, was identified using a combination of diverse bioinformatic tools since the family members share very low sequence similarity with previously characterized nucleases (10). Thus, no similarity to other single-strand-specific nucleases (18) or structure-specific nucleases (19) was observed although the activity of Bax1 indicates a functional relationship.

We identified Bax1 as an active Mg$^{2+}$-dependent endonuclease which acts preferentially as a monomer (Supplemental Data, Figure S3). Our incision studies provide initial insights to a possible mechanism how Bax1 incises DNA and how it may play an important role in NER. Bax1 is a structure specific endonuclease cutting DNA in the single-stranded region 4 to 6 nts next to the dsDNA/ssDNA junction. Other substrates such as dsDNA, a 5’ overhang or a substrate containing mismatches are not recognized. The endonuclease activity of Bax1 requires at least one divalent cation, preferably Mg$^{2+}$. Furthermore, we identified individual amino acids within Bax1 that are essential for catalysis and or DNA binding. Finally we have shown that this endonuclease activity is suppressed in the presence of XPB.

We verified that T. acidophilum Bax1 physically interacts with the superfami-ly 2 (SF2) helicase XPB in the absence of DNA as demonstrated by analytical gel filtration and analytical ultracentrifugation (Figure 1). XPB is able to unwind dsDNA along the 3’-5’ direction (20) upon detection of a helical distortion in DNA caused by bulky DNA lesions (21). Whether XPB or the 5’-3’ helicase XPD performs the DNA damage verification step has not been resolved (8,21). However, after the presence of an NER substrate has been confirmed, the damage has to be removed by a structure-specific endonuclease, which specifically recognizes the 3’ overhang produced by XPB’s helicase and XPG’s endonuclease activity. In this study, Bax1 was shown to be a structure-specific endonuclease, incising DNA at the scissile 3’ overhang.

Due to their close genomic proximity it is likely that XPB and Bax1 are transcribed and translated concomitantly (7). We propose that XPB and Bax1 form a complex which subsequently binds to a DNA substrate. XPB then locally unwinds the DNA at the site of the lesion, thus providing a 3’ overhang as a substrate for Bax1. As the XPB/Bax1 complex
does not seem to exhibit any nuclease activity on its substrate (Figure 4), we suggest that XPB hands over the DNA to Bax1 and either dissociates or adopts a different conformation to enable Bax1 to incise the DNA. This proposed mechanism would ensure that the sequential process of damage recognition, followed by verification, is completed prior to incision. In this way, Bax1 would form only a short-term pre-incision complex and subsequently cleave the phosphodiester bond. Therefore, XPB and Bax1 would not only interact physically but also functionally associating helicase and nuclease activity.

The archaeal homologs of XPG and XPF are structure-specific endonucleases in the NER pathway (22-24). In contrast to Bax1, which cuts DNA in the single-stranded region, both XPG and XPF incise DNA in the double-stranded region of a DNA substrate close to the dsDNA/ssDNA junction. XPG has been shown to incise 3' to a damaged site and remove a 5' overhang (25), whereas XPF/ERCC1 makes the 5' incision and also cleaves 3' overhangs (22). The presence of two endonucleases of opposite polarity ensure the complete removal of the damaged oligonucleotide (26). Interestingly, *T. acidophilum* seems to lack an XPF homolog in contrast to many other archaeal organisms (3). Moreover, genome analysis of *T. acidophilum* did not uncover an additional NER mechanism, for example the UvrABC system, which is present not only in prokaryotes, but also in several archaea (27). However, database research revealed that *T. acidophilum* contains a Hef protein. Hef consists of two domains, an N-terminal helicase domain and a C-terminal nuclease domain, which exhibits high similarity to the XPF/Rad1/Mus81 nuclease family (28) with respect to their sequences and to their incision properties. Although Hef was suggested to act preferentially on stalled replication forks, both XPF and Hef are structure-specific endonucleases cleaving DNA in the double-stranded region 5' to a branched DNA structure (29). Thus, it could be either Hef or Bax1 which replace the missing XPF protein in *T. acidophilum* as both endonucleases are able to remove 3' overhangs and thus could mediate the 5' incision.

