

FAST PHOTOCHEMICAL OXIDATION OF PROTEINS COUPLED WITH MASS  
SPECTROMETRY REVEALS CONFORMATIONAL STATES OF  
APURINIC/APYRIMIDIC ENDONUCLEASE 1

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## DEDICATION

This work is dedicated with love to my grandfather. A man of few words that with actions has taught me perseverance and hard work. His continuous love and support gives me strength for the everyday. Following his example, I strive to excel in life with love, respect and humility in order to make a positive impact in this world.

~ Aqui dejo mi huella gordito ~

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Fast photochemical oxidation of proteins (FPOP) is an emerging footprinting method that utilizes hydroxyl radicals. The use of hydroxyl radicals create stable labeled products that can be analyzed with mass spectrometry. The advantage of FPOP over other methods is the fast acquisition of results and the small amount of sample required for analysis. Protein structure and protein- ligand interactions have been studied with FPOP. Here we evaluated (1) the reproducibility of FPOP, (2) the effect of hydrogen peroxide concentration on oxidation and (3) the use of FPOP to evaluate protein- nucleic acid interaction with Apurinic/Apurinic endonuclease 1 (APE1) protein. APE1 is a pleotropic protein that has been crystallized and studied widely. The 35641.5 Da protein has two major functional activities: DNA repair and redox function. An intact protein study of APE1 showed consistent global labeling by FPOP and a correlation between oxidation and hydrogen peroxide concentration. Furthermore, analysis of APE1 with DNA was done in hopes of probing the DNA binding site. Although the oxidation observed was not sufficient to define the complex pocket, a dramatic effect was seen in residue oxidation when DNA was added. Interestingly, the internal residues were labeled collectively in all APE1 experiments which indicates partial unfolding of the protein as previously suggested in the literature. Hence, these findings establish the use of FPOP to capture protein dynamics and provide evidence of the existence breathing dynamics of APE1.

Lisa, Jones PhD., Chair

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## LIST OF ABBREVIATIONS

|          |   |
|----------|---|
| AA       | amino acid  |
| ACN      | acetonitrile  |
| AGC      | automatic gain control                                    |
| AP       | apurinic/apyrimidic                                       |
| APE1     | apurinic/apyrimidic endonuclease 1                        |
| BER      | base excision repair                                      |
| BLG      | $\beta$ -lactoglobulin                                    |
| DTT      | dithiothreitol  |
| FA       | formic acid   |
| FPOP     | fast photochemical oxidation of proteins                  |
| HCl      | hydrogen chloride   |
| HDX      | hydrogen deuterium exchange                               |
| IAA      | iodoacetamide   |
| LC       | liquid chromatography                                     |
| MS       | mass spectrometry   |
| MS/MS    | tandem mass spectrometry                                  |
| NEM      | N-ethylmaleimide  |
| NMR      | nuclear magnetic resonance                                |
| PBS      | Phosphate buffer saline                                   |
| ROS      | reactive oxygen species                                   |
| SASA     | solvent accessible surface areas                          |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |

|     |                 |
|-----|-----------------|
| TOF | time of flight  |
| THF | tetrahydrofuran |

## Chapter 1. INTRODUCTION

### 1.1 Protein Footprinting

#### 1.1.1 Proteins

Proteins are important elements of living system that perform a variety of vital biological functions and provide cell structure. Proteins, which are made of one or more polypeptide chains, have amino acids (AA) as their building blocks. AA are linked through the formation of an amide or peptide bond involving the carboxylic acid group of one amino acid and the amino group of the next AA. There are twenty naturally occurring AA that give rise to the unique properties and chemical diversity of proteins (33).

Proteins have four levels of architecture that describe their conformation. The primary structure is the linear AA sequence of the protein. Secondary structure includes structural elements such as alpha helices and beta sheets that are formed through hydrogen-bonding between atoms in the peptide backbone. Tertiary structure is the arrangement of secondary structural elements to form a unique three-dimensional structure. Quaternary structure applies to proteins made up of more than one polypeptide chain; it shows the organization of the polypeptide chains.

The primary structure of a protein is unique but does not provide sufficient information to accurately predict its three-dimensional structure unless it is closely related to a protein of known structure. There are 500,000 protein sequences known and only 51,535 structures reported (37). Protein structure is crucial for understanding the protein's functions and interactions with other macromolecules. Hence, it is important to gain more information on the structure of proteins.

#### 1.1.2 Protein Structural Methods

The scarcity of protein structures led to the development and use of diverse techniques. There are now various techniques that integrate experimental data with computational methods to determine a protein structure. Among the most important are NMR and X-ray Crystallography, which can provide near atomic level resolution.

### 1.1.2.1 Nuclear Magnetic Resonance

Nuclear Magnetic Resonance (NMR) is a spectroscopic technique that employs radio frequency energy. With the use of a magnetic field, the molecules' nuclei emits certain energy frequencies that are absorbed and measured (37). The analysis of proteins by NMR gives a compilation of the conformations that are consistent with through-bond and through-space NMR measurements in solution (34). The technique allows for the study of kinetic reactions and provides structural analysis with atomic resolution (37). It provides the advantage of the study of proteins in solution. However, it requires high concentrations of protein and is limited by protein size (34).

### 1.1.2.2 X-Ray Crystallography

X-ray crystallography is the technique that provides the most precise 3-D image of a protein structure. The technique uses ordered protein crystals, X-rays, a detector and computer software to visualize a protein at high resolution. Crystals are produced after slow precipitation of an aqueous solution under favorable conditions for the protein (35). The crystals are bombarded with high energy X-rays from a home source or synchrotron source (36). The intensities, which are related to the structure factor amplitudes of the diffracted X-ray, are measured on a CCD or pixel array detector, but the phase information, which would allow for direct reconstruction of the object that gave rise to the diffraction cannot be measured directly (35, 37). This gives rise to the phase problem in X-ray crystallography. Diffraction data are processed and merged to produce a unique dataset, i.e. one intensity value for each diffracted X-ray. Then the phase problem must be solved by either molecular replacement or experimental phasing methods. Once an atomic model for the structure has been obtained, it is subjected to crystallographic refinement. The one serious limitation for X-ray crystallography is that the molecule of interest must form single crystals that diffract to high enough resolution to be useful for evaluation of structure. With this technique, it is possible to obtain protein structures at atomic level resolution.

### 1.1.3 Protein Footprinting Coupled with Mass Spectrometry

Footprinting is a technique to obtain detailed information defining interacting surfaces of biological molecules. Footprinting was initially used for the study of DNA-protein interactions (27). In this technique, DNA-protein complexes are exposed to hydroxyl radicals. Unprotected DNA is cleaved while the binding surface remains protected. The fragments are analyzed by gel electrophoresis. In this manner, the region of DNA that is protected from digestion indicates protection through protein binding (33).

While analysis of footprinting by denaturing urea gels electrophoresis provides a sufficient level of detail for the DNA that interacts with the protein, the converse is not true for mapping the region of the protein that interacted with the DNA through the use of SDS-PAGE. The coupling of footprinting with mass spectrometry provides detailed information on proteins. Today there are several methods that provide high resolution protein structural mapping and analysis.

#### 1.1.3.1 Hydrogen Deuterium Exchange

Hydrogen deuterium exchange (HDX) is a powerful method for protein structure mapping coupled with mass spectrometry (MS). The method relies on the use of deuterium ( $D_2O$ ) to modify proteins and footprint interactions (15). The protein is exposed to deuterium, digested and analyzed. The exposure to deuterium replaces solvent accessible amide protons of the protein (27). A low pH of 2.5 and low temperature conditions quench the back-exchange during proteolysis (15, 28). The protein is then digested by pepsin before separation and analysis by LC/MS (28). The mass shift of peptides can be detected by MS analysis (27). The more backbone amides that are solvent accessible then the more mass uptake and mass shift observed. Although the method is limited by back-exchange, HDX is very valuable for probing secondary structure of proteins, mapping protein- protein interfaces, and identifying conformational dynamics in proteins (15, 27, 32).

#### 1.1.3.2 Covalent binding

Covalent binding is an established method for in-solution probing of macromolecules structure (27). A covalent target specific probe is reacted with the

protein (1). The protein is then purified and analyzed by MS. The accessibility of the target site determines the reactivity of the probe (28). Thus, covalent binding provides information on protection and accessibility of the molecule which enables the study of tertiary and quaternary structure of a protein (15, 27).

#### 1.1.3.3 Hydroxyl-radical mediated footprinting

Hydroxyl radical-mediated footprinting is a chemical approach that uses hydroxyl radicals to modify solvent accessible surfaces in macromolecules (23, 28). Radicals are used because of their water-like properties and high reactivity (27). The production of hydroxyl radicals is inexpensive, and the modifications are covalent. The stable covalent oxidation products are able to be detected and analyzed with LC/MS (8). This method minimizes the quantity of protein required compared to other techniques (11).

The reactivity of hydroxyl radicals is non-specific, however, the labeling efficiency for amino acids is not equal. When in solution amino acids have different reactivity to hydroxyl radicals, with sulfur containing residues being the most reactive as presented in Table 1. Cysteine is the most reactive residue and over 1000-fold more reactive than the least reactive glycine (28). Another highly reactive residue is methionine which has been previously shown to be oxidized by a theorized electron-transfer mechanism (21).

| Residue | Code | Rate (M-1 S-1) |
|---------|------|----------------|
| Cys     | C    | 3.50E+10       |
| Trp     | W    | 1.30E+10       |
| Tyr     | Y    | 1.30E+10       |
| Met     | M    | 8.50E+09       |
| Phe     | F    | 6.90E+09       |
| His     | H    | 4.80E+09       |
| Arg     | R    | 3.50E+09       |
| Ile     | I    | 1.80E+09       |
| Leu     | L    | 1.70E+09       |
| Val     | V    | 8.50E+08       |
| Pro     | P    | 6.50E+08       |
| Gln     | Q    | 5.40E+08       |
| Thr     | T    | 5.10E+08       |
| Lys     | K    | 3.50E+08       |
| Ser     | S    | 3.20E+08       |
| Glu     | E    | 2.30E+08       |
| Ala     | A    | 7.70E+07       |
| Asp     | D    | 7.50E+07       |
| Asn     | N    | 4.90E+07       |
| Gly     | G    | 1.70E+07       |

Table 1. Reaction rates of hydroxyl radicals and amino acids at near neutral pH.

The side chain of residues impacts the reactivity and the oxidation products. The most common modifications by hydroxyl radical modifications are shown in Table 2. The reaction of the hydroxyl radical with the different amino acids creates a peroxy radical that then gets modified to a hydroxyl radical and sometimes into a carbonyl modification (28). The most common modification incorporates a hydroxy group and shifts the net mass by +16 Da. Some residues have a favored oxidation pathway that creates a mass change signature for that residue. For example Cys and Met have an oxidation signature of +48-Da cysteine sulfonic and +16-Da methionine sulfoxide (28).

| Side Chain | Modification and mass change (Da)                            |
|------------|--|
| Cys        | sulfonic acid (+48), sulfinic acid (+32), hydroxy (-16)      |
| Met        | sulfoxide (+16), sulfone (+32), aldehyde (-32)               |
| Trp        | hydroxy- (+16, +32, +48, etc.), pyrrol ring-open (+32, etc.) |
| Tyr        | hydroxy- (+16, +32, etc.)                                    |
| Phe        | hydroxy- (+16, +32, etc.)                                    |
| His        | oxo- (+16), ring-open (-22, -10, +5)                         |
| Leu        | hydroxy- (+16), carbonyl (+14)                               |
| Ile        | hydroxy- (+16), carbonyl (+14)                               |
| Val        | hydroxy- (+16), carbonyl (+14)                               |
| Pro        | hydroxy- (+16), carbonyl (+14)                               |
| Arg        | deguanidination (-43), hydroxy- (+16), carbonyl (+14)        |
| Lys        | hydroxy- (+16), carbonyl (+14)                               |
| Glu        | decarboxylation (-30), hydroxy- (+16), carbonyl (+14)        |
| Gln        | hydroxy- (+16), carbonyl (+14)                               |
| Asp        | decarboxylation (-30), hydroxy- (+16)                        |
| Asn        | hydroxy- (+16)   |
| Ser        | hydroxy- (+16), carbonyl (-2- or +16-H <sub>2</sub> O)       |
| Thr        | hydroxy- (+16), carbonyl (-2- or +16-H <sub>2</sub> O)       |
| Ala        | hydroxy- (+16)   |

Table 2. Modifications of residues by hydroxyl radicals.

There are a number of methods for generating hydroxyl radicals. There are two particular techniques that are the most prevalently used (Figure 1). The first technique is Fenton chemistry, which uses the ferrous ion (II) to decompose hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Figure. 1 top) (12). The second technique involves the radiolysis of water with X-rays or gamma rays (Figure. 1 bottom) (3). In recent years, laser-induced photolysis of H<sub>2</sub>O<sub>2</sub> has gained interest in the field. It provides the advantage that it reduces exposure time to hydroxyl radicals and minimizes secondary reactions (28).

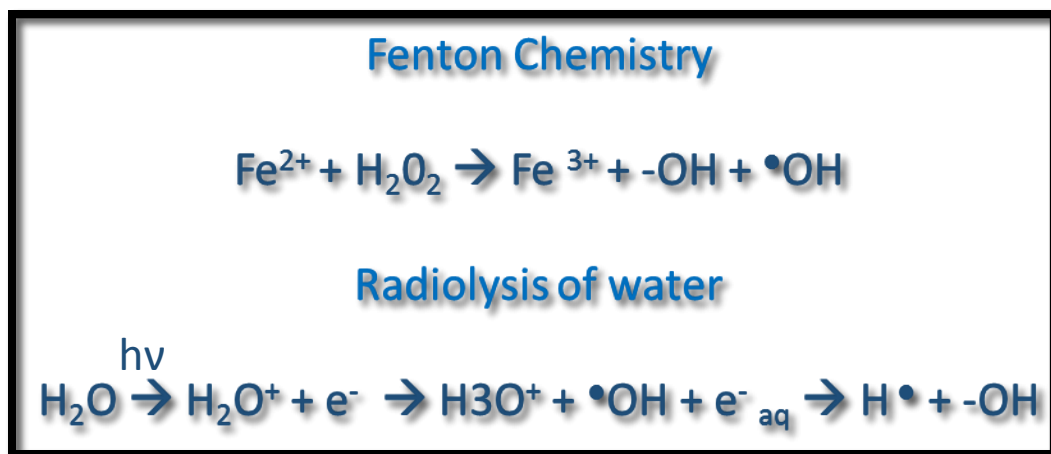


Figure 1. Hydroxyl radical generation via Fenton chemistry and Radiolysis of water.

