Domain Mapping of Human Apurinic/Apyrimidinic Endonuclease

We recently described the pre-steady state enzymatic binding kinetics of apurinic/apyrimidinic endonuclease (AP endo). In this report we describe the domain structure of the enzyme in solution determined by mild protease digestion in the presence and absence of substrate, product, and an efficient competitive inhibitor (HIDP). AP endo is a 35.5-kDa protein with a high degree of homology to its prokaryotic counterpart, exonuclease III (Exo III), except for the amino terminus, which is lacking in the prokaryotic enzyme. The entire conserved region plus an additional 20 residues unique to the eukaryotic enzyme was inaccessible to trypsin and V8 protease, indicating that it forms a tight globular structure. In contrast, the amino-terminal 35 residues were readily accessible to all the proteases investigated, leading us to conclude that they associate poorly with the rest of the structure and constitute a highly fluid region. When AP endo was boiled with SDS and cooled prior to the addition of V8 protease, several acidic residues within the globular domain became protease-accessible, indicating rapid renaturation except along the nuclease fold with restoration of globular conformation for the carboxy terminal two-thirds of the molecule. Of all the proteases tested, only chymotrypsin was able to cleave internal to the globular portion without prior denaturation. Although AP endo cleaved with chymotrypsin retained full enzymatic activity, the activity was lost when the digested peptides were recovered after denaturation by heat and/or boiling in SDS, precipitation, and renaturation or when fragments were recovered from an SDS gel and renatured. Thus, the protein is probably held together strongly by noncovalent interactions that maintain enzymatic function after protease nicking. The three major chymotrypsin cleavage sites, Tyr-144, Leu-179, and Leu-205, became strikingly less accessible to protease digestion in the presence of abasic site-containing DNA. Since the three residues form a spherical triangle on the surface of the molecule on one side of the nuclease fold, there must be multiple means by which DNA containing an abasic site associates with the enzyme. The most likely explanation is that substrate and product, both of which were present during proteolysis, bind differently to the enzyme. Finally, the two cysteine residues thought to be involved in the redox reaction of AP endo with Jun protein were entirely inaccessible to proteolysis even after prolonged exposure of AP endo to reducing agents. Consequently, if AP endo plays a role in the physiological function of Jun, it must undergo major conformational changes in the process. Alternatively, the two cysteines could maintain an appropriate conformation such that other residues participate directly in the redox activity.

Apurinic/apyrimidinic endonuclease (AP endo) is a critical enzyme in the repair of abasic sites in DNA. These sites can arise through spontaneous loss of a nucleobase, through the action of N-glycosyl hydrolases, or through oxidative damage of DNA (1, 2). The crystallographic structure of human AP endo has been determined at 2.2-Å resolution, revealing a 2-fold symmetric a/b fold with a single metal ion bound at the active site (3). The three-dimensional structure of the human enzyme is similar to the prokaryotic homologue, exonuclease III (Exo III), from Escherichia coli (4, 5). Not only does the amino acid alignment of AP endo with Exo III reveal a high degree of overall sequence similarity, but also the tertiary structure of the putative active site (the nuclease fold) is conserved (5). However, the crystal structures of both AP endo and Exo III were obtained in the absence of DNA so that there is still no clear picture as to the precise interaction of the abasic site substrate or its cleavage product with the enzyme. Furthermore, the amino-terminal 60 residues of AP endo are missing in Exo III, and the first 35 amino acid residues were truncated from AP endo in order to obtain crystallization. Hence, the relationship of the amino terminus to the remainder of the protein and of the protein to its substrate and product is unknown. In this report we present the results of experiments using limiting proteolysis that provide information on the dynamic structure of human AP endo and its interaction with DNA containing an abasic site.

