Application of Protein Footprinting, Crosslinking, and Mass Spectrometry in Mapping Ape1 Contacts During BER Progression

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Overview

• DNA damage resulting in abasic (AP) sites are generated spontaneously, from normal cellular processes, and from exposure to environmental agents.

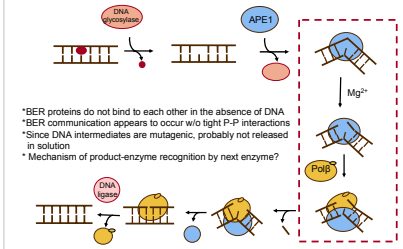
• Cytotoxic and mutagenic AP DNA damage is repaired via the Base Excision Repair (BER) pathway. BER is a multi-step process involving the concerted effort of dedicated DNA repair enzymes. The key enzyme in BER is AP endonuclease (APE1), which recognizes and cleaves the backbone 5′ to the abasic site.

• APE1 is essential for survival and development. Knockout mice lacking any key BER gene result in embryonic lethality. BER gene mutations have been linked to cancer and neurodegeneration (ALS).

• The mechanism of DNA hand-off between BER enzymes is not well understood. Determining the amino acid residues engaged in protein-protein interactions will allow us to understand how the BER pathway is coordinated.

• Using combined MS3D (footprinting, crosslinking, and MS detection) methods, our goal is to identify APE1 inter and intramolecular contacts involved in binary and ternary nucleoprotein complexes formed during BER progression.

BER pathway



*BER proteins do not bind to each other in the absence of DNA
*BER communication appears to occur w/o tight P-P interactions
*Since DNA intermediates are mutagenic, probably not released in solution
* Mechanism of product-enzyme recognition by next enzyme?

Materials and Methods

APE1 and POLβ proteins were expressed in *E.coli* cells and purified by FPLC. Phosphodiester and phosphothioate DNA substrates were commercially synthesized. FDNA incision and binding assays were performed according to established protocols.[2]

41FDNA 5′TAG ACG GAT GAA TAA TGA GGG **F** AGA AGT TGG ATT TGG TAG T3′
3′ATC TGC CTA CTT ATT ACT CCC G TCT TCA ACC TAA ACC ATC A5′

41Fgap 5′TAG ACG GATGAA TAA TGA GGG3′ AGA AGT TGG ATT TGG TAG T3′
3′ATC TGC CTA CTT ATT ACT CCC G TCT TCA ACC TAA ACC ATC A5′

5′**F**

* **F** = tetrahydrofuran (abasic site analog)

STRUCTURE PROBING

• APE1:FDNA complexes were formed by incubating 1 µg APE1 with 15 pmol 41FDNA duplex for 5 min on ice in 15-µL buffer (50 mM HEPES (pH 7.5), 50 mM KCl, 4 mM EDTA). For phosphothioate FDNA, buffer contains 10 mM $MgCl_2$. Equimolar POLβ was added to form the (1:1:1) ternary complexes.

• *N*-hydroxysuccinimidobiotin (BT) was used to investigate Lys reactivity in the proteins.[3] The complexes were incubated with 50x molar excess of probe for 30 min on ice and quenched with 10 mM glycine Disuccinimidyl suberate (DSS) and disuccinimidyl glutarate (DSG) were used for crosslinking APE1 and APE1:FDNA. Proteins were resolved by SDS-PAGE, excised and processed for in–gel digestion with either trypsin or Glu-C.[3]

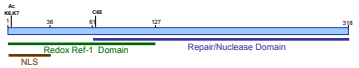
MASS SPECTROMETRY

• Peptides were analyzed by ABI Voyager MALDI-TOF MS. Complementary ESI- MS and MS/MS analyses were also performed using Micromass Q-TOF Ultima MS equipped with an in-house nanospray assembly and a Waters Cap-LC system. Tryptic peptides were separated with a Magic C18 RP-HPLC column (Michrom).

• MS peak reduction were performed with Data Explorer or Mascot Distiller software. Data analysis was performed using web-based implementations of Links and MS2Links available through the C-MS3D portal:

https://ms3d.ca.sandia.gov:11443/cms3d/portal

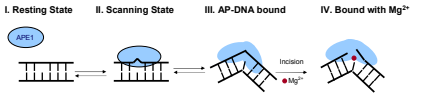
APE1 structure & functions



• AP site incision, endonuclease activity
• 3′ phosphodiesterase activity (3′ repair removal of obstructive 3′ ends)
• Intermediary transcriptional regulator in signal transduction processes
• Oxidative signaling

• High-res structures for APE1(43-318) and APE1-DNA complex
• N-terminal region (1-58) predicted to be intrinsically disordered by DISEMBL[1]
• Post-translational modifications (redox (C65), acetylation (K6, K7))
• Based on previous biophysical characterization (fluorescence and CD studies), APE1 is believed to undergo dynamic changes during its interaction with DNA.