## 1.2 Fast Photochemical Oxidation of Proteins

### 1.2.1 Fast Photochemical Oxidation of Proteins

Another method for producing hydroxyl radicals is the Fast Photochemical Oxidation of Proteins (FPOP). FPOP employs an excimer laser at 248 nm for the photolysis of hydrogen peroxide to generate hydroxyl radicals (8). A major advantage of FPOP is the speed of labeling which is on the microsecond timescale. This technique allows for the capture of a snapshot of a macromolecule by oxidizing surface accessible residues (6) (Figure 2). The irreversible labeling allows for the structural analysis of a protein with tandem MS to the amino acid residue level (5). Therefore, FPOP provides a rapid and simple analysis of structure, interactions, and dynamics of a protein and its ligands (6, 21).

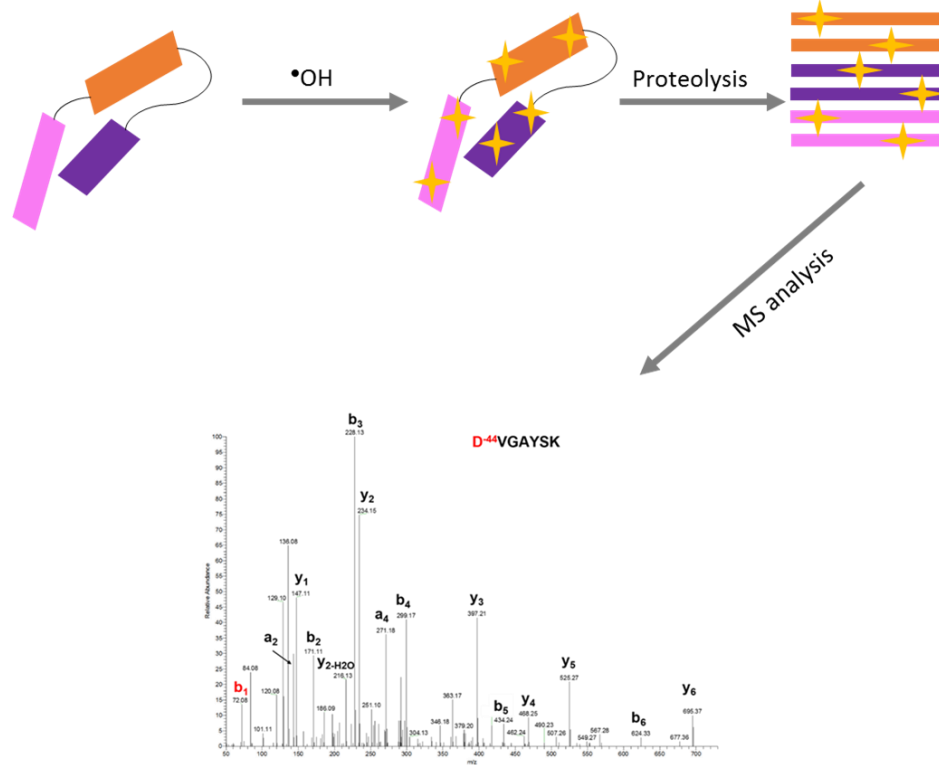


Figure 2. FPOP oxidation combined with MS flowchart.

FPOP utilizes an excimer laser to generate hydroxyl radicals via photolysis of hydrogen peroxide (11). The KrF laser generates a 17 ns pulse of 248 nm that strikes the sample as it flows at a constant rate (6, 8). The samples are pumped, via syringe, through a flow tube that feeds into the quenching reaction (Figure 3).

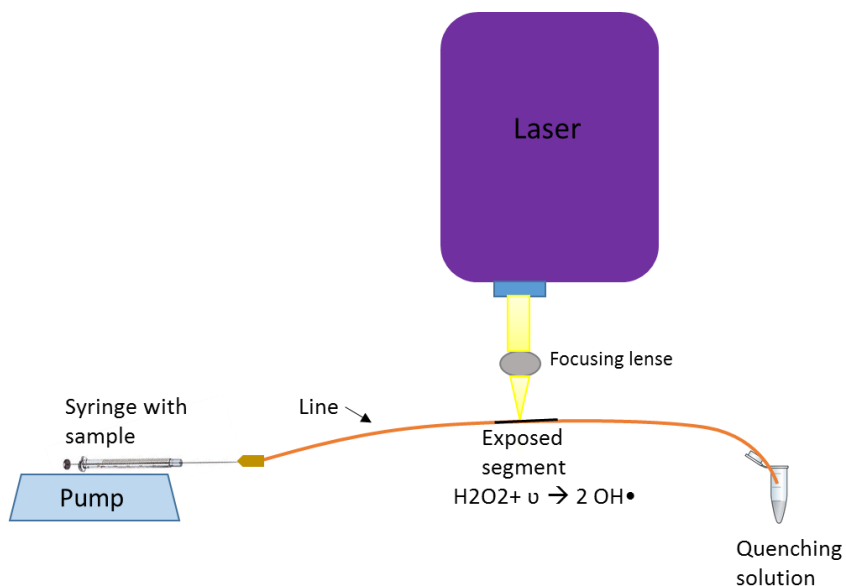


Figure 3. FPOP flow set up diagram

The high reactivity of hydroxyl radicals imparts the possibility of over-oxidation. FPOP sample preparation is designed to protect samples from this. Glutamine is used as a scavenger to remove excess radicals (8). In the presence of glutamine, radical decay is very fast leading to labeling on the microsecond time scale. To prevent secondary oxidation reactions, samples are collected in a tube that contains a quench solution of the enzyme catalase and amino acid methionine (6). Catalase quenches excess hydrogen peroxide while methionine quenches hydroxyl radicals (5, 28). Samples can then be processed and analyzed.

FPOP sample analysis is performed by tandem MS. Since FPOP is an irreversible labeling method, it is amenable for high resolution chromatography (12). MS coupling allows for the quantification and resolution of peptides. Further, data analysis with programs, such as Mascot, allows for identification of peptides and amino acids that are modified by FPOP (5).

Studies have explored the use of FPOP for diverse protein studies. Zhang Hao et al. (30) utilized FPOP for the study of the calmodulin protein binding to skeletal muscle. FPOP results of calmodulin were comparable with existing NMR structural information and extended to identification of residues involved in protein-ligand complex (30).

Furthermore, Jones et al (12) studied antibody- antigen binding of thrombin for epitope mapping and obtained comparable results between FPOP and HDX. Since FPOP is an emerging technique, the use of FPOP is still being explored and is in expansion. But existing studies have shown that the potential of FPOP for high-throughput structural analysis and proven to be applicable to drug discovery in combination with other structural techniques (30).

FPOP is a fast and simple method to probe solvent accessible residues that is ideal for the study of protein structure. Thus, FPOP was used in the structural study of the apurinic/aprimic endonuclease 1(APE1) protein. Preliminary studies address the effect of different concentrations of hydrogen peroxide on global oxidation of APE1, the reproducibility of residue labeling in independent experiments, and the effects of DNA-binding on residue labeling by using FPOP.

#### 1.2.2 Advantages of FPOP

The use of FPOP in the structural analysis of protein has advantages over other footprinting methods such as HDX. HDX probes the protein amide backbone (32). The back-exchange of HDX provides a limitation in the protein size requires instrumentation to achieve residue level analysis (27). Also the use of HDX exposes proteins to non-physical conditions that can lead to unfolding of proteins (15). Alternatively, FPOP uses a fast pulsed laser and radical scavengers to prevent damage of the protein (8). Side chain residues are probed with hydroxyl radicals that form irreversible covalent bonds. Thus laser photolysis of hydrogen peroxide and the formation of covalent bonds are the strengths of FPOP (28). Nonetheless, although there are advantages of FPOP over HDX, these methods provide different information and cannot be compared.

## Chapter 2. HUMAN APURINIC/APYRIMIDINIC ENDONUCLEASE 1

### 2.1 Background –DNA replication & repair

#### 2.1.1 DNA Replication

Human evolution and progression is due to the ability to reproduce and inherit characteristics of progenitors. DNA allows the precise transmission of genetic information to subsequent generations (1). Via DNA replication genetic information is transferred from generation to generation. DNA replication duplicates the genetic material for cell division. It is accomplished by enzymatic machinery and polymerases. The semiconservative process uses the parent strand to copy the base sequence and synthesize a daughter strand in the 5' to 3' direction (3). The parent and daughter strands make a complete complementary double stranded final product. In this manner, genetic material is conserved and passed on.

#### 2.1.2 DNA Repair Pathways

The replication of DNA machinery is fast and has many check points. Nonetheless, there are mistakes that occur during replication despite mechanisms to ensure fidelity, and the DNA is constantly subjected to damage from both endogenous and exogenous sources. The major endogenous source of damage is reactive oxygen species produced during normal cellular metabolism in aerobic organisms (3). Left unrepaired, damage to DNA is mutagenic and can lead to diseases such as cancer. There are several pathways that help repair DNA damage: mismatch repair, base excision repair (BER), nucleotide excision repair, non-homologous end joining repair and homologous repair (37). Each of these pathways repairs specific errors and damages. Of particular interest for apurinic/apyrimidic endonuclease 1 (APE1), which will be discussed later, is the BER mechanism.

##### 2.1.2.1 Base excision repair

The base excision repair pathway mostly repairs incorrect and damaged bases caused by DNA damage (14). DNA is susceptible to damage from both environmental sources and normal cellular activity. The most abundant damaging agents of DNA are

reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radicals and singlet oxygen (3). ROS are produced by X-rays,  $\gamma$ -rays, other radiation exposure, chemicals and drugs (3, 14). But regular cellular mechanism also produces ROS as byproducts of metabolism and sometimes oxidative stress arises (14). Damaging agents like ROS cause lesions that destabilize DNA and lead to genetic instability (20). Hence, repair of AP sites is crucial to maintain genetic integrity.

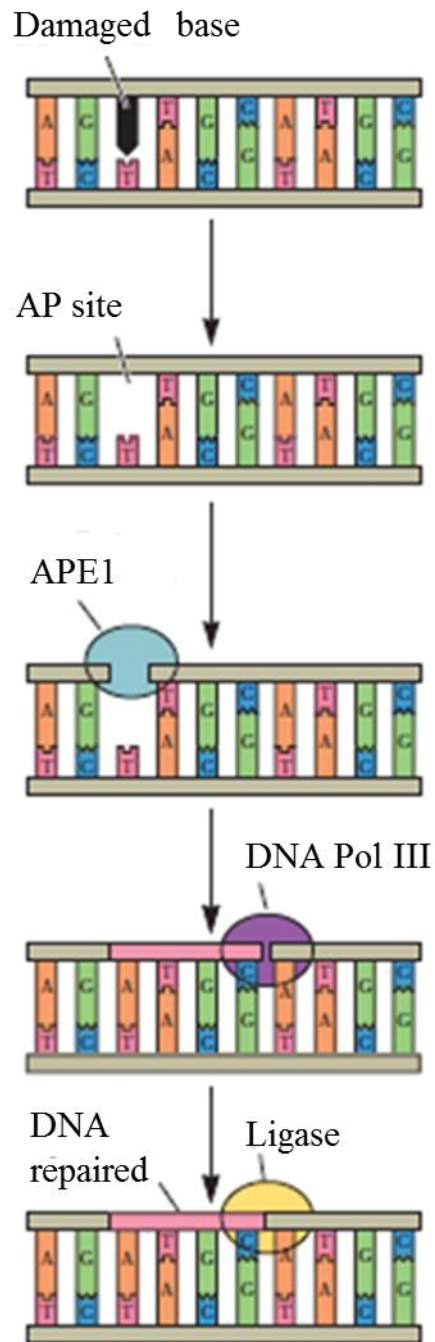


Figure 4. BER mechanism adapted from Berg et al (1).

Base excision repair (BER) is the pathway that repairs these chemically damaged or modified bases. The repair pathway involves five steps with the help of various proteins as depicted in Figure 4. A) The damaged base has to first be removed by a DNA glycosylase creating an AP site (3). B) The AP site is then recognized by an AP

endonuclease (APE1) that nicks the DNA backbone. A priming 3'-OH and 5' deoxyribose phosphate are generated (14). C) The break in the backbone allows DNA polymerase III (or DNA polymerase beta in mammals) to remove the AP site. D) DNA polymerase III repairs the gap, and finally E) DNA ligase seals the gap (37).

## 2.2 Apurinic/apyrimidic edonuclease 1

### 2.2.1 APE 1/Ref-1

Apurinic/apyrimidic endonuclease 1 (APE1) is a pivotal protein also called redox factor 1 (Ref-1), and APEX (17, 23). APE1 consists of 318 amino acids and weights 35.5 kDa (18). It folds into a sandwich-like structure of  $\alpha$ -helices on the outside and  $\beta$ -sheets in between as shown in Figure 6. It is localized in the nucleus and within the mitochondria in different cells according to the function involved and the environment (18, 23).