EXPERIMENTAL PROCEDURES

Domain Mapping—AP endo was prepared by expression of the protein from plasmid pXC53 as described (6). The protein was digested at 23 °C with several proteases for the time intervals and with the concentrations listed in the text. Trypsin (bovine pancreas, either soluble or covalently attached to acrylic beads) and chymotrypsin (bovine pancreas, soluble) were obtained from Sigma, while V8 protease (Endoprotease Glu-C) was obtained from Boehringer Mannheim. Trypsin digestion was performed in a buffer consisting of 50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 50 mM KCl, and 1 mM urea. V8 protease digestion was performed in the same buffer lacking urea or with the urea substituted by 0.1% SDS. When SDS was included in the buffer, the protein was boiled for 3 min and cooled to room temperature before addition of the protease. Chymotrypsin digestion was performed in the same buffer as trypsin except that urea was omitted and the pH was maintained at 8.0. After each reaction was terminated by the addition of 6× protein loading buffer (7) and boiling, the products, resolved by SDS-polyacrylamide gel electrophoresis.

The abbreviations used are: AP endo, apurinic/apyrimidinic endonuclease; PAGE, polyacrylamide gel electrophoresis.
amid gel electrophoresis on 12.5% or 16% gels, were visualized with Coomassie Blue (7). When digests were performed in the presence of DNA, the oligonucleotide was added 5 min before the addition of the protease. The β-elimination product of the abasic site containing oligonucleotide, HDP, was prepared by heating as described previously (6). Recovery of Peptides from SDS-PAGE—Peptides to be eluted from SDS-PAGE were visualized by KCl staining, excised, homogenized, eluted in 50 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 150 mM NaCl, and acetone-precipitated as described by Hager and Burgess (8). The acetone precipitate was dissolved in 10 μl of 6 mM guanidine HCl in 50 mM Hepes-NaOH, pH 7.4, 20% glycerol, 0.1 mg/ml bovine serum albumin, 150 mM NaCl, 1 mM dithiothreitol, and 0.1 mM EDTA. The protein solution was shaken for 20–60 min on a rotary shaker, diluted 50-fold in the same buffer without guanidine HCl, and left at room temperature overnight to allow reformation of secondary and tertiary structure. On two occasions digested and undigested AP endo were boiled in protein loading buffer, acetone-precipitated, renatured, and assayed directly. Recovery of the undigested control protein and the digestion products after boiling in protein sample buffer or after elution from SDS-PAGE was estimated from the enzymatic time course over a 10,000-fold range after boiling in protein sample buffer or after elution from SDS-PAGE.

To generate the abasic site at position 21, the single-stranded oligomer, labeled at the 5′-end of the U-containing strand, was annealed with its homologue. After the double-stranded oligomer was treated with uracil DNA glycosylase (1 unit/100 pmol of U-containing oligonucleotide; Epicentre Technologies, Madison, WI) for 20–30 min at 37 °C in the presence of 0.1 mM NaBH₄, the uracil DNA glycosylase was heat-inactivated at 75 °C for 5 min. Steady-state enzyme activities were determined as described earlier (6) using 500 nm substrate and 0.5 μM enzyme over a 10–60-s time interval. Single-turnover binding assays were performed using 0.4 nM enzyme and 4 nM substrate. Substrate was mixed with enzyme in the presence of 4 mM EDTA, which permits the substrate to bind but prevents enzymatic cleavage, for varying periods of time (0–30 s). Divalent cation (10 mM MgCl₂, final concentration) was then added simultaneously with trap, consisting of HDP + heparin (6), so that the enzyme was limited to a single turnover. Cleavage was terminated by the addition of EDTA to a final concentration of 87 mM. Product (20-mer) was resolved from substrate (45-mer) by gel electrophoresis on denaturing 15% polyacrylamide gels containing 8 M urea (6, 9), followed by quantitation of substrates and products by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA). To obtain binding constants, simulations using the HOPKINSIM program (10) were fitted to the data.