However, in consideration of several other archaea, the question arises why in addition to XPF another endonuclease seems to be involved in archaeal NER as it is associated to the helicase XPB. This situation resembles the prokaryotic NER system in *Escherichia coli*, which contains the UvrC homolog Cho in addition to UvrC performing both 3' and 5' incisions (30). Cho conducts the 3' incision at DNA lesions which are poorly incised by UvrC and thus leads to a broader substrate range for NER (30). A similar mechanism could have evolved also in archaea employing Bax1 as a complementary endonuclease.

In stark contrast to the structure-specific endonucleases, restriction endonucleases are known to be sequence-specific. Nevertheless, a BLAST search revealed a small patch of 14 amino acids in Bax1, which shares 60% sequence identity between the type II restriction enzyme *TspRI* (31) and Bax1. The residues within this patch presumably are part of the active site where one or two divalent cations are coordinated to catalyze the cleavage of a phosphodiester bond. This finding together with a multiple sequence alignment of Bax1 homologs from other archaea uncovers conserved residues which might play an essential role in DNA binding as well as in catalysis. To verify the importance of a residue, point mutants were generated and analyzed in incision assays. A reduced incision activity compared to WT Bax1 indicates that these residues are crucial for Bax1 to act effectively on its DNA substrate either resulting from a decreased DNA binding affinity, from the inability to coordinate Mg$^{2+}$ or from a lower capability to stabilize reaction intermediates. So far, stable complex formation of Bax1 and DNA could not be detected. Therefore it was not possible to further distinguish between residues involved in DNA binding and residues involved in catalysis. The nature of the mutated amino acids and comparisons to the active sites of other endonucleases, however,
sheds light onto their possible role in the incision reaction.

The crystal structure of UvrC’s C-terminal domain from *Thermotoga maritima*, visualizes how a metal ion is coordinated by the catalytic triad, consisting of residues D367, D429 and H488, either directly or indirectly through water molecules (16). Similar catalytic triads, i.e. DDH, DDD and DDE, are present in *Pyrococcus furiosus* Argonaute (32), *Bacillus halodurans* RNase H (33) and *E. coli* Tn5 transposase (34), respectively. The latter two were shown to bind two divalent cations in their active site. The first metal ion facilitates the formation of a hydroxide ion which in turn performs a nucleophilic attack of the scissile phosphate. The second metal ion fulfills the role of a Lewis acid stabilizing the reaction intermediate via interaction with the 3’ oxygen (16,35). UvrC and Argonaute most likely also conduct a two-metal-ion mechanism as suggested by the strong homology in the active site (16,32,35). Analyzing the crystal structure of UvrC’s N-terminal domain reveals how the residues Y29, E76 and N88 coordinate one Mg2+ ion (17). In this case, the Mg2+ ion acts as a Lewis acid, and it was speculated that Y29 facilitates the formation of a nucleophilic hydroxide catalyzing the cleavage of the phosphodiester bond.

In Bax1, residues E124, D132, Y152 and E155, which are highly conserved among other archaea, provide the functional groups required for binding metal ions. In addition, we could demonstrate that mutating these amino acids to alanine strongly reduces incision efficiency. Therefore, we hypothesize that the above mentioned residues coordinate either one or two Mg2+ ions thus catalyzing the incision reaction. Interestingly, Bax1 from *S. solfataricus* lacks the residue which corresponds to E124 in *T. acidophilum* Bax1 (Figure 5A). To ensure a functional *S. solfataricus* Bax1, another acidic residue would have to take over the role of E124 in *T. acidophilum* Bax1. Assuming that Bax1 plays a similar role in archaea as Cho does in prokaryotes, *S. solfataricus* would still be vital even with an inactive form of Bax1, but would have a more restricted substrate range compared to *T. acidophilum*.