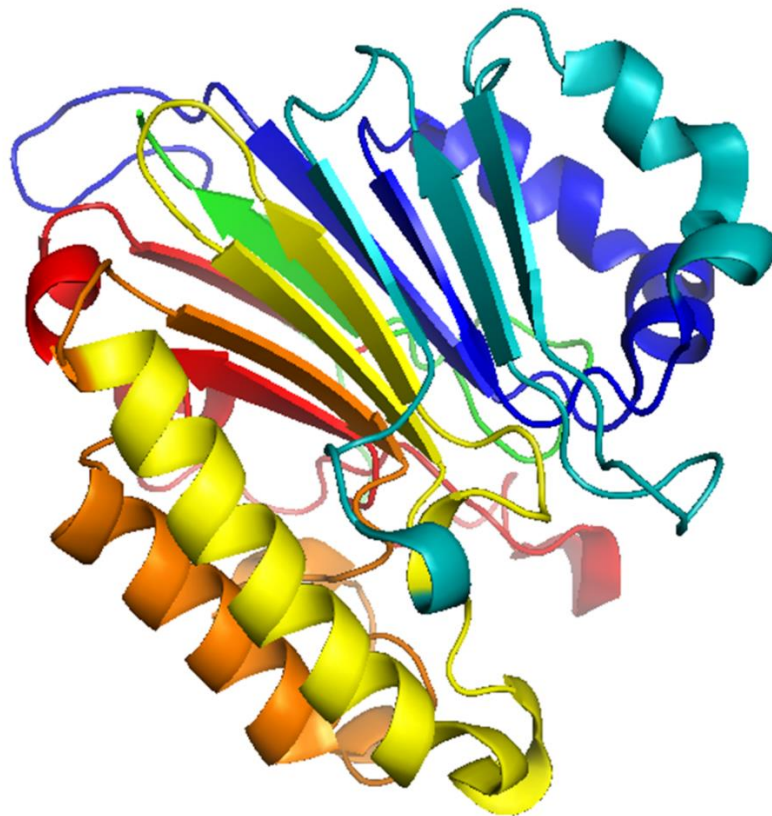


Figure 5. APE1 cartoon depiction, Pymol image from PBD 4IEM. One color was assigned per 50 amino acids.

APE1 is a pleiotropic protein that is necessary for cell survival (25, 31). The activities of APE1 influence many vital biological processes. The many functions of APE1 include cleavage of RNA abasic sites, indirect binding of  $\text{Ca}^{2+}$  responsive elements, DNA repair and reduction of transcription factors (13, 24). In this last role, APE1 serves as a redox factor. APE1 has also been found to have a role in mitochondria genome protection (16). Knock out and knockdown experiments have shown that APE1 loss leads to cell growth arrest, apoptosis, and impairment of mitochondrial function (25). Functionally important residues are as well as their association with either redox or repair functions are shown in Figure 6 (20).

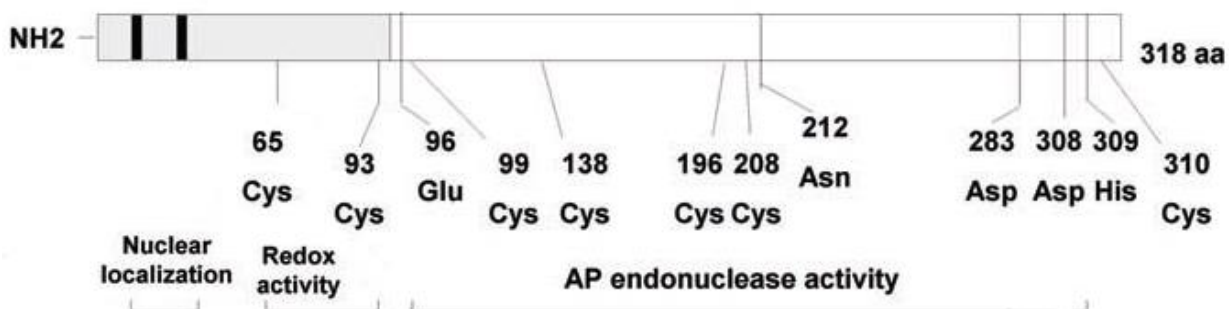


Figure 6. APE1 functional domains and AA of interest, adapted from Luo et al (17).

Overall, both redox and repair functions make APE1 an essential protein because they are crucial for survival and cell growth (25).

#### 2.2.1.1 DNA Repair function

As previously mentioned, unrepaired AP sites lead to cytotoxicity. AP sites are generated spontaneously following removal of a damaged base by DNA glycosylases. From the family of AP endonucleases, APE1 is responsible for 95% of BER repairs of AP sites (13). APE1 scans the DNA and binds at the AP site. Structures of both APE1 alone and in a complex with DNA have been reported (2, 7, 9, 19, 20, and 26).

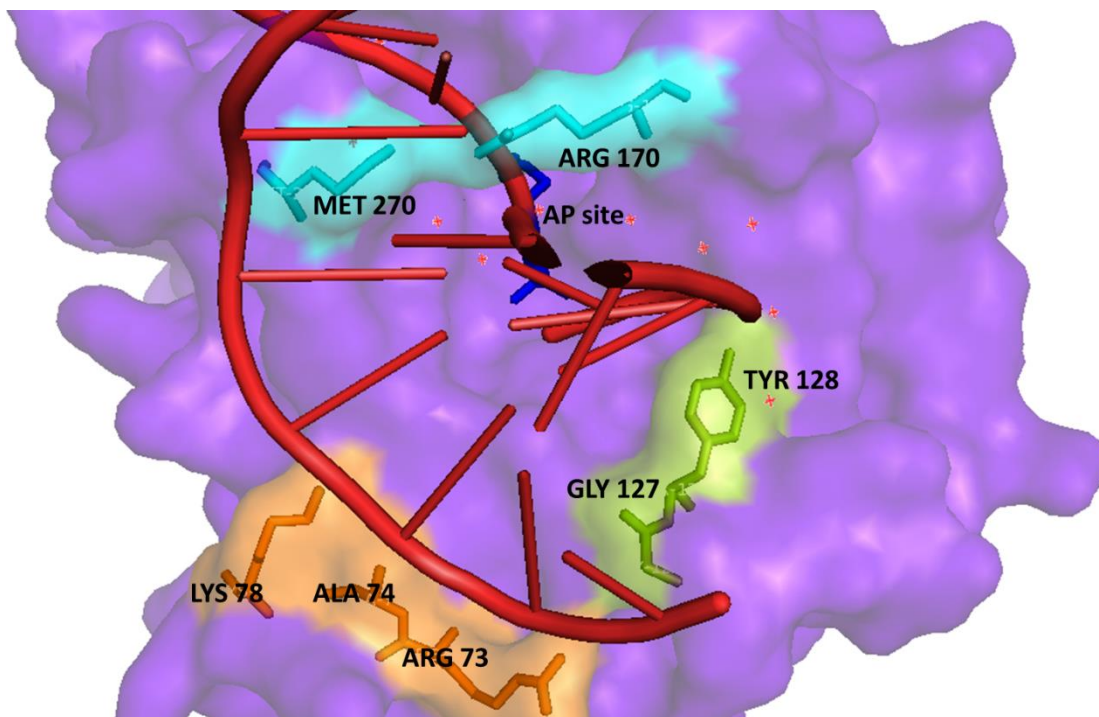


Figure 7. APE1 with DNA and interacting residues. A) Anchoring and kinking of DNA: residues that contact phosphates in the DNA backbone (orange), and residues that interact with the minor groove (green). B) Double loop insertion in the AP site (blue): Met 270 through the minor groove and Arg 177 through the major groove (20).

APE1 distorts the DNA within the active sites and nicks the AP site (13, 16). The nick of the DNA backbone is catalyzed by an Asp-283-His309-H<sub>2</sub>O triad acid-base reaction (14). Glu96 and Asp210 along with Mg<sup>2+</sup> stabilize the DNA and its phosphate charges (16, 18). Figure 7 shows the residues that interact with the AP site and the DNA. APE1 interacts with minor and major groove of DNA to position Met270 and Arg177 at the AP site (20).

#### 2.2.1.2 Redox function

APE1 also performs reduction/oxidation (redox) of transcription factors. The redox function of APE1 is critical for cell survival (22). Oxidizing conditions can lead to the formation of aberrant disulfide bond formation in proteins with resultant loss of function. APE1 reduces transcription factors and activates them. Therefore, APE1 indirectly is a transcriptional regulator (25).

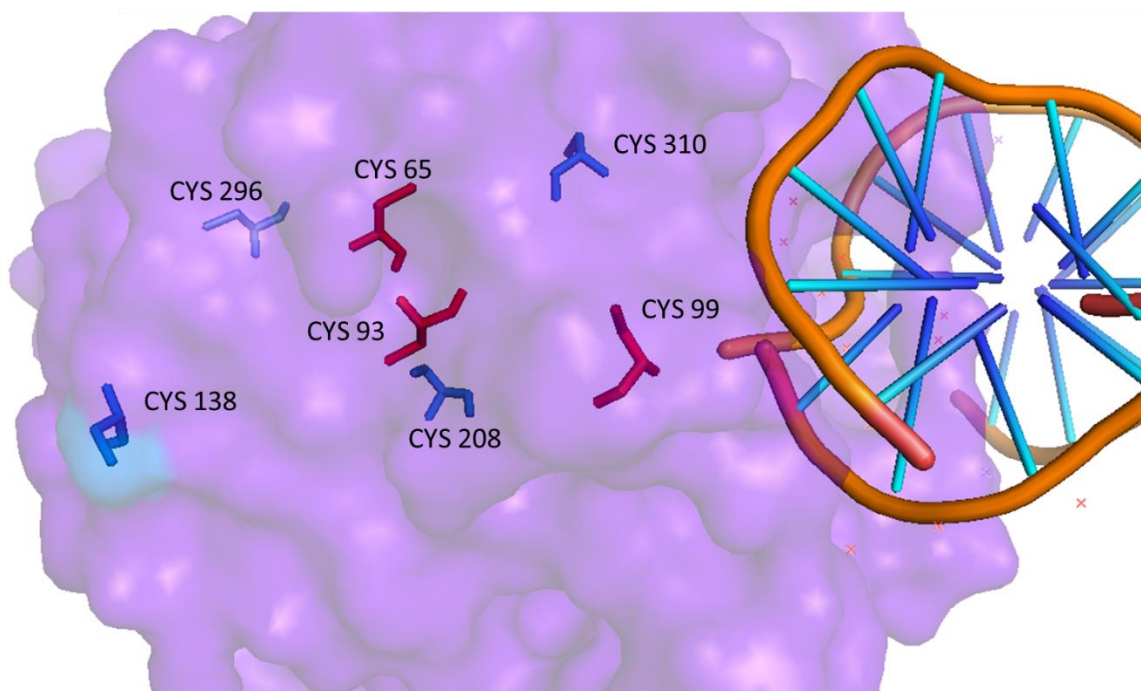


Figure 8. APE1 cysteine residues position

Reduction of transcription factors requires formation of a mixed disulfide bond formation via a thiol-mediated reaction. APE1 has seven cysteine residues (Figure 8). The redox function has been shown to require three Cys residues, namely 65, 93, and 99, which function in a partially unfolded state of the protein in which the nucleophilic Cys 65 is now accessible (17, 22). This work led to the hypothesis that APE1 exists in solution as a mixture of partially and fully folded states. The fully folded proteins has endonuclease activity specific for duplex DNA containing an AP site. Of the three Cys residues, 65 and 93 are buried residues; 99 is a solvent accessible (22, 31). Cys65 acts as the nucleophilic cysteine; substitution of this residue results in the loss of redox activity. Cys93 and Cys99 reduce APE1 to its initial state; substitution of either of these residues results in a decrease in redox activity (18). However the proximity of these residues is not as the usual C-X-X-C motif for reduction (22). Further studies with E3330 inhibitor corroborate the hypothesis that APE1 must partially unfold to act as a redox factor (22).

### 2.2.2 APE1 & diseases

Many pathways are influenced by APE1. Its diverse functions and interactions with proteins involved in DNA repair and growth signaling have a great effect on the

microenvironment and regulation of biological processes. Thus, the pleiotropy of APE1 makes it a potential promoter of beneficial responses as well as unfavorable disease-causing responses; APE1 is known to play a role in inflammation and angiogenesis (13). But it is also being studied as a potential target for the treatment of age-related macular degeneration, diabetic retinopathy and various cancers (13).

In cancer, upregulated levels of APE1 are observed and believed to be associated with chemotherapy resistance (31). APE1 reduces transcription factors such as AP-1, NF- $\kappa$ b, HIF-1 $\alpha$  and p53 (20, 22). Reduction of these transcription factors stimulates transcription of cancer promoting genes. Overexpression of APE1 induces cellular proliferation and represses cell death; which highlights once more the necessity of APE1 for survival (25).

#### 2.2.2.1 Redox inhibitors as therapeutics

APE1's redox function regulates signaling within tumors as well as influencing the tumor microenvironment. Hence, it has become a good candidate target for drug development (25). Resveratrol and soy isoflavones are shown in literature as natural occurring redox inhibitors (13). However the inhibitor that has brought attention is the synthetic compound E3330.

E3330 inhibits the redox but not the repair activity of APE1 (13). The E3330 compound is believed to stabilize an unfolded or partially unfolded state of APE1 based on N-ethylmaleimide labeling (NEM) and thermal melting studies (22, 31). The studies also support that the partially or locally unfolded state that E3330 induces on APE1 allows for Cys65 and Cys93 to be exposed and form a disulfide bond (22). Therefore, E3330 has been studied as a potential therapeutic for diseases with APE1 overexpression and also as a way to study the redox mechanism of APE1.

#### 2.2.3 Evidence of partially unfolding of APE1

In a study by Su et al (22) the labeling of the critical residue Cys65 provided evidence of a partially unfolded conformation of APE1. Experiments of APE1 with E3330 inhibitor with HDX and a NEM experiments were performed. Two species of NEM adducts were observed: +2 NEM and +7 NEM (22). Given that there are only two

solvent accessible residues, the +7 NEM species represents modification of buried cysteine residues. Furthermore, the analysis of these two species with HDX show a considerable shift in mass for the +7 NEM adduct (22). This represents the increased accessibility of the protein amide backbone. Altogether, the results indicate a conformational change of APE1 that increases surface accessibility and allows probing of buried residues.