Results

Native AP Endo Contains a Flexible Amino-terminal Portion and a Tight Globular Domain—AP endo was treated with limiting concentrations of trypsin (0.1 μg), which resulted in degradation of the amino-terminal 32 residues without cleavage in other parts of the protein (Figs. 1 and 2). The next basic residue, Lys-35, remained inaccessible to cleavage. Increasing the concentration of trypsin to 1 μg resulted in degradation of AP endo to small peptides. Similarly, V8 protease (0.02–1.0 μg) removed the terminal 36 residues after a 2-h exposure but failed to cleave Glu-39 (data not shown). Exposure to V8 protease (1 μg) for as long as 24 h at 37 °C failed to cleave the endonuclease further. Several other conditions known to optimize proteolysis by V8 protease (14) failed to promote further digestion. These included: 50 mM potassium phosphate (pH 6.5) with or without added KCl, 50 mM ammonium carbonate (pH 8.0) with 1 mM CaCl₂ instead of MgCl₂, and 50 mM Heps-NaOH (pH 7.4) with 1 mM CaCl₂ instead of MgCl₂. Exposure to chymotrypsin, which is described in detail below, resulted in partial loss of the amino terminus up to the first 31 residues (Fig. 1) and cleavage at two specific internal residues. Because the first 36 residues of the amino terminus are readily accessible to proteolysis by all the proteases examined, they are flexible and only loosely associated with the remainder of the molecule.

The Flexible Portion Does Not Extend to the Cysteine Residues Thought to be Involved with Jun Interaction—Note that Cys-65 and Cys-93, the two cysteine residues required for AP endo acting as a redox factor for Jun, were not accessible to

![FIG. 1. Limiting proteolytic digestion of AP endo. AP endo (7.2 μg) in 20 μl was mixed with different proteases for the times and concentrations indicated. The products were resolved by SDS-PAGE (12.5% gel). Trypsin (0.1 μg), lanes 1–5; V8 protease in the presence of SDS (0.02 μg), lanes 6–10; or chymotrypsin (concentration as indicated), lanes 11–16. Lanes 1, 6, and 11, no added protease; lanes 2 and 7, 15-min incubation; lanes 3 and 8, 30-min incubation; lanes 4 and 9, 60-min incubation; and lanes 5 and 10, 120-min incubation. Lanes 12–15, 120-min incubation with 0.01, 0.1, 1.0, and 5.0 μg of chymotrypsin respectively.](image-url)

Domain Mapping of Human AP Endonuclease

TRYPsin V8 + SDS CHYMOTRYPTISn

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

35.5 25.0 12.7 35.5 19.5 12.8

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The Globular Domain of AP Endo Can Be Loosened by Boiling in Detergent to Make the Nuclease Fold Accessible—Since V8 protease functions in the presence of SDS, we chose to examine whether AP endo could be degraded after being boiled in the presence of detergent (Figs. 1 and 2). The results are depicted visually in Fig. 5A. When AP endo was boiled in the presence of 0.1% SDS and cooled before V8 protease was added, it lost its enzymatic activity (Fig. 3) and several subdomains became more accessible to proteolysis (Figs. 1, 2, and 5A). The most prominent proteolytic sites were Glu-96, Glu-216, and Asp-283. Glu-96 and Asp-283 lie along the nuclease fold, whereas Glu-216 is exposed to the exterior surface (3) and is solvent-accessible. The surprise was that Glu-216 was not cleaved in the absence of detergent. Furthermore, Asp-283 is involved in catalysis (13), whereas Glu-96 is the divalent cation binding site (3). One nearby residue that might have been expected to cleave in the presence of detergent but did not cleave was Glu-101 located in the loop between β2 and α3. Structurally, the enhanced accessibility argues either for greater flexibility along the nuclease fold than other regions of the protein or a failure to renature along the nuclease fold so that key residues extending into the region become sensitive to added V8 protease.