Residues Y128, D130, E134 are also suitable for binding metal ions regarding their functional groups, but mutation to alanine has a milder effect on their incision activity. Thus we conclude that amino acids Y128, D130, E134 support coordination of the Mg2+ ion or play an important role with respect to the formation of a stable active site but are not essential for catalysis. H488 in the C-terminal domain of UvrC seems to fulfill a similar role (16).

In conclusion, this study suggests an alternative mechanism for NER in some archaeal species in which Bax1 is loaded at the site of a damaged nucleotide by XPB and then functions to produce the incision reaction in the free 3’ overhang 4-6 nts from the dsDNA/ssDNA junction. Current experiments are underway reconstituting this full activity on a defined DNA substrate.

**REFERENCES**


FOOTNOTES

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1The abbreviations used are: CD, circular dichroism; CS, Cockayne syndrome; dsDNA, double-stranded DNA; NER, nucleotide excision repair; nts, nucleotides; PAGE, polyacrylamide gel electrophoresis; ssDNA, single-stranded DNA; SV, sedimentation velocity; TTD, trichothiodystrophy; WT, wildtype; XP, xeroderma pigmentosum;

FIGURE LEGENDS

Figure 1 T. acidophilum Bax1 forms a complex with XPB. (A) Size exclusion chromatography was performed to verify the presence of a stable XPB/Bax1 complex. Bax1 was first analyzed individually (shown in black), followed by a 1:1 stoichiometric mixture of Bax1 and XPB (shown in gray). Peak fractions were analyzed via SDS-PAGE to confirm complex formation. (B) Both Bax1 and the purified XPB/Bax1 complex were subjected to analytical ultracentrifugation in separate experiments to support the finding that XPB and Bax1 interact in solution (same color coding as in A).

Figure 2 T. acidophilum Bax1 is an active structure-specific endonuclease. (A) The DNA substrate NDT/NDB22 (shown in (C)) was labeled at the 5’ end of the upper strand and used at a concentration of 10 nM. Arrows indicate full-length DNA as well as the incision product. Increasing concentrations of Bax1 lead to increasing amounts of the incision product. (B) Bax1 incises 4 to 6 nts away from a dsDNA/ssDNA junction. Incision assays employing diverse DNA substrates, which differ with respect to the length of the 3’ overhang indicate that Bax1 cuts specifically 4 to 6 nts to the dsDNA/ssDNA junction in the ssDNA region. The DNA ladder M was generated by mixing each 10 nM of 5’ labeled ssDNAs of known length, in this case 50 nts, 40 nts, 32 nts, 30 nts, 26 nts and 19 nts. (C) Overview of the different substrates used for the incision assays. Arrows depict where Bax1 cuts the DNA substrates.

Figure 3 The endonuclease activity is specific to Bax1. (A) Bax1 elution profile after size exclusion chromatography. Absorptions at 260 nm and 280 nm are shown in gray and black, respectively. The SDS-gel below indicates the Bax1 content of the different fractions. Both peaks contain Bax1 and revealed a monomer-dimer equilibrium according to a calibration of the column. (B) Fractions of the monomer peak were subjected to both SDS-PAGE and incision assays. The incised product was quantified and visualized in a bar chart confirming a direct correlation of the amount of Bax1 and the formation of the DNA fragment.
Figure 4 The presence of XPB inhibits the endonuclease activity of Bax1. The incision assay performed with increasing amounts of the purified XPB/Bax1 complex does not reveal an incision product as observed for Bax1 alone (highlighted by an asterisk). The XPB/Bax1 complex was assumed to contain the two proteins in a 1:1 stoichiometric ratio according to analytical size exclusion chromatography and analytical ultracentrifugation. 10 nM substrate NDT/NDB22 was used throughout.