## Chapter 3. MATERIALS & METHODS

### 3.1 Reagents

Phosphate buffered saline (PBS), and formic acid (FA) were purchased from Sigma Aldrich, St. Louis, MO, USA). L-glutamine, L-methionine, catalase, acetonitrile (ACN), hydrogen peroxide, acetone, 0.1% FA in water, 0.1% FA in ACN, were purchased from Fisher Chemical (Fair Lawn, New Jersey), Purified water was obtained from in-house Milli Q Advantage AO system (Millipore, Billerica, MA). Pierce™ Trypsin Protease MS-Grade 5 x 20 µg (90057), and Pierce Universal Nuclease for Cell Lysis 5kU, 20 µl (88700) are all from Fisher Scientific (Waltham, MA). Digestion buffer, cell lysis buffer, dithiothreitol (DTT), and Iodoacetamide (IAA) came as part of the Pierce™ Mass Spec Sample Prep Kit for Cultured Cells (84840) purchased from Thermo Fisher Scientific (Waltham, MA). Full-length human APE1 was purified as previously described by Zhang et al (24) The DNA used for this study was a 26-mer AP site-containing DNA duplex, 5'-AATTCACCGGTACC(THF)CCTAGAATTCG-3' and complementary strand 3'-TTAAGTGGCCATGGTGGATCTTAAGC- 5' purchased from Midland Certified Reagent Company, Inc. (Midland, TX) by the Georgiadis laboratory in the Department of Biochemistry and Molecular, Indiana University School of Medicine (Indianapolis, Indiana).

### 3.2 FPOP labeling

APE1 with and without DNA samples were provided by the Georgiadis Lab (IU School of Medicine). Samples were provided in 35 µl aliquot volumes with 10 µM of APE1 alone or in complex with 20 µM of 26-mer DNA in 50 mM HEPES pH 7.5, 50 mM KCl, 1 mM DTT buffer solution. Additionally glutamine was added to each sample for a final concentration of 40 mM. Hydrogen peroxide, a 5 µl volume was added immediately before FPOP with varying concentrations per the experimental parameters: final concentrations 3.75 mM, 7.5 mM and 15 mM).

The KrF excimer laser (GAM Laser Inc, Orlando, FL, USA) was set to a frequency of 10 Hz and wavelength of 248 nm which does not overlap with absorbance of biological molecules (38). Samples were infused by a 100 µl Hamilton syringe at a

rate of 33.8  $\mu\text{l}/\text{min}$  through a flow tube made out of fused silica tubing (Polymicro Technologies, Phoenix, AZ, USA) with a 360  $\mu\text{m}$  outer diameter (OD) and 150  $\mu\text{m}$  inner diameter (ID). The coating was removed by burning with a household lighter to create a window for irradiation.

The sample was pumped at a constant flow rate and fed into a quenching solution containing methionine and catalase. Samples were vortexed, immediately placed on ice and stored at 4  $^{\circ}\text{C}$  for further processing. For each experiment, there were control (not irradiated) and FPOP (irradiated) samples for each condition tested. Triplicates or quadruplicates FPOP and control samples were prepared in the same manner, and infused on the same day to ensure consistency.

### 3.3 Alkylation and Digestion

Each sample was digested for MS analysis following a modified procedure of Pierce<sup>TM</sup> Mass Spec Sample Prep Kit for Cultured Cells, Document 2522.1. Enzymatic digestion of DNA in the sample was done by adding 1.25 units of nuclease incubated for 15 min at room temperature. The sample was centrifuged at 16,000  $\times g$  for 10 min at 4  $^{\circ}\text{C}$  (Survival Legend micro 21 centrifuge, Thermo Fisher Scientific, Asheville, NC)). Pierce cell lysis buffer was added to a final volume of 100  $\mu\text{l}$ . Disulfide bonds were reduced by adding 500mM DTT to a final concentration of 10mM. Samples were incubated at 50  $^{\circ}\text{C}$  in a water bath (FS30D water bath, Fisher Scientific) for 45 min and were allowed to cool at room temperature for 10 min. Thiol groups were protected by adding IAA to the sample to a final concentration of 50 mM and allowed to incubate for 20 min at room temperature covered with aluminum foil.

Following the alkylation by IAA, 460  $\mu\text{l}$  of 100% acetone pre-chilled at -20  $^{\circ}\text{C}$  was added to the sample and allowed to precipitate at -20  $^{\circ}\text{C}$  overnight. The sample was then centrifuged at 16,000  $\times g$  for 10 min. The acetone was carefully removed, and the pellet was washed with 50  $\mu\text{l}$  of 90% pre-chilled acetone and centrifuged as previously described for 5 minutes. The acetone was removed and samples were air dried for 3-4 min. The samples were resuspended in 100  $\mu\text{l}$  of the Pierce digestion buffer. Trypsin was reconstituted in 1 mM hydrogen chloride (HCl), and 2  $\mu\text{g}$  of trypsin was added to each

sample. The samples were incubated at 37 °C (HeraTemp incubator, Thermo Fisher) and digested overnight.

Digestion was quenched with formic acid final concentration of 5 %. The samples were then dried in a speed vacuum (Savant speed vacuum SC110 with refrigerated condensation trap in conjunction with a dual seal pump from the Welch Scientific Company). Samples were resuspended with 2 µl of 0.1% FA in ACN and 18 µl of 0.1% FA.

### 3.4 Mass Spectrometry

An UltiMate 3000 RSLC and Q-Exactive Orbitrap MS (Thermo Fisher Scientific, Mississauga, ON) were used for the analysis of the sample. The sample was loaded onto a 35 cm column in house bomb-packed with media magic 5 micron C18 particles (Michrome Bioresources Inc, Auburn, CA, USA). The spectra of eluting peptides were obtained at high resolution of 70,000 with a scan range of 350 to 200 *m/z*. MS/MS was set to select the top 15 abundant peptides eluting at a normalized collision energy of 30% of maximum and resolving power of 17,500. Ions with charged states of 1, 7, 8, > 8 and unassigned charges were rejected. Ion trap automatic gain control (AGC) target was set to 3e6 for MS and 1e5 for MS/MS with an underfill ratio of 5 % and an intensity threshold of 3.3e4. The gradient was linear from 4% solvent B (0.1% FA in ACN) to 45% solvent B over an eloquent flow of 0.300 µl/ min. The method has a total runtime of 110 min and 87 min retention time. Blanks were run in between each sample.

### 3.5 Data Acquisition & Analysis

Raw data files from each experiment were searched against a FASTA database containing the sequence for APE1 (Uniprot #P27695) and the sequence from the cRAP database ([thegpm.org/crap](http://thegpm.org/crap)) [accessed on 02/27/2015] using Proteome Discoverer version 1.4 (Thermo Fisher Scientific, Mississauga, ON). As shown in Figure. 9, different filters were set for the search to achieve high confidence results. Protein spectrum match (PSM) search results were filtered to yield a false discovery rate (FDR) of 1%, and PSM ambiguity was set to “not rejected nor unconsidered”. Only PSMs with peak area and ranks of 1 & 2 were selected. MS<sup>2</sup> spectra searches were done by Sequest HT and Mascot

(Matrix Sciences Ltd., London, UK). Precursor tolerance was set to 10 ppm with fragment ion tolerance of 0.02 Da. Xcorr in Sequest HT was set to 2.1 and an ion score of 30 was set for Mascot. Data was exported to Excel and condensed with manual PowerPivot.

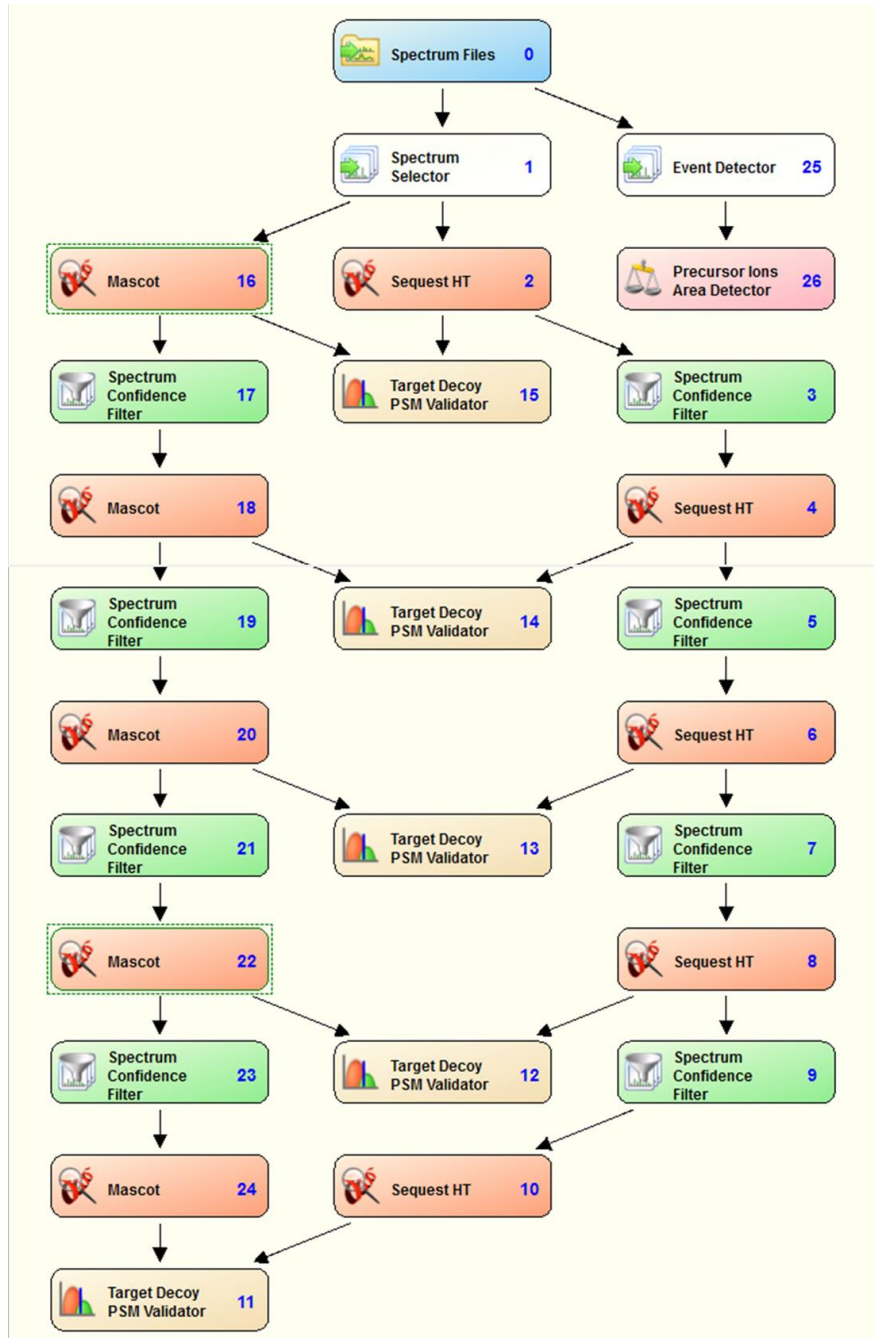


Figure 9. Proteome Discoverer version 1.4 workflow provided by Aimee L. Rinas.

## Chapter 4. INVESTIGATION OF APE1 WITH FPOP AND MS

### 4.1 Labeling reproducibility of FPOP at a residue level using APE1 protein

#### 4.1.1 Introduction

The utilization of FPOP to label proteins is a promising method for mapping protein-protein interactions and protein folding. Proteins are rapidly labeled through the use of hydroxyl radicals and a pulsed laser with minimal damage to the sample (8). However, there are a number of variables that affect the production of radicals by laser-induced photolysis of hydrogen peroxide including fluctuating laser energy and residue reactivity differences leading to some variation in exactly which residues are labeled within a protein from one experiment to the next. Here, we examined reproducibility and the effect of DNA-binding on FPOP labeling of APE1 at the residue-level. We also examined the effect of varying the hydrogen peroxide concentration on FPOP labeling at a global level to determine the extent of labeling in APE1.

#### 4.1.2 Experiment Design

##### 4.1.2.1 Intact protein analysis

APE1 samples were prepared and oxidized as described in the Methods section. Three different hydrogen peroxide final concentrations were tested: 3.75 mM, 7.5 mM and 15 mM, with duplicates of each condition. Analysis was performed with Accurate-Mass Q-TOF 6520 coupled with 1200 Series LC from Agilent Technologies and a C4 reverse phase column. The Time-of-Flight (TOF) spectra mass range was set to 300-1700 m/z. The total run time was 12 min at a flow rate of 0.2 ml/min with a 4% to 60% solvent B (0.1% FA in ACN) gradient at 6 min followed by a 60% to 100% solvent B gradient. The Agilent Mass Hunter Workstation Data acquisition and Mass Hunter Qualitative Analysis Version 3.0 were used to acquire and process data.

##### 4.1.2.2 Reproducibility experiments

Three independent experiments were performed on non-consecutive days over a period of three months. For each, triplicate FPOP and control samples were prepared, oxidized and analyzed as described in the Methods section. A final concentration of 7.5

mM hydrogen peroxide was used. Data analysis was done individually following completion of each experiment.

#### 4.1.3 Results & Discussion

##### 4.1.3.1 Intact protein analysis

MS spectra of each sample were deconvoluted and overlaid for analysis by visual comparison. For the analysis of FPOP reproducibility at different concentrations of duplicates of each hydrogen peroxide concentration were overlaid separately in Figure 10b. The deconvoluted spectra for each concentration of hydrogen peroxide include four peaks corresponding to approximate masses of 35641 Da (unmodified protein), 35810 Da (plus 169 or ~10 OH), and 35965 Da (plus ~20 OH). The unmodified peak mass or native peak ~ 35641 Da agrees with the actual molecular weight 35641.5 Da of APE1. Also a smaller peak of 35597 Da (minus ~45 Da) is observed before the native peak. This loss of mass is due to the double ring opening of His (28).