Chymotrypsin Cleaves within the Tight Globular Domain—Chymotrypsin was the only protease that readily cleaved the globular domain without the addition of denaturants (see Figs. 1, 2, 5B, and 6). In fact, to slow the protease, urea was omitted, Mg²⁺ was substituted for Ca²⁺ (14), and the pH was adjusted to 8.0. When these alterations in digestion conditions were made, three major digestion products were observed: a 19.5-kDa fragment beginning at Gly-145 and extending to the carboxyl terminus, a 19.5-kDa fragment beginning at the amino
from each mixture (buffer the abasic site-containing oligonucleotide was a mixture of cleavage product and starting substrate. After a zero time aliquot was withdrawn 5 min without SDS, heated to 78 °C for 5 min, or boiled in SDS

undigested in this experiment. Enzyme that had been boiled for accounted for by the fact that 35% of the protein remained

gestated sample (16% of the mock-digested control) could be

"Experimental Procedures." The activity recovered in the di-

precipitated with acetone, and renatured as described under

digested, was boiled with SDS-PAGE sample loading buffer,

shortened by trypsin digestion using trypsin attached to a solid

APEndo (3.6 μg) was incubated for 5 min at room temperature with protease digestion buffer, lanes 1-5; double-stranded oligonucleotide, lanes 6-10; double-stranded oligonucleotide containing the β-elimination product at position 21 of the upper strand, lanes 11-15; or double-stranded oligonucleotide containing an abasic site at position 21 of the upper strand. Note that because of the presence of Mg²⁺ in the protease digestion buffer the abasic site-containing oligonucleotide was a mixture of cleavage product and starting substrate. After a zero time aliquot was withdrawn from each mixture (lanes 1, 6, 11, and 16), chymotrypsin was added and samples were withdrawn at time intervals between 20 and 120 min. Lanes 2, 7, 12, and 17, 20-min incubation, 0.25 μg of protease; lanes 3, 8, 13, and 18, 60-min incubation, 0.25 μg of protease; lanes 4, 9, 14, and 19, 120-min incubation, 0.25 μg of protease; lanes 5, 10, 15, and 20, 120-min incubation, 0.67 μg of protease. Products were resolved by SDS-PAGE (12.5% gel).

Fig. 4. Accessibility of Tyr-144, Leu-179, and Leu-205 to chymotrypsin digestion in the presence and absence of DNA. AP endo (3.6 μg) was incubated for 5 min at room temperature with protease digestion buffer, lanes 1-5; double-stranded oligonucleotide, lanes 6-10; double-stranded oligonucleotide containing the β-elimination product at position 21 of the upper strand, lanes 11-15; or double-stranded oligonucleotide containing an abasic site at position 21 of the upper strand. Note that because of the presence of Mg²⁺ in the protease digestion buffer the abasic site-containing oligonucleotide was a mixture of cleavage product and starting substrate. After a zero time aliquot was withdrawn from each mixture (lanes 1, 6, 11, and 16), chymotrypsin was added and samples were withdrawn at time intervals between 20 and 120 min. Lanes 2, 7, 12, and 17, 20-min incubation, 0.25 μg of protease; lanes 3, 8, 13, and 18, 60-min incubation, 0.25 μg of protease; lanes 4, 9, 14, and 19, 120-min incubation, 0.25 μg of protease; lanes 5, 10, 15, and 20, 120-min incubation, 0.67 μg of protease. Products were resolved by SDS-PAGE (12.5% gel).

terminus and extending to Leu-179, and a 12.8-kDa fragment

beginning with Val-206 and ending at the carboxyl terminus (Figs. 1 and 2). The two ~19-kDa fragments, unresolved by SDS-PAGE, were identified during amino acid sequencing in a ratio of approximately 3:1 (19.9-kDa fragment:19.5-kDa fragment). In keeping with the flexibility of the amino-terminal portion, substantial quantities of digestion products ending in Leu-179 and beginning at Arg-18, Ala-29, and Lys-32 were also observed. Although cleavage on the carboxyl side of aromatic amino acids as well as leucine and alanine is standard for chymotrypsin (14), the cleavage after Lys-31 is likely due to trace contamination with trypsin found in most commercial preparations of chymotrypsin and the extreme sensitivity of the amino-terminal domain to trypsin digestion. Note that Lys-141 and Lys-203, two potential trypsin cleavage sites in the amino-terminal domain to trypsin digestion. Note that Lys-141 and Lys-203, two potential trypsin cleavage sites in protein sample buffer had no activity. At the same time, when the 19.5-kDa and 19.8-kDa fragments were eluted from an SDS-PAGE, renatured, and assayed for enzymatic activity, we recovered 0% of the control activity recovered from undi-
gested enzyme eluted from the same gel. Since the two ~19-
kDa fragments overlapped and encompassed the entire mole-
cule, it was theoretically possible that activity could have been