Figure 5 (A) Conservation of Bax1 among archaeb. Multiple sequence alignment of Bax1 (T. acidophilum) and related proteins from Picrophilus torridus, Pyrococcus furiosus, Pyrococcus abyssi, Haloquadratum walsbyi, Sulfolobus solfataricus and Methanosarcina acetivorans depict the level of sequence conservation among different archaeb. The arrows point to sites where point mutations were introduced, dots correspond to similar residues, colons to conserved residues and asterisks to identical residues, respectively. Numbering above the alignment corresponds to the T. acidophilum sequence.
(B) Conserved residues of Bax1 and TspRI endonucleases. The alignment of Bax1 and TspRI endonuclease reveals a high local sequence similarity indicating conserved and thus probably important residues for Bax1 endonuclease activity.
(C) Incision activity of Bax1 mutants. Quantification of three independently conducted incision assays with DNA substrate NDT/NDB26 (2 nM) reveals different cutting efficiencies for diverse Bax1 variants (8 µM each). Error bars represent the standard deviation of three independent measurements.

Figure 6 Mg$^{2+}$ is crucial for Bax1 incision activity. Incision assays were performed in the presence of different divalent cations or the chelating reagent EDTA (10 mM each) using the DNA substrate NDT/NDB22 (10 nM). Only in the presence of MgCl$_2$ a specific incision product is formed.
Figure 2

A

![Image of gel electrophoresis with lanes labeled - 1 2 2.5 3 4 5 7.5 10 μM Bax1 showing bands for full length and incised DNA.]

B

![Image of gel electrophoresis with lanes labeled 5 10 μM Bax1 and sub-lanes labeled NDT/NDB22 M 5 10 μM Bax1 and sub-lanes labeled NDT/NDB26 M and NDT/NDB30 M, showing bands for full length and incised DNA.]

C

| 5’ | GACTACGTACTGTACGTCGTCCCATCTCTACCGCAATCAGGCCACGATCTGC | 3’ | NDT |
| 3’ | CTGATGCATGACAATGCCGAGGG | 5’ |
| 5’ | GACTACGTACTGTACGGCCATCTCTACCGCAATCAGGCCACGATCTGC | 3’ | NDT |
| 3’ | CTGATGCATGACAATGCCGAGGGTAGTAG | 5’ | NDB26 |
| 5’ | GACTACGTACTGTACGTCGTCCCATCTCTACCGCAATCAGGCCACGATCTGC | 3’ | NDT |
| 3’ | CTGATGCATGACAATGCCGAGGGTAGAGATG | 5’ | NDB30 |
Figure 3

A

\[ \text{Absorption [mAU]} \]

Elution Volume [ml]

\[ \text{A}_{280} \]

\[ \text{A}_{260} \]

Bax1

B

\[ \text{Incised DNA [%]} \]

no protein, B9, C3, C4, C5, C6, C7, C8
Figure 4

- 1 2 2.5 3 4 5 7.5 10 10*

μM XPB/Bax1

*Full length DNA

*Incised DNA

*Bax1
Figure 5

A

Bax1  
T. acidophilum   AEHFSVSPQDIEEAIYGDMDSENVLDCPSITPEHLNRRNYNEQL
Picrophilus torridus  AESFNIDYIDIAIYADKDESILERPYNTEGKLIREYNEQL
Pyrococcus furiosus  ATYFNTPEEIERAMFADREEEKILTRVPGEISEELIRRYNLSLL
Pyrococcus abyssii  AQEFNTSIEIEERAVFADREEERVLVKIPEISEEELIKRYNLSLL
Halocarcula walsbyi  GDEFELETENESNLWADREDHAVLRSEPEITTPCNELRQYNISLT
Sulfolobus solfataricus  GDGFHVDP----KAMYEDLDVEKKIVELPKFSPEDLLKIIYNLK
Methanosarcina acetivorans  AKEKSLVPELEKSLWADLEENQFLKEFNPLSPAELLRQYNISLT

Sequence conservation

.  .  :  :  *  :  :  .  :  *  :  **  :

B

Bax1  124  EEAITYGDMDSENVLDA  137
TspRI  194  DERVYGDMSSDNILD  208

C

Incised DNA [%]

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<th>Incised DNA [%]</th>
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<tr>
<td>WT</td>
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<td>F116A</td>
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Heide M. Roth, Ingrid Tessmer, Bennett Van Houten and Caroline Kisker

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