As expected the native peak has the highest intensity representing higher concentration of unmodified APE1 detected. The preceding peaks are representation of the different oxygen species due to FPOP oxidation. The addition of a hydroxyl (+16) is the most common oxidation and the second highest peak after the native peak followed by two hydroxyl (+32) and three hydroxyl (+48) additions. The next oxidation species with significant intensities were identified at 35810 Da and 35965 Da representing +10 hydroxyl and +20 hydroxyl accordingly. These heavy labeling suggests there is a substantial solvent accessible surface which allows multiple hydroxyl additions.

##### a) APE1 without FPOP

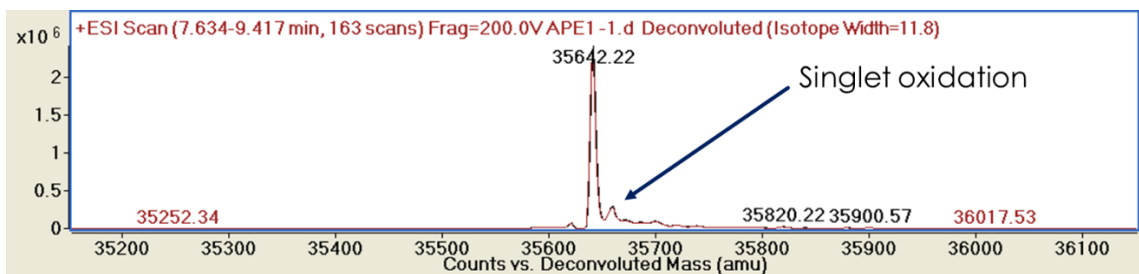
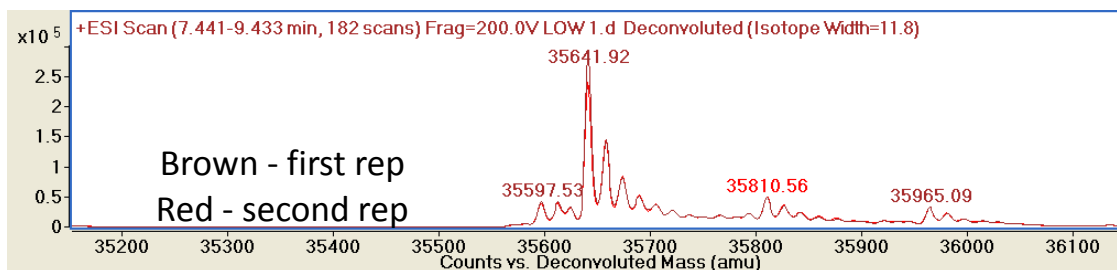
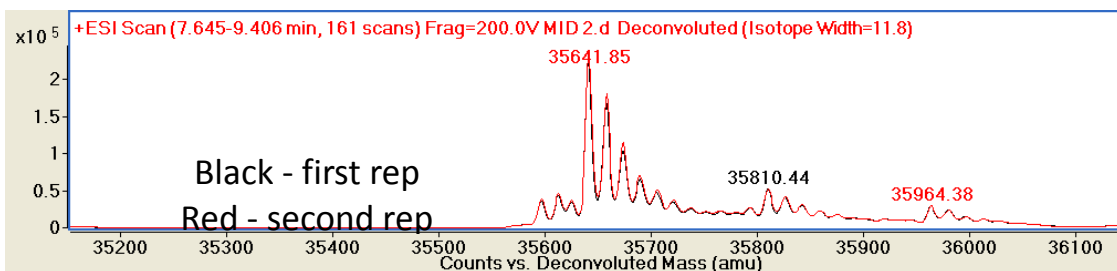


Figure. 10 Results of intact protein experiment - Continued.

b) Low concentration – 3.75 mM of hydrogen peroxide



Mid concentration – 7.5 mM of hydrogen peroxide



High concentration – 15 mM of hydrogen peroxide

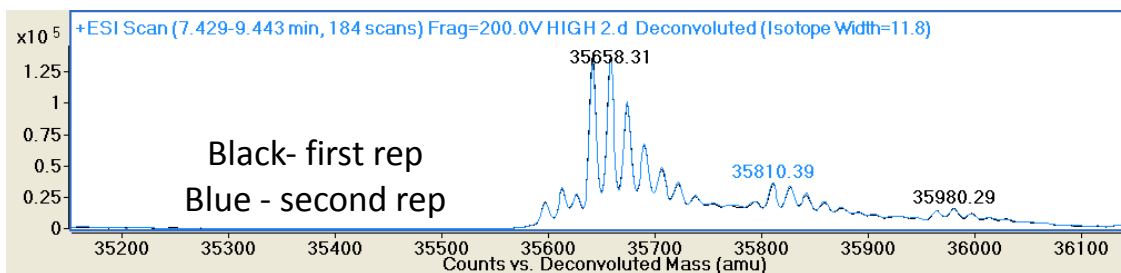
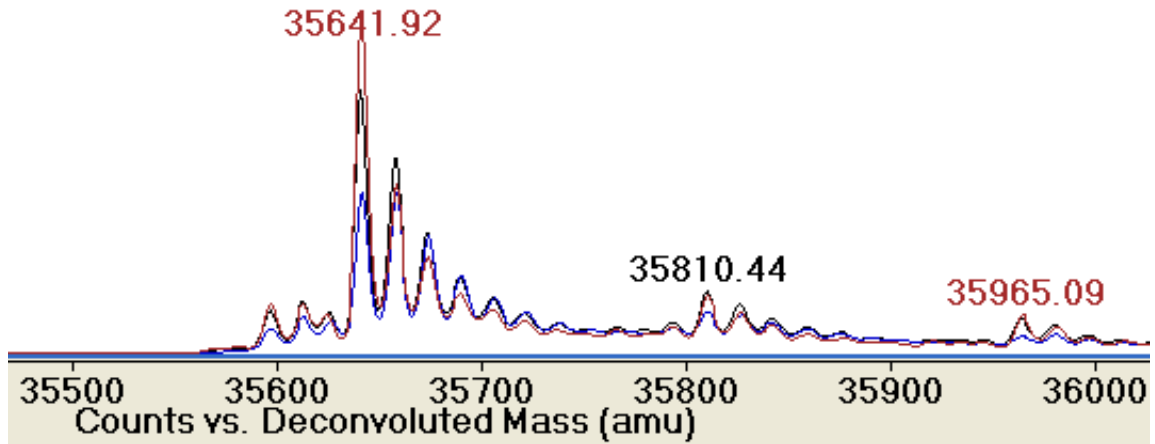
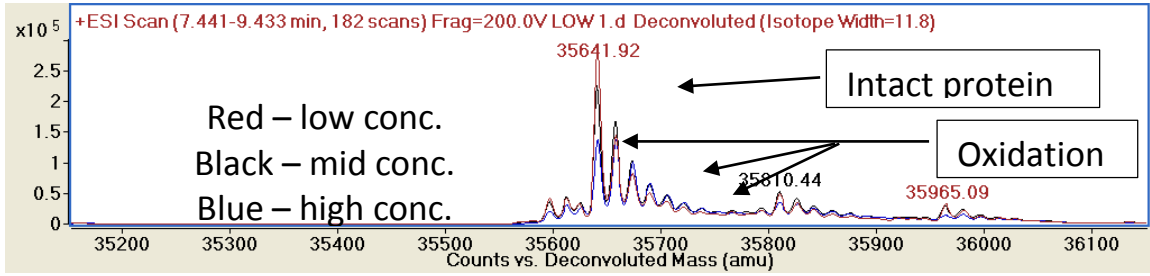


Figure. 10 Results of intact protein experiment - Continued.

c) Comparison of first replicates different concentrations.



Comparison of second replicates different concentrations

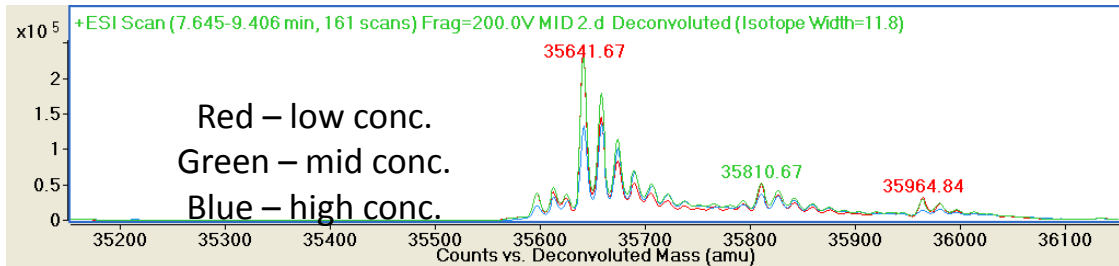


Figure. 10 Results of intact protein experiment - Continued.

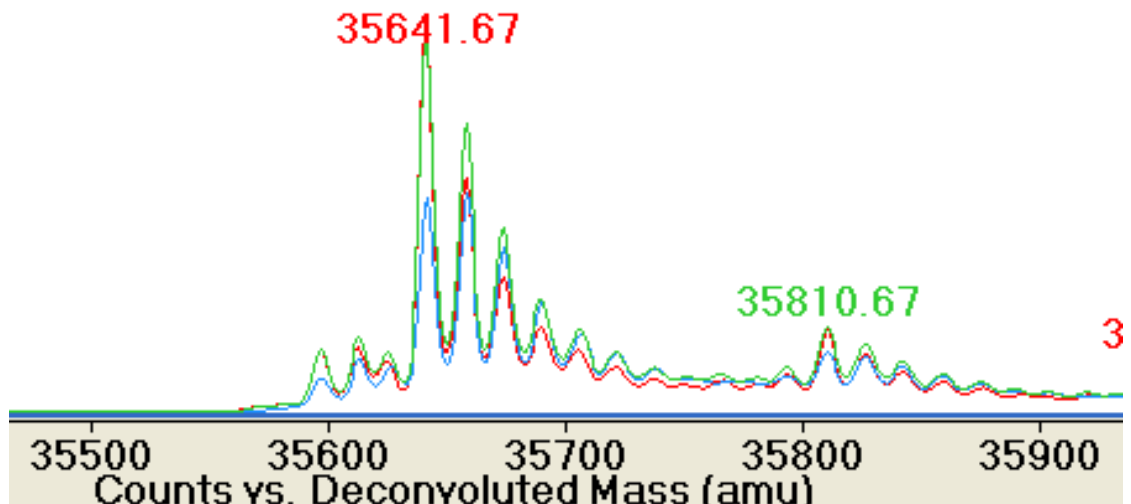


Figure. 10 Results of intact protein experiment: a) APE1 without FPOP oxidation, b) hydrogen peroxide condition duplicate oxidation precision, and c) comparison of three hydrogen peroxide conditions oxidation extent chromatograph large and magnified view.

The effects of hydrogen peroxide concentration were compared by overlaying the three concentrations considered (Figure. 10c) and comparing the ratio of modified to native peak intensities. The low concentration (3.75 mM), represented in red, has high intensity for the unmodified peak and as oxidation states appear becomes the lowest intensity. The opposite is seen with the high concentration shown in blue. The high concentration (15 mM) has the lowest intensity in the unmodified peak but the highest in the oxidative species peaks. While the middle concentration (7.5 mM) intensities lie between the range of the high and the low concentration for all peaks. Overall as the concentration of hydrogen peroxide was increased, the peak height for the oxidative species increased while that of the native protein decreased. Thus, the results indicate more oxidation occurs with higher concentrations of hydrogen peroxide.

#### 4.1.3.2 Reproducibility of FPOP

For peptide level analysis, data files were normalized to catalase, and averaged with Carbamidomethyl modification being excluded. Percent oxidation was calculated against the oxidation of control samples. A Proteome Discoverer peptide search, presented in Figure. 12, had an acceptable match coverage of 81.11% for the first

experiment (expt), for the 2<sup>nd</sup> expt 82.33% and for the 3<sup>rd</sup> expt 80.82%. The percentage coverage is the representation of APE1 peptides identified by tandem MS. The coverage among experiments was consistent although oxidation of peptides differed in the three experiments (Figure. 11). This would indicate that there is variation in labeling from one FPOP experiment to the next. To further investigate the extent and position of oxidative labeling in these experiments, the data were analyzed at the residue level.