Proposed Pathway for APE1 Recognition and Catalysis*



"Resting" state of APE1 in the absence of DNA.

APE scans DNA, exhibiting weak interactions with normal DNA until it encounters AP site.

"Pre-incision bound" state. APE1 is bound to non-incised AP site. This conformation is captured by sequestering metal ions from the APE1-DNA complex.

"Post-incision bound" state. APE1 is bound to incised AP-DNA and Mg^{2+}. This conformation could serve as a loading mechanism for POLβ.

*Based on earlier fluorescence and CD studies (unpublished data)

Results

1. Protein-DNA complexes of APE1

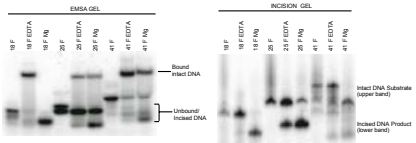


Figure 1. Protein-DNA complexes of APE1 with ^{32}P end-labeled AP-DNA (18, 25, 41 mer) in the presence and absence of Mg^{2+}. Incision occurs in the presence of Mg^{2+}. Position of DNA-protein complexes, and incised DNA is shown. In the presence of Mg^{2+}, APE1(State IV) binds to the incised product of 25mer (or larger) AP-DNA.

2. Identification of lysines protected by DNA and protein contacts

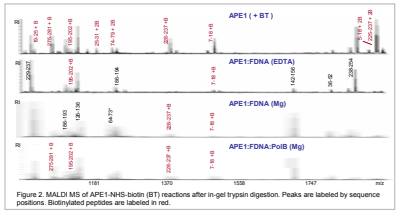


Figure 2. MALDI MS of APE1-NHS-biotin (BT) reactions after in-gel trypsin digestion. Peaks are labeled by sequence positions. Biotinylated peptides are labeled in red.

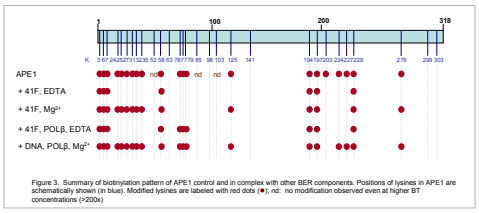


Figure 3. Summary of biotinylation pattern of APE1 control and in complex with other BER components. Positions of lysines in APE1 are schematically shown (in blue). Modified lysines are labeled with red dots (●); nd: no modification observed even at higher BT concentrations (>200x)

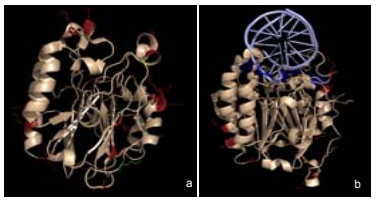


Figure 4. High-resolution structures of a) APE1 and b) APE1:FDNA complex. Lysines modified in free and DNA-bound APE1 are highlighted in red. Solvent accessible lysines that were not modified are shown (green, panel a), as well as lysines protected by DNA in the complex (blue, panel b). The position of A43 is also shown (yellow) for reference. Figures are generated with PyMOL using PDB ID 2ISI and 1DE9.

NHS-biotin readily modified most of the solvent accessible lysines in APE1, generally in agreement with the calculated solvent accessibilities of lysines in the crystal structure. Lysines in the DNA binding sites of APE1 were protected from biotinylation in the presence of FDNA.

In the pre-incision bound state, we also observed protection of lysines that are not involved in DNA binding (K24, K25, K27 and K31). These N-terminal lysines, however, were modified when the Mg^{2+} is added in solution, and presents direct evidence for a conformational change in the Ref-1 domain upon formation of the catalytically active APE:FDNA complex.

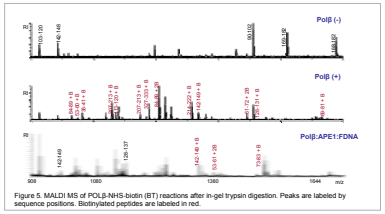


Figure 5. MALDI MS of POLβ-NHS-biotin (BT) reactions after in-gel trypsin digestion. Peaks are labeled by sequence positions. Biotinylated peptides are labeled in red.