restored by renaturation. However, this did not occur. In short, the chymotrypsin data indicated that, even if the protein was

nicked by proteolysis, hydrogen bonding and other noncovalent interactions maintained sufficient tertiary structure to protect enzymatic activity. However, if the protein was heated or treated with detergent to disrupt noncovalent interactions or if the protease fragments were dissociated after nicking, either by treatment with detergent or gel separation, activity could not be recovered.

Cleavage at Tyr-144, Leu-179, and Val-206 Is Blocked by the Presence of DNA, Particularly an Oligonucleotide Containing an Abasic Site—We investigated the effect of the presence of 45-mer double-stranded oligonucleotide on digestion by trypsin, V8 protease, and chymotrypsin. Although there was no effect on digestion by the first two proteases, there was a striking effect on chymotrypsin digestion (Fig. 4). The moiety at position 21 of the oligonucleotide differed in each case: a G/U pair, for which the enzyme has a relatively poor binding affinity (6, 15); a phosphodeoxyribose (the β-elimination product of an abasic site), which is an efficient inhibitor of AP endo (6); or an abasic site. To ensure complete binding, the ratio of oligonucleotide to AP endo during the incubation was 6:1. In the case of the oligonucleotide containing an abasic site, both product and substrate were present because AP endo was active during digestion due to the presence of Mg²⁺ required for proteolysis. All three oligonucleotides delayed digestion of full-length AP endo. (In Fig. 4, compare the retention of the 35.5-kDa starting material in lanes 2–5 with that in lanes 7–10, 12–15, and 17–20.) However, only the presence of substrate/product delayed the appearance of the doublet band containing the 19.5- and 19.8-kDa fragments. (In Fig. 4 compare the rate of disappear-

cence of substrate and the amount of ~19-kDa fragments in the same lanes.) Note also the lack of appearance of either a
22.7-kDa fragment or a 15.7-kDa fragment that would have signaled decreased accessibility at either Leu-179 or Leu-205 without affecting the other sites. Although total degradation continues to occur during proteolysis, we feel that the presence of oligonucleotide, particularly substrate/product, has a profound effect on the accessibility of Tyr-144, Leu-179, and Leu-205 to chymotrypsin by blocking all three sites simultaneously.

**DISCUSSION**

Limiting proteolysis is an extremely useful tool in describing functional and structural regions of a protein molecule in solution (16, 17). Whereas x-ray crystallography provides details of a particularly thermodynamically stable conformation, domain mapping by limiting proteolysis provides information on dynamic structure. We have shown that AP endo contains two major regions, an amino-terminal flexible portion of approximately 36 amino acids, which is readily accessible to protease digestion, and the remainder of the molecule, which forms a tight globular domain that is inaccessible to most proteases. After boiling in 0.1% SDS, the enzyme loses its activity and the tight globular domain becomes somewhat more flexible or re-