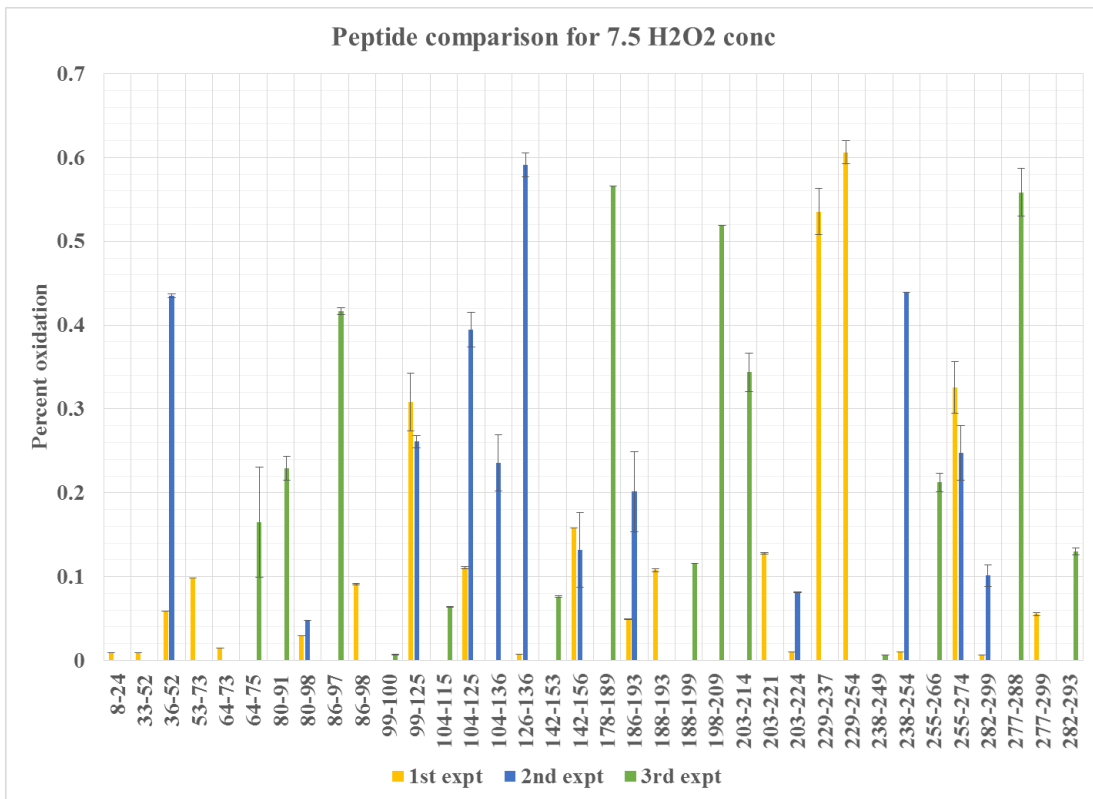


Figure 11. Peptide level oxidation of independent triplicate analysis



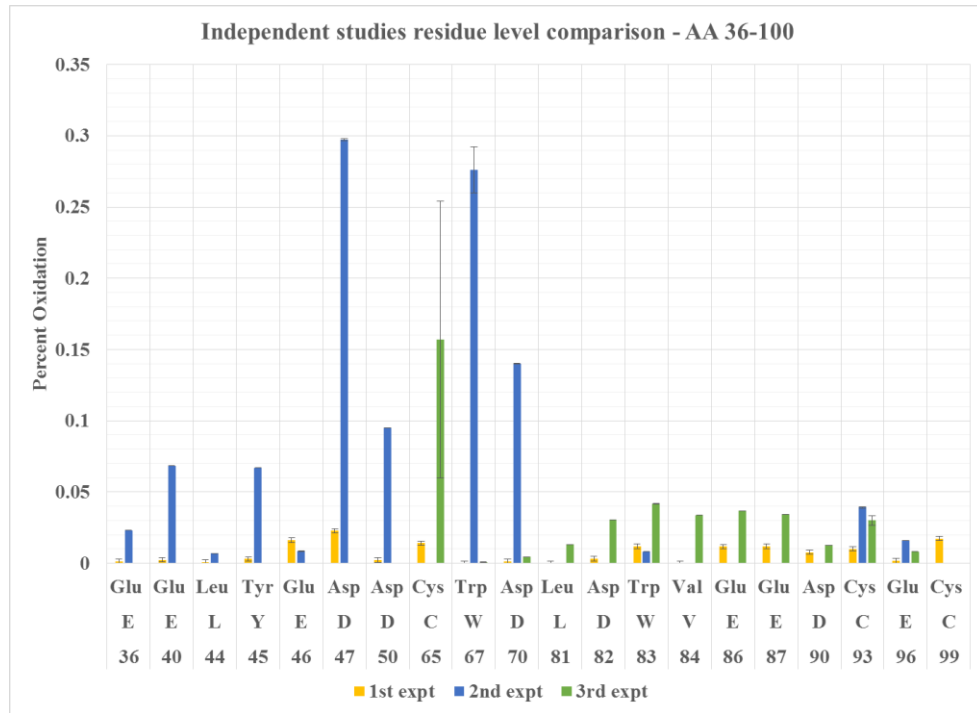
Figure 12. Peptide level database match coverage

For residue level analysis, residues with 0.01 or greater percent oxidation were considered significant, and controls were used to normalize for non-FPOP oxidation. In Figure. 13 a-d the results show differences in the residues oxidized in each experiment. The segments of residues 107-128 and 264-272 seem to have uniform oxidation exposure and labeling in all three experiments. However, these residue segments and other residues oxidized in all three experiments have a low level of oxidation that lie below the 0.01 percent threshold.

On the other hand there is significant labeling on residues that are structurally buried according to SASA values. The SASA values were calculated by POPs wiki using 4IEM PBD with DNA and waters removed. SASA values of 0.11 or less were considered buried or internal. Buried residues labeled in at least one experiment are Trp188, Asp219, Ala263, Tyr264, Arg281, Tyr284, Phe285 and Cys296 with Cys65, Trp67 and Glu236 being preferentially labeled. Cys93 is the only buried residue that was labeled in all three experiments. Given that the labeling occurs within a microsecond, these results suggest that the internal residues labeled in this experiment were in fact accessible.

Also the labeling of a small number of buried residues rule out the possibility of unfolded protein being detected. It could be possible that for some unknown reason, some proteins denature and unfold. An unfold protein would acquire more FPOP oxidation due to the increase of SA. If the abundance of unfolded proteins is low then MS setting would not select it since the settings are to identify only top 15 peptides. However if the abundance of unfolded protein is large then in the analysis we would see more buried residues that had been oxidized. Thus the few buried residues labeled confirm that unfolded proteins were not detected by MS analysis.

a)



b)

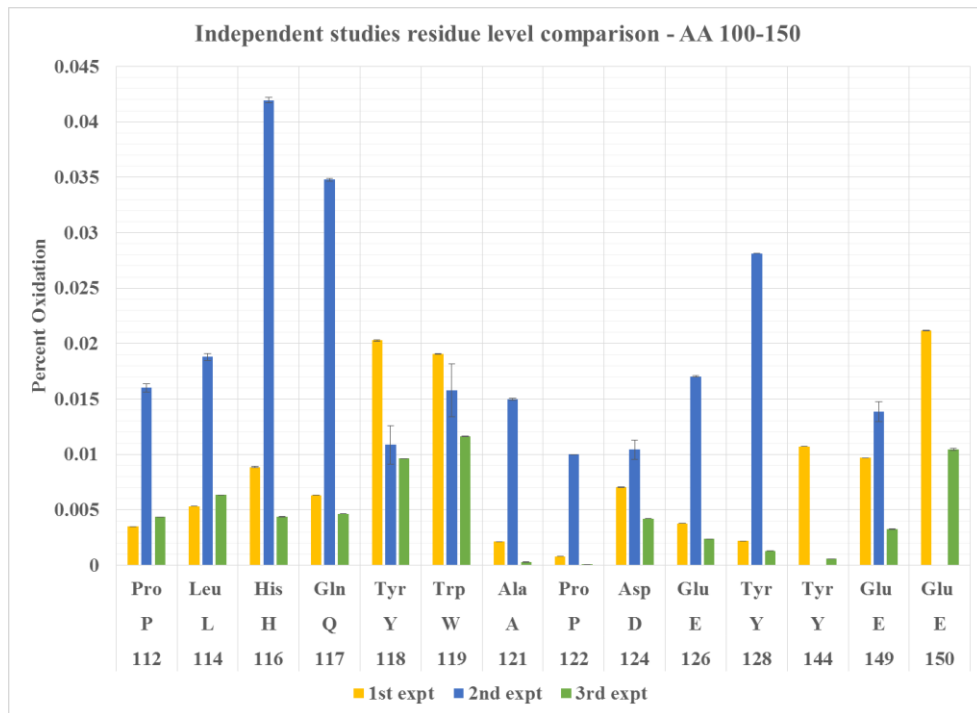
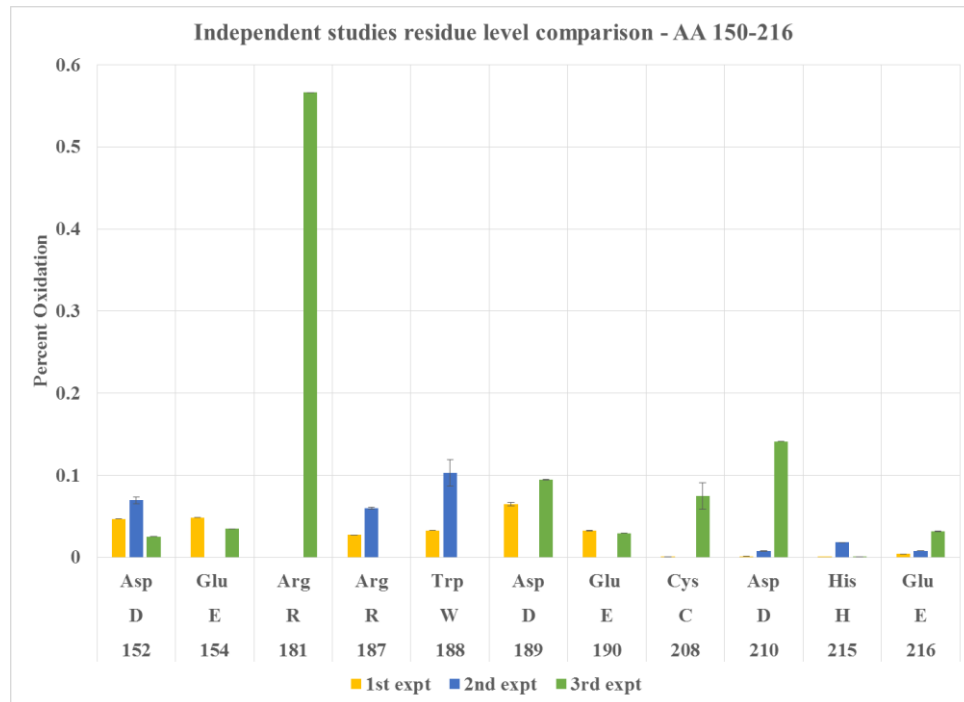


Figure. 13 Residue level oxidation of independent triplicate analysis of APE1 -  
Continued

c)



d)

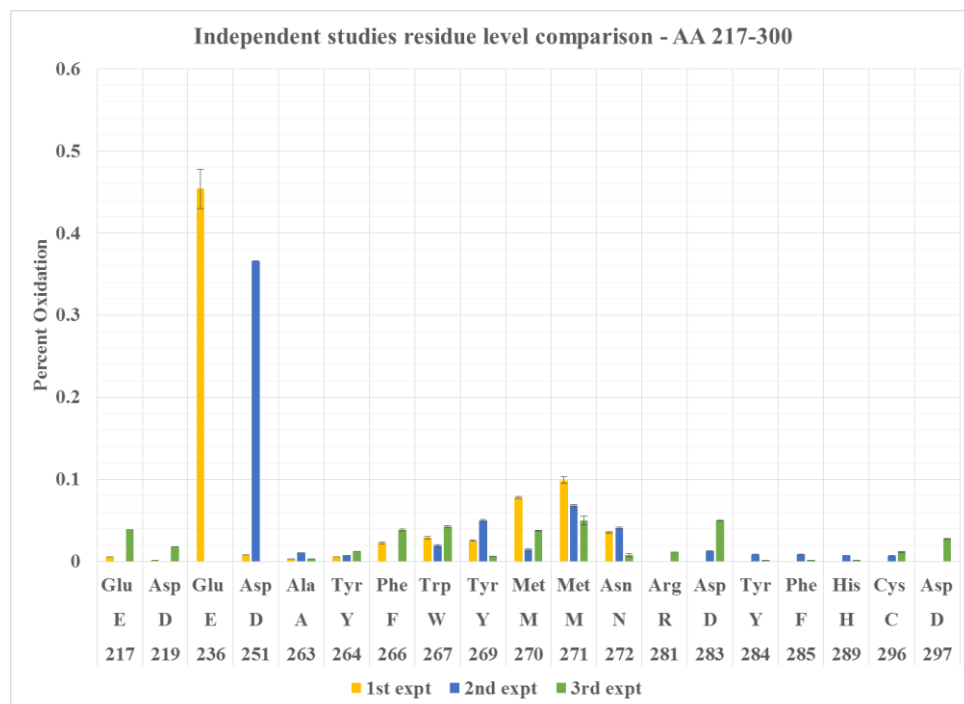


Figure. 13 Residue level oxidation of independent triplicate analysis of APE1. A-D graphs show chronological order of APE1 residues. Scale adjusted in each graph for better view.

## 4.2 Structural analysis of APE1-DNA complex with FPOP

### 4.2.1 Introduction

FPOP has been previously used to map antigen-antibody complexes by Jones et al (7). This study demonstrates the use of FPOP to map epitopes and established FPOP coupled with mass spectrometry as a valuable method for structural studies of complexes. Here, we aimed to study APE1-DNA complexed with FPOP coupled to tandem MS as a test case for use of this method to study protein-nucleic acid interactions. Crystal structures have been reported for both APE1 alone and in complex with DNA (2, 7, 9, 19, 20, and 26). We also aim to investigate the structural interacting information that can be obtained with the use of FPOP coupled to tandem MS. Residue level data may provide new insights on the interactions of APE1 with DNA and identify conformational changes that were not captured in the crystallographic work. Such studies may ultimately enable advances in drug discovery targeting APE1 especially in the field of cancer biology.

### 4.2.2 Experiment Design

Triplicate samples of APE1 and APE1 complexed with DNA with corresponding controls were prepared, oxidized, and analyzed as described in the Methods section. The final concentration of hydrogen peroxide was of 7.5 mM for both conditions. Protein and complexed were analyzed in parallel. The DNA sequence includes tetrahydrofuran (THF) as a mimic of an AP site. As shown in previous work, APE1 efficiently cleaves this substrate in the presence of  $Mg^{2+}$  (19).

### 4.2.3 Results & Discussion

Raw data files were normalized to catalase, and averaged. Percent oxidation was calculated against the oxidation of control samples. FPOP oxidation greater than 0.01 was considered significant. Results were calculated and plotted in Excel. Proteome discoverer search was of 80.82% peptide match and 65% oxidation coverage is seen in Figure 14a

for APE1. For peptide level, the results show a greater number of peptides being preferentially oxidized in APE1 alone than when it is complexed with DNA. This would be expected since complexing with DNA hide many residues and protects them from being modified. Also interestingly peptides 80-98, 188-193, 228-237 and 238-274 are preferentially oxidized in the DNA complex. These peptides are mapped in Figure 14b.

