Biochimica et Biophysica Acta 1854 (2015) 505–516

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Critical determinants for substrate recognition and catalysis in the *M. tuberculosis* class II AP-endonuclease/3′–5′ exonuclease III

Taran Khanam, Ankita Shukla, Niyati Rai, Ravishankar Ramachandran *

Molecular and Structural Biology Division, CSIR-Central Drug Research Institute, Sector 10, Jankipuram Extension, Lucknow, Uttar Pradesh 226031, India

ARTICLE INFO

Article history:
Received 15 December 2014
Received in revised form 2 February 2015
Accepted 25 February 2015
Available online 5 March 2015

Keywords:
Base excision repair
DNA repair
XthA
AP site recognition
DNA damage

ABSTRACT

The *Mycobacterium tuberculosis* AP-endonuclease/3′–5′ exodeoxyribonuclease (MtbXthA) is an important player in DNA base excision repair (BER). We demonstrate that the enzyme has robust apurinic/apyrimidinic (AP) endonuclease activity, 3′–5′ exonuclease, phosphatase, and phosphodiesterase activities. The enzyme functions as an AP-endonuclease at high ionic environments, while the 3′–5′-exonuclease activity is predominant at low ionic environments. Our molecular modelling and mutational experiments show that E57 and D251 are critical for catalysis. Although nicked DNA and gapped DNA are fair substrates of MtbXthA, the gap-size did not affect the excision activity and furthermore, a substrate with a recessed 3′-end is preferred. To understand the determinants of abasic-site recognition, we examined the possible roles of (i) the base opposite the abasic site, (ii) the abasic ribose ring itself, (iii) local distortions in the AP-site, and (iv) conserved residues located near the active site. Our experiments demonstrate that the first three determinants do not play a role in MtbXthA, and in fact the enzyme exhibits robust endonucleolytic activity against single-stranded AP DNA also. Regarding the fourth determinant, it is known that the catalytic-site of AP endonucleases is surrounded by conserved aromatic residues and intriguingly, the exact residues that are directly involved in abasic site recognition vary with the individual proteins. We therefore, used a combination of mutational analysis, kinetic assays, and structure-based modelling, to identify that Y237, supported by Y137, mediates the formation of the MtbXthA–AP-DNA complex and AP-site incision.

1. Introduction

Mycobacterium tuberculosis is a dreaded human pathogen that effectively counteracts the hostile macrophage environment in the host. The macrophage itself contains antimicrobial free radicals, reactive oxygen, and nitrogen species that can cause permanent changes and aberrations to the genomic DNA of the bacteria [1]. The plethora of changes includes miscoding base alterations, DNA strand breaks, and generation of abasic sites [2]. Analysis of the *M. tuberculosis* genome has led to the identification of the full complement of genes involved in oxidative DNA damage repair, nucleotide excision repair (NER) and base-excision repair (BER) [3]. On the other hand, genes participating in Mismatch repair have not yet been identified [4,5]. The *M. tuberculosis* genome is GC rich (~65%), and is therefore susceptible to modifications involving cytosine and guanine respectively. Modified bases are promutagenic and have to be repaired using dedicated machinery [6]. Generally, lesions are recognised and excised by DNA glycosylases [7–11]. These enzymes hydrolyse the N-glycosidic bond between altered bases and sugar resulting in the generation of apurinic/apyrimidinic (AP) sites in DNA. As reported in various studies [12,13], the presence of abasic sites can obstruct metabolic processes like replication and transcription. Additionally, their accumulation is mutagenic and cytotoxic, necessitating prompt repair [14]. Abasic sites are consequently acted upon by class II AP endonucleases that cleave the phosphodiester bond and produce a nick [6,15,16]. Subsequently, enzymes that are present downstream like the 5′-deoxyribose phosphodiesterase, DNA polymerase, and DNA ligase act on the nicked DNA to restore the integrity of the original sequence [17].