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**FIG. 5.** Three-dimensional structure of AP endo, showing the protease-accessible residues after V8 + SDS digestion (A) and chymotrypsin digestion (B). Ribbon diagram in stereo image with relevant amino acid residues shown in three dimensions. A, cleavage sites identified by V8 plus SDS. Note that Glu-216 is on the exterior of the molecule, while Glu-96 and Asp-283 face the nuclease fold. B, cleavage sites identified by chymotrypsin. Tyr-144 and Leu-179 are clearly solvent-accessible. Leu-205, which appears to be somewhat less solvent accessible from visual inspection of the crystal structure, nevertheless has space for a H2O molecule adjacent to it and is also accessible by limiting proteolysis. The three-dimensional structure was visualized from the crystal coordinates by means of RASMOL (27).
natures partially, exposing several residues along the nuclease fold and one on the exterior surface to V8 protease. Even so, it is striking that after boiling in detergent, large regions of the molecule renature so efficiently that they are still relatively inaccessible to digestion. The only protease able to digest the protein within the globular domain is chymotrypsin. In this case digestion provides three major fragments involving cleavage at Tyr-144 or cleavage at Leu-179 and Leu-205. This is the first dynamic structural information available for this enzyme.

Perhaps the most striking result to emerge from this study is the alteration in the accessibility of the three major chymotrypsin cleavage sites in the presence of 45-mer oligonucleotide, particularly the 45-mer containing an abasic site. The three residues form a spherical triangle on one side of the nuclease fold (see space-filling residues in Figs. 5B and 6). The distance (arc length along a great circle$^3$) from Leu-179 to Leu-205 is 29.7 Å, the distance from Leu-179 to Tyr-144 is 34.4 Å, and the distance from Leu-205 to Tyr-144 is 15.8 Å. These distances correspond to approximately one turn of a DNA helix for the first two pairs and one-half a turn of the helix for the third pair (18). Given the distances between the three residues, it is unlikely that a single oligonucleotide could block all three sites simultaneously. Hence, we need to account for at least two modes of interaction. The blockage at Leu-179 is consistent with the model proposed by Gorman et al. (3), whereby the DNA binds across one aspect of the nuclease fold (See Fig. 6, arrows as aligned with Fig. 3 in Ref. 3). Leu-179 being part of α helix α5 proposed to bind the major groove of DNA. There were no major protease cleavage fragments originating in the loop on the side of the nuclease fold opposite from Leu-179 in the absence of DNA, and no new fragments appeared during proteolysis in the presence of DNA. Therefore, we could not observe directly whether DNA binds across the nuclease fold, which would occur if α helix α5 interacted with the minor groove of DNA in analogy with DNase I (19). The surface of the nuclease between Leu-179 and Leu-205 is occupied by α helices α6 and α9 (see Figs. 5B and 6). However, the surface of the nuclease between Leu-179 and Tyr-144 is occupied by several regions that are relatively disordered, including Glu-150, His-151, Asp-152, Glu-154, Gly-176, Asn-174, Pro-173, Val-180, and Arg-181 that might provide an alternative binding site for a DNA helix and enable the substrate to swivel about Leu-179. By far the more intriguing distance is the half a helical turn from Tyr-144 to Leu-205. The region between Tyr-144 and Leu-205 is occupied by parts of β sheets β5, β6, and β7 as well as α helix α6, containing a number of conserved amino acids facing the exterior surface of the molecule. Furthermore, the straight line distance through the nuclease fold from Tyr-144 to Glu-96, the divalent cation binding site required for enzymatic activity (3), is 16 Å or again approximately one-half of a helical turn of DNA.

How then would an abasic site interact with the active site residues of AP endo? Even in the absence of enzyme, the presence of an abasic site lowers the melting temperature (20, 22) and confers conformational changes to the adjacent and opposing base pairs (20, 21). An abasic site causes perturbations in the helix for one-half a helical turn on the strand opposite the abasic site in the 5’ direction (23) as shown by accessibility to nuclease S1 and P1. Addition of a second abasic site on the opposite strand disturbs the nicking reaction, especially when the abasic site is 3 base pairs 5’ away from the initial site on the opposite strand, or slightly less than one-half a helical turn.

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$^3$Arc length along a great circle (Ar) was calculated as $Ar = \text{Rcos}^{-1}(1 - L^2/2R^2)$, where $R$ is the average radius of the ideal sphere, estimated by measuring the distance from 5 random external residues on opposite sides of the molecule and dividing by 2, and $L$ is the linear distance between two residues obtained from RASMOL (27