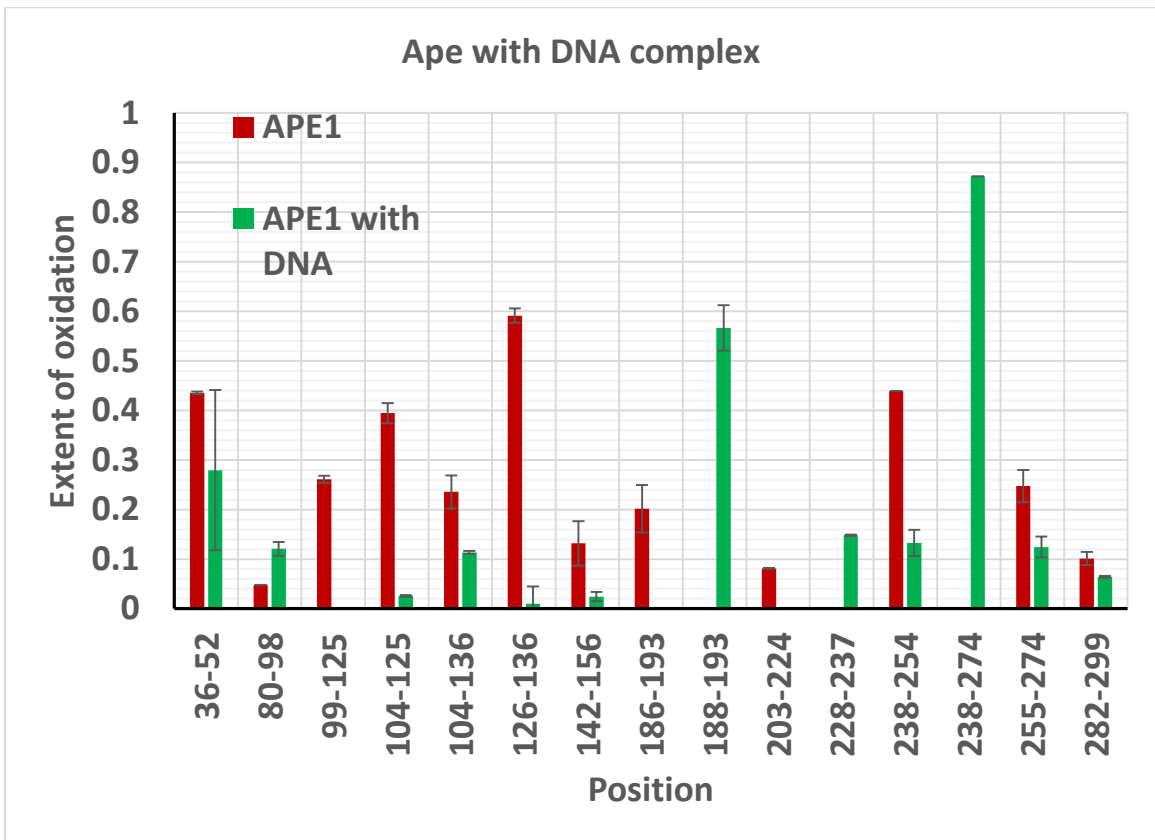


Figure 14. Peptide level FPOP results - Continued

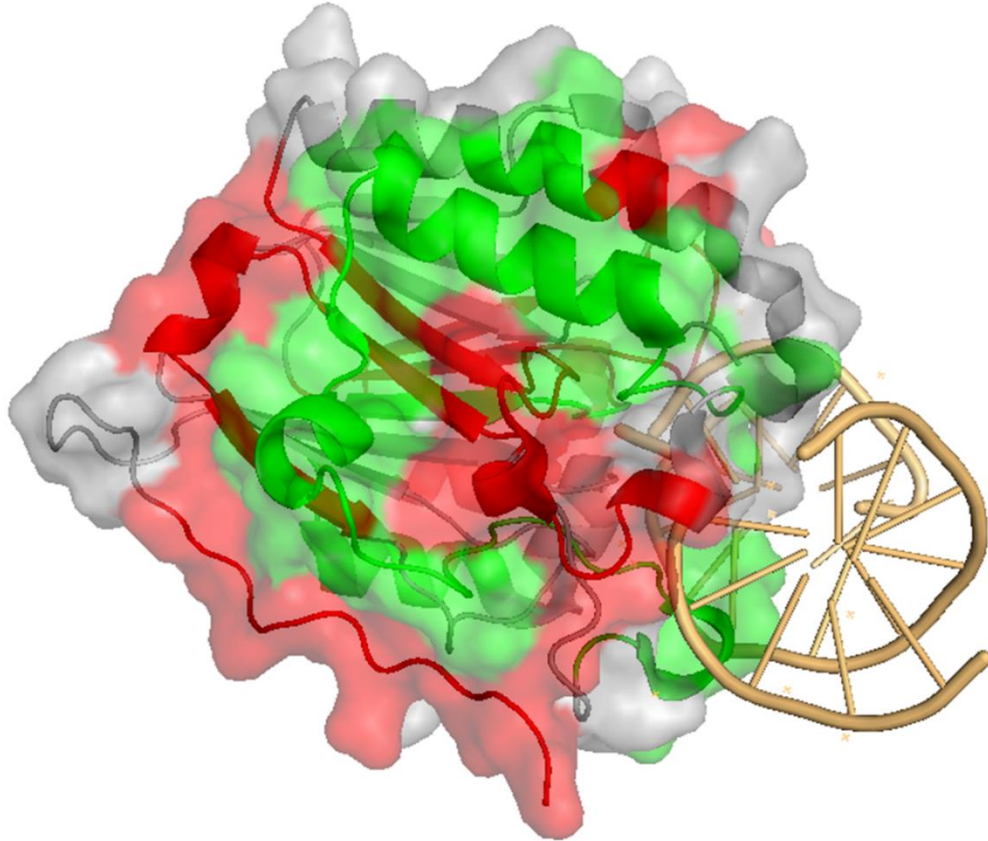
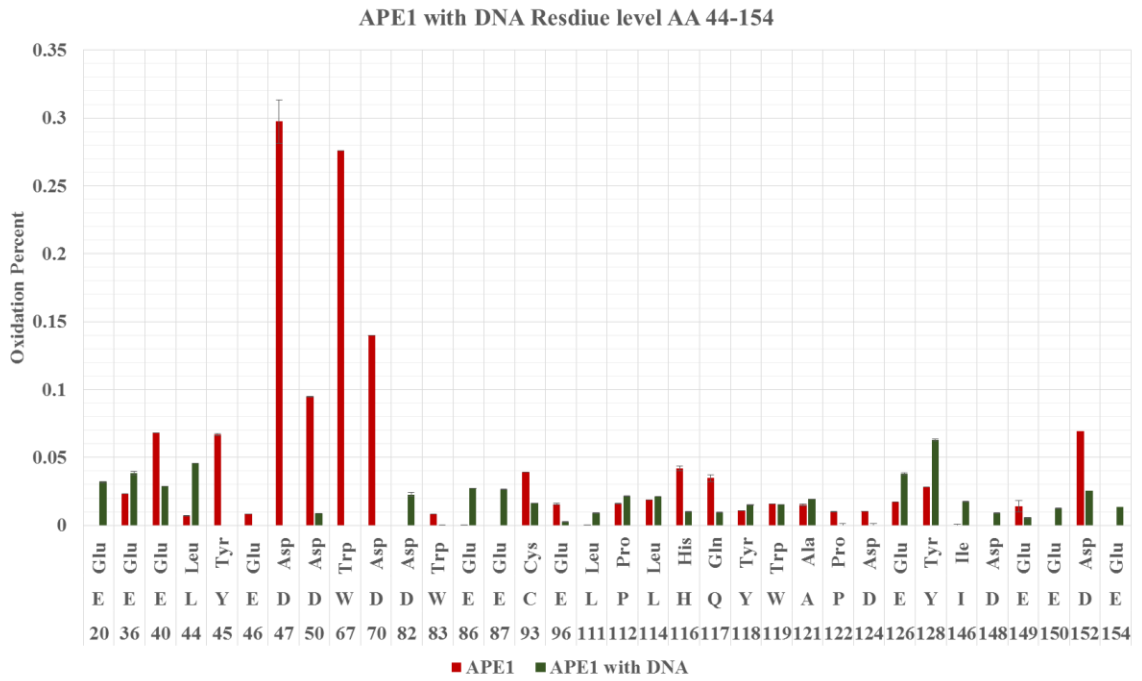


Figure 14. Peptide level FPOP results: a) bar graph representation and b) peptides mapped into 4IEM structure with preferentially labeled APE1 peptides in red and preferentially labeled with DNA peptides in green.

At the residue level, in general more residues were oxidized in APE1 with DNA versus APE1 (Figure 15 a-b). APE1 alone had 11.6% oxidation coverage with 37 total residues labeled and 9.3% of SASA residues labeled. The APE1 with DNA had 13.5% oxidation coverage with 43 residues labeled and 14.6% labeling of SASA residues. A total of 23 residues are labeled in both conditions with Pro112, Leu114, Trp119, Ala 121, Glu216 and Met270 having approximately equal oxidation. These residues lie in two localized oxidation areas between residues 111-128 and 266-272 where oxidation is seen in both conditions, implying the residues are on the surface and readily available. Overall the oxidation of highly reactive residues like Trp, Tyr and Phe is seen; but oxidation of less reactive Glu and Asp is also observed. Thus, FPOP oxidation fairly represents residues of different reactivities.

a)



b)

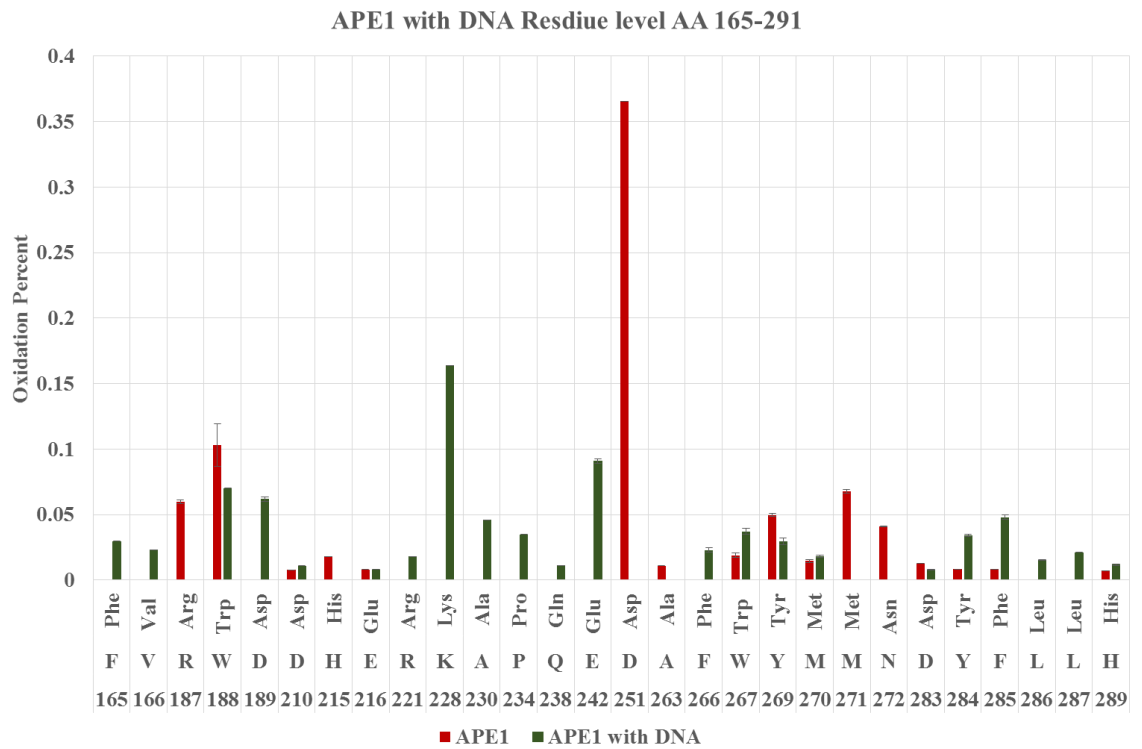


Figure 15. Residue level FPOP results bar graph results

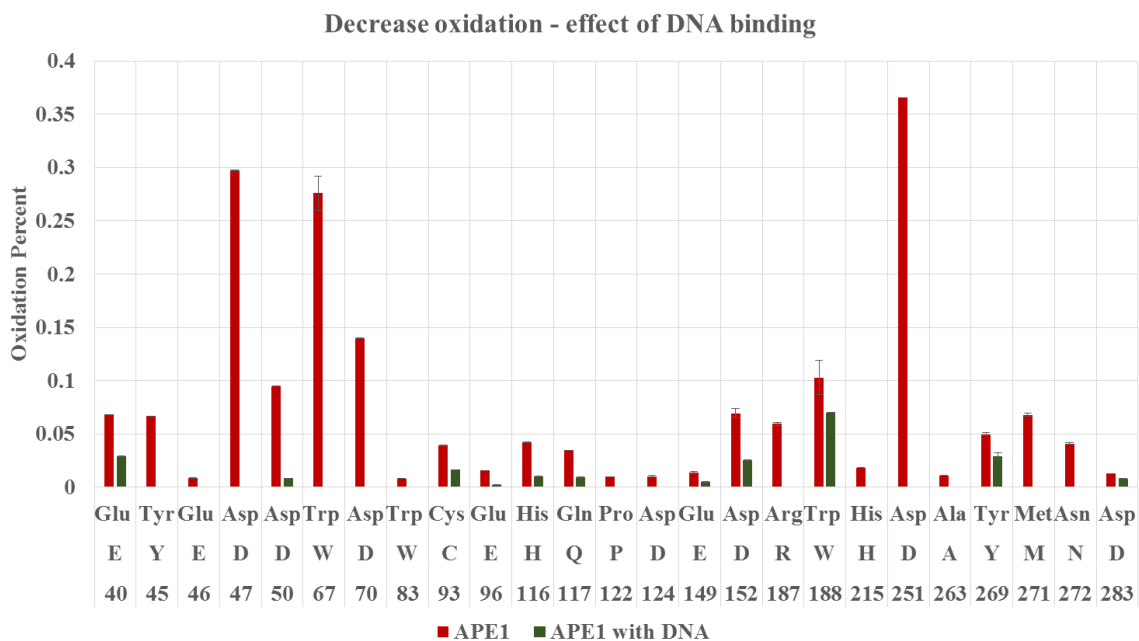
The results also showed unexpected labeling of buried residues in both conditions. The SASA values were calculated by POPs wiki using 4IEM and a value of 0.11 or less were considered buried. For APE1 alone, SASA values were calculated with the DNA and waters were removed from the PBD file. The list of buried residues labeled at each condition is compiled in Table 3. The buried residues all have high hydroxyl radical reaction rates with Trp being the highest and showing an oxidation percent greater than 0.05 for both Trp67 and Trp188. For the residues to be oxidized by FPOP, in particular the labeling of Cys93, it would mean that APE1 undergoes a conformational change that makes these residues accessible. Hence, this result provides insightful structural information of APE1.