AP endonucleases have been classified into XthA and Nfo families based on sequence homology and structural conservation studies involving *Escherichia coli* exonuclease III (ExoIII) or endonuclease IV (EndoIV) respectively [18]. XthA is expressed constitutively and accounts for over 80% of the total AP endonuclease activity in *E. coli*, while Nfo accounts for ~10% of the activity [19]. Two human homologs of ExoIII, Ape1 and Ape2 have been characterised [20–22], and it has been reported that Ape1 accounts for more than 95% of the total cellular AP endonuclease activity [23]. Other homologs from organisms like *Saccharomyces cerevisiae* (Apn2) and *Pyrococcus furiosus* (PfuApe) [19,24] have also been characterised. Additionally, it has been found that *E. coli* mutants defective in this enzyme are hyper sensitive to UV radiation and H_2O_2 [6,25]. XthA deficient strains of *Salmonella typhimurium* exhibit attenuation of infection in a mouse model and the results point to the importance of abasic site repair for bacterial survival [26].

* Corresponding author. Tel.: +91 522 2771940; fax: +91 522 2771941.
E-mail address: r_ravishankar@cdri.res.in (R. Ramachandran).

http://dx.doi.org/10.1016/j.bbapap.2015.02.019
1570-9639/

The significance of DNA repair in the pathogenesis of *M. tuberculosis* has also been studied in detail [27]. Furthermore, the *xtha* gene has been found to be highly resistant to changes in clinical tuberculosis strains and supports its important role in pathogenesis [28].

The ExoIII family of class II AP endonucleases consists of versatile enzymes [29,30]. Apart from recognising AP sites and cleaving at the 5′ side of the abasic lesion, they are known to possess 3′-phosphatase, 3′-phosphodiesterase, and other activities. These activities help remove 3′-blocking groups that arise at single-strand breaks in DNA upon oxidative damage [31]. These proteins also exhibit 3′–5′ exonuclease and ribonuclease H activities, the functional significance of which is unknown in *E. coli* [32]. On the other hand, human Ape2 has been shown to possess 3′–5′ exonuclease activity and also it preferentially acts on mismatched base pairs [33]. Recent studies have highlighted a novel role for Ape1 in RNA metabolism where, amongst other things, it was found to interact with rRNA and cleave abasic RNA [34].

It is intriguing that amongst a clutch of conserved aromatic residues near the catalytic site, only one or two are important for substrate recognition in the respective XthA homologs [18,35,36]. The exact residues from amongst these conserved residues also vary from protein-to-protein [35] and cannot be predicted a priori. In the initial part of the present work, we report a detailed characterisation of the XthA enzyme and show that it possesses robust AP endonuclease, 3′–5′ exonuclease, phosphatase and phosphodiesterase activities displaying varied substrate specificities. A variety of modified DNA substrates, along with mutation analysis, were used to understand the determinants of AP-site recognition/incision and we found that the DNA elements play no role in the same. Mutational analysis of aromatic residues located vicinal to the catalytic site revealed that Y237 acts as a primary AP site recogniser while Y137 has an ancillary role in MtbXthA.

2. Materials and methods

2.1. Cloning of MtbXthA and its mutants

The MtbXthA (Rv0427c) gene was amplified by standard PCR techniques and cloned between the BamHI and HindIII restriction sites of pET23a (Novagen). The resultant plasmid was designated as pET23a-MtbX. Eight point mutations, viz. Y173S, Y234S, Y235S, Y234S/W235S, Y237S, F242S, E57A and D251A were generated using pET23a-MtbX as the template using standard protocols [37]. A double mutant E57A/D251A was generated using the single mutants, D251A or E57A, as the template during the PCR reaction. All expression constructs were sequenced to verify their integrity. The forward and reverse primers for the constructs are listed in Table S2.

2.2. Overproduction and purification of proteins

pET23a-MtbX and respective mutants were transformed into *E. coli* BL21(DE3) cells (Novagen) and grown in LB medium supplemented with 0.1 mg/ml ampicillin until A_{600} 0.5. Protein expression was induced by adding 1.0 mM isopropyl β-D-thiogalactopyranoside and the cells were grown further at 20 °C for 16 h. Subsequently, cells were harvested by centrifugation at 7000 rpm, resuspended in 40 mM Tris–HCl (pH 9.0), 500 mM NaCl, and 0.1% N-Lauryl Sarcosine and 5 mM imidazole (Buffer A) supplemented with 10 mM PMSF and 0.2 mg/ml lysozyme and left on ice for 30 min. Harvested cells were sonicated and the lysate was incubated on ice for 30 min to allow solubilisation of insoluble recombinant protein followed by centrifugation at 13,000 rpm for 30 min at 4 °C. The supernatant loaded onto a nickel NTA column (Amersham Biosciences) equilibrated with buffer A, and protein was eluted using 10–500 mM imidazole gradient. Purified fractions were pooled and dialyzed against the dialysis buffer (40 mM Tris–HCl (pH 8.0), 50 mM NaCl and 2 mM DTT). The dialyzed protein was subsequently precipitated using ammonium sulphate (40% saturation) and re-dissolved in a minimum volume of buffer B (40 mM Tris–HCl (pH 8.0), 50 mM NaCl and 2 mM dithiothreitol and 10% glycerol). This was then loaded onto a Superdex S200 gel filtration column (Amersham Biosciences) equilibrated with buffer B. The purity of the protein was evaluated by running 12% SDS-PAGE gels.