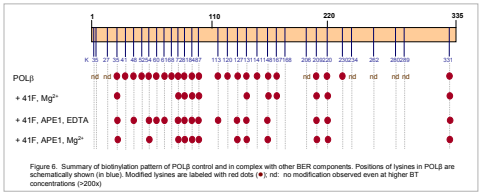


Figure 6. Summary of biotinylation pattern of POLβ control and in complex with other BER components. Positions of lysines in POLβ are schematically shown (in blue). Modified lysines are labeled with red dots (●); nd: no modification observed even at higher BT concentrations (>200x)

3. Identification of crosslinked lysines in APE1

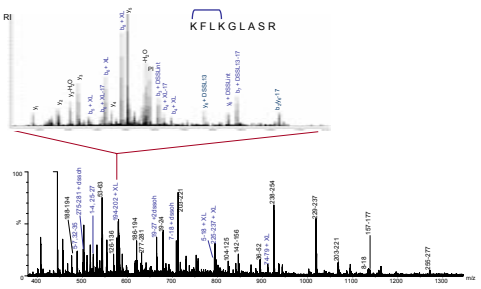


Figure 7. ESI- MS of APE1-DSS reaction after in-gel trypsin digestion. Peaks are labeled by sequence positions. Modified peptides are labeled in blue (XL for crosslink and dsoh for monoadduct). Inset shows assigned MS^2 spectra consistent with the crosslink assignment.

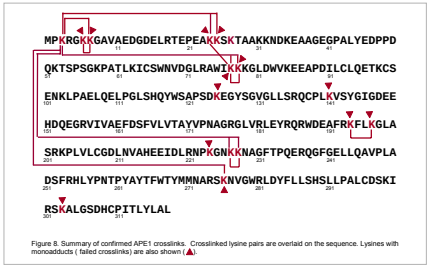


Figure 8. Summary of confirmed APE1 crosslinks. Crosslinked lysine pairs are overlaid on the sequence. Lysines with monoadducts (failed crosslinks) are also shown (▲).

The same lysine pairs in APE1 were crosslinked by DSG and DSS. As expected, adjacent lysines were cross-linked with the highest efficiency.

Preliminary crosslinking of DNA-bound APE1 (with EDTA) yielded almost similar crosslinks:

CROSSLINK	APE1	APE1:DNA
K3-K7	+	+
K3-K24	+	-
K3-K77	+	+
K3-K227	+	-
K3-K276	+	+
K6-K7	+	+
K24-K77	+	-
K77-K78	+	-
K194-K197	+	+
K227-K228	+	-

General Conclusions

• We have probed APE1 surface topology at various conditions corresponding to different steps of the BER pathway. In the pre-incision complex, we observed protection of lysines located in the DNA binding loops, as well as protection of lysines that were not previously shown to interact with DNA (K24, K25, K27 and K31). These N-terminal lysines, however, were modified in the post-incision complex, providing evidence for a localized conformational change in the Ref1 domain upon formation of the catalytically active complex.

• It is possible that APE1 employs this conformational change as a well-timed signal to recruit down-stream BER proteins. Interestingly, residues 1-35 of APE1 have been implicated in binding XRCC1, an BER accessory protein.[5]

• Footprinting of the catalytically active BER ternary (APE1:FDNA:POLβ) complex showed APE1 lysines involved in DNA binding modified with lower intensity, while POLβ DNA contacts were protected. Other than K54, K127, and K167 of POLβ, we did not observe any significant difference in protein footprints.

• With very few differences in the footprints, it is difficult to infer the putative protein-protein interface. It is possible that POLβ rapidly displaces APE1 for the DNA binding contacts by "sandwiching" the DNA, making minimal protein-protein interactions.

• We are now further defining APE1 contacts and the Ref1 interaction using other footprinting reagents, as well as using other DNA substrate intermediates (gapped and nicked FDNA)[6] to form and freeze DNA-protein complexes.

•We have also identified crosslinks between the Ref1 domain and the C-terminal domain of APE1. The observation of crosslinks between K3-K77, K3-K224 and K3-K276 reveals that the N-terminus is situated near the DNA binding pocket. We are looking at employing other crosslinking reagents to increase the number of distance constraints for subsequent APE1 modeling.

• We are in the process of validating other intramolecular crosslinks and comparing crosslinking profiles of APE1 in complex with DNA in order to model the DNA-mediated conformation changes in the Ref1 domain.

References and Acknowledgements

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