| APE1 internal residues labeled               | APE1-DNA complex internal residues labeled |
|--|--|
| Trp67, Cys93, Trp188, Asp283, Tyr284, Phe285 | Ile146, Trp188, Tyr284, Asp283 and Leu286  |

Table 3. List of Internal Residues Labeled by FPOP in APE1& APE1-DNA complex

The impact of DNA binding was assessed by observing oxidation changes when DNA binds APE1. In general, residues are preferentially labeled in only one condition. Figure 16a presents all the residues that were preferentially labeled in APE1. The oxidation effect of these residues upon DNA binding shows a dramatic decrease with most residues oxidation being null in the presence of DNA. Figure 16b presents the residues preferentially labeled in APE1 with DNA. Inversely these residues have a favorable effect with an increase of oxidation upon DNA binding. For buried residues that are found in both conditions, Trp188 oxidation decreased with DNA binding while Tyr284 and Phe285 oxidation was quadrupled in the presence of DNA. This could mean that Tyr284 and Phe285 becoming more solvent accessible with DNA binding. Comprehensively, the results indicate that the binding of DNA has a great impact, and creates a major effect on both buried and surface residues oxidation.

a)



b)

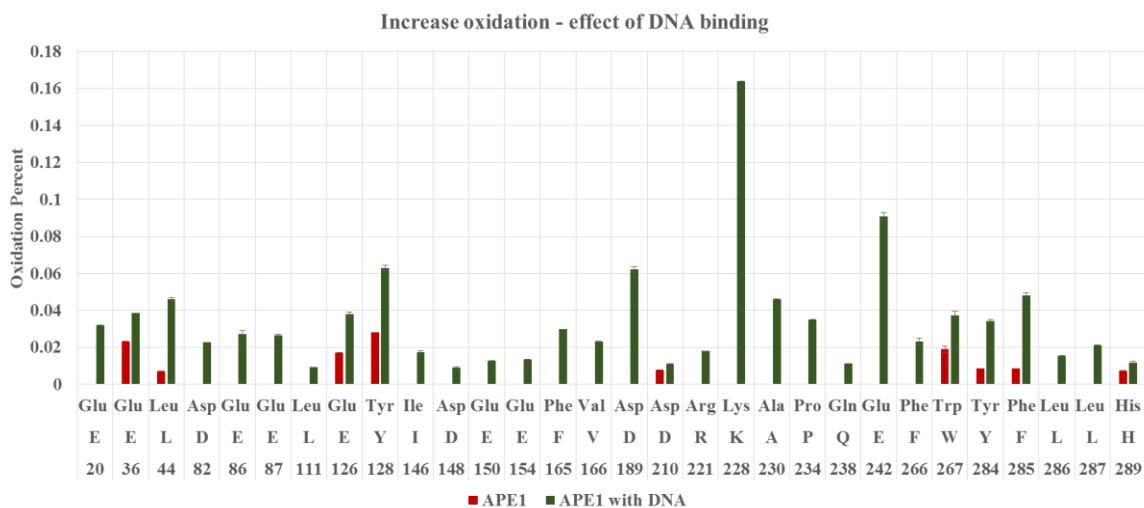


Figure 16. Impact of DNA binding on residue oxidation

Previously presented data on Figure 16a also allowed the investigation of DNA interacting residues. The residues interacting with DNA would be preferentially oxidized in the absence of DNA. Although the DNA-binding surface within APE1 is relatively large accommodating approximately 10 base pairs of duplex, only five residues were

preferentially labeled: Asp70, Glu96, Tyr269, Met271, and Asn272. These residues lie within a region of the protein that protects them from labeling in the presence of bound DNA as seen in Figure17. Thus, while this differential labeling is consistent with the crystallographic results, it is insufficient to fully define the DNA-interacting surface of the protein.

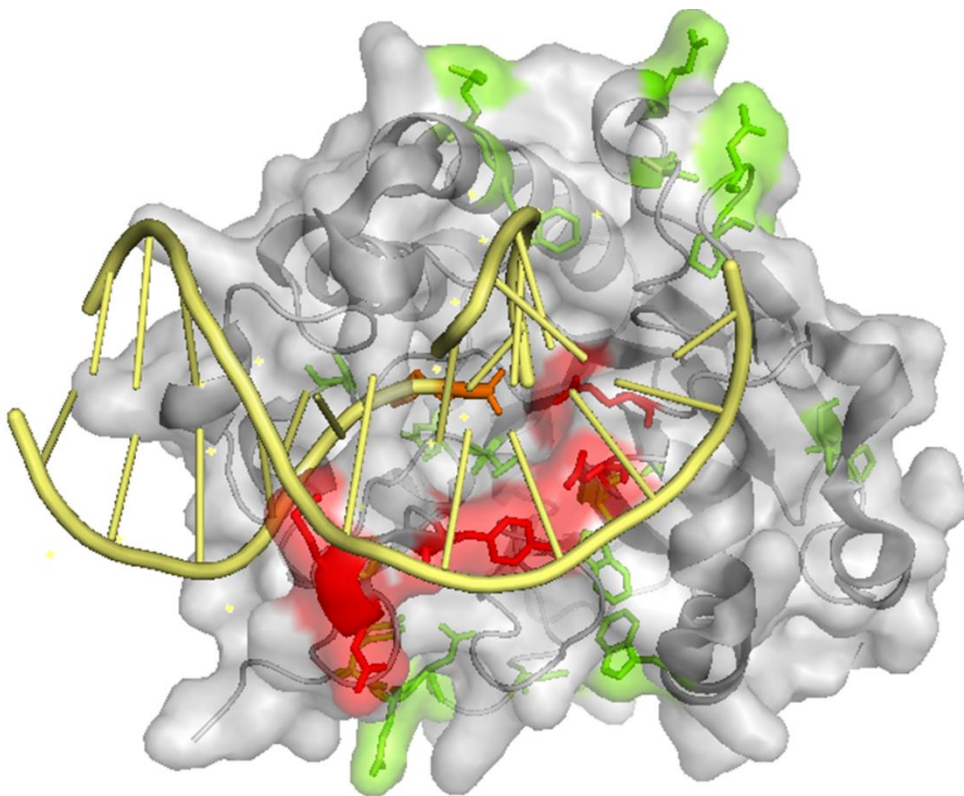


Figure 17. APE1 residues that interact with DNA. In green are residues preferentially labeled in APE1 and DNA interacting residues are labeled in red. DNA structure with AP site in orange added for visual confirmation.

#### 4.3 Conclusion

FPOP is a fast method ideal for structural studies of protein. The use of radicals is one of the advantages of FPOP, but the inexact behavior could lead to inconsistent results. In the intact protein experiment, the same peaks and oxidative species were detected in all three hydrogen peroxide conditions. This shows that at a given hydrogen

peroxide concentration the global levels of oxidative labeling for APE1 are very consistent for duplicate samples. The results also demonstrate increases in oxidation as hydrogen peroxide concentration is increased; thus establishing a correlation between hydrogen peroxide concentration and FPOP oxidation.

In the three non-consecutive independent experiments processed using the same procedure, residue labeling was not consistent for all three experiments. Since oxidation occurs within micro-seconds, the inconsistent labeling may reflect a dynamic population of APE1 molecules. But, perhaps of more interest is the consistent labeling of buried residues. These results support conclusions from previous work in which APE1 was proposed to exist in both fully folded and partially unfolded states. Altogether the intact protein and independent experiments provide evidence that FPOP oxidation is fairly reproducible in labeling approximately the same percentage of surface and buried residues even if the residues labeled in each case are not the same. However, assessment of oxidation extent is required. In order to complement the results presented here, an investigation of potential over-oxidation by FPOP is will be performed.

Furthermore, APE1 complexed with DNA was used to investigate the use of FPOP coupled with MS for protein-nucleic acid interaction and structural interactions. Results showed that only five residues that interact with DNA were protected. Of these Asp 70 and Glu 96 are critical for the initial capture of  $Mg^{2+}$  and catalysis (30). This shows consistency with the crystallographic data available. Nonetheless, the oxidation observed does not fully define the DNA-interacting surface of the protein.

Collectively in all of the APE1 experiments to date, buried residues including Cys 65, Trp 67, Cys93, Trp 188, Asp 283, and Tyr 284 within APE1 were labeled in the absence of DNA. However, upon addition of DNA these residues were protected from labeling. This result is consistent with fully folded APE1 interacting with the DNA. In addition, several residues were labeled preferentially in the presence of bound DNA consistent with local conformational changes not observed in the crystal structures of APE1 bound to DNA.

Based on our results, here we theorize that DNA binding changes the dynamics of APE1. Entropy is loss when DNA binds and interactions free up which allow for

oxidation of buried residues. In Figure 18, we depict that complex formation of APE1 decreases fluidity of the top half of the protein while making the lower half more fluid. Changes in fluidity and exposure of residues of APE1 would allow for the cysteine residues to become accessible and participate in redox reactions. On the other hand, the binding of DNA would decrease fluidity of the complex surface and the lodge of the proper residues for the AP endonuclease function. Specifically, the oxidation of redox critical Cys65 supports that APE1 complex formation increases dynamics of sites remote from the interacting surface. Hence, breathing dynamics of APE1 would allow it to perform separately its dual functions.

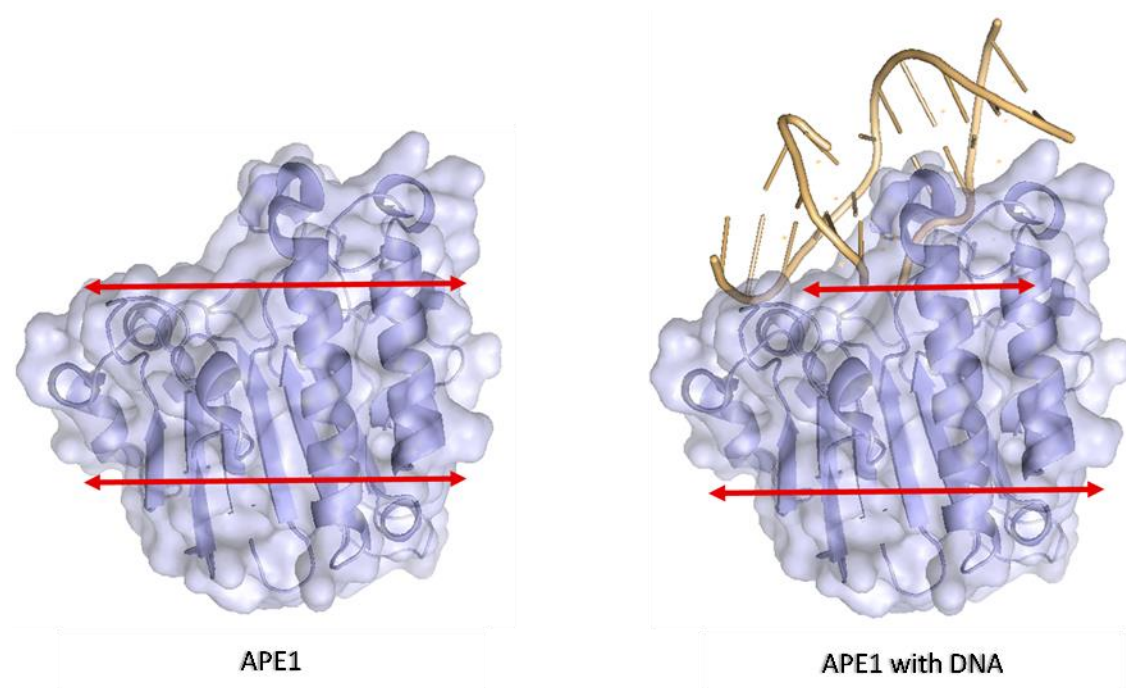


Figure 18. APE1 diagram of breathing dynamic.

In conclusion, labeling of the APE1-DNA complex did not provide substantial data to probe the interactions. However, the results confirm that FPOP is a useful method for protein study and establishes it as a method to capture protein's dynamics that can't be observed in crystals structures. Additionally, the results showed labeling of buried residues which confirms the theory of Georgiadis et al. that APE1 exists in a secondary conformation, and we proposed APE1 possessing breathing dynamics that alter the

protein's fluidity when DNA binds. As a potential follow up to our theory; protection factor (PF) analysis that incorporates SASA and residues reactivity with FPOP oxidation, can be done to quantitatively structurally characterize our results and map local accessibility of different regions of APE1(33).

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