2.3. AP endonuclease assay

DNA substrate N1 containing an abasic site analogue tetrahydrofuran (THF), was used for AP site incision activity assays. Substrate N2 that contained propanediol, tetrahydrofuran, ethane or 2′ deoxyribose as the abasic residues was used for studying the effect of abasic site structure on the AP site incision activity of MtbXthA. For the preparation of the duplex DNA containing a 2′ deoxyribose as abasic residue, first an oligonucleotide containing a 2′ deoxyribose was prepared by enzymatic treatment of an oligonucleotide harbouring 2′ deoxyuridine with uracil N-glycosylase (New England BioLabs Inc.). This was followed by annealing with the complementary oligonucleotide [38]. To analyse whether the base opposite the abasic site plays any role in AP site recognition, DNA substrate N3 which contained different bases (A/T/G/C) opposite the abasic site was used. The oligonucleotide N4 containing abasic site analogue THF was used as a ssDNA substrate to analyse whether undamaged DNA strand plays any role in AP site recognition. All duplex DNA substrates were generated by annealing of oligonucleotide pairs listed in Table S3. All oligonucleotides were purchased from M/s Integrated DNA technologies Inc.

A standard AP site incision reaction mixture (20 μl) contained 5 nM DNA substrate and 1 nM MtbXthA protein in 40 mM Tris–HCl (pH 7.8), 50 mM NaCl, 10 mM $MgCl_2$, 2 mM DTT, 100 μg/ml BSA. Reactions were assembled on ice, incubated at 37 °C for 10 min, and quenched by adding 10 μl stop solution (98% formamide, 10 mM EDTA, 0.15% xylene cyanol and 0.15% bromophenol blue). Samples were heated for 5 min at 100 °C and chilled on ice before loading. Reaction products were resolved on 12% polyacrylamide gels containing 8 M urea. Gels were scanned using ImageQuant LAS 4000 and band intensities were measured and quantified using ImageQuantTL 8.1 software. For the optimisation of pH, salt and $MgCl_2$ concentrations respectively, the buffers were supplemented with indicated values of the variable component in each case.

2.4. 3′–5′ Exonuclease assay

For 3′–5′ exonuclease activity assays, a 3′ recessed DNA substrate X1 was used. Substrate X2 that either harboured a nick or contained 1, 2, 3 or 4 nucleotide-gaps was used in exonuclease assay to assess substrate specificity of MtbXthA. Also, oligonucleotides X3, X4 and X5 which are ssDNA, 3′ overhang containing heteroduplex DNA and 3′ blunt duplex DNA, respectively were used as substrates. For the proof reading assay, DNA substrate X6 was used and this consists of all possible combinations of 16 different base pairs at the primer–template junction. All duplex DNA substrates were generated by annealing of oligonucleotide pairs listed in Table S3.

A standard 3′–5′ exonuclease assay reaction mixture (20 μl) contained 5 nM DNA substrate and 1 nM MtbXthA protein in 40 mM Tris–HCl (pH 7.8), 50 mM NaCl, 2 mM $MgCl_2$, 2 mM DTT, 100 μg/ml BSA. Reactions were assembled on ice, incubated at 37 °C for 20 min, and were terminated by adding 10 μl of the stop solution. Samples were heated for 5 min at 100 °C and chilled on ice before loading. The reaction products were resolved on 12% polyacrylamide gels containing 8 M urea. Gels were scanned using ImageQuant LAS 4000 and band intensities were measured and quantified using ImageQuantTL 8.1 software. The pH, NaCl and $MgCl_2$ concentration were optimised using buffers supplemented with indicated values of the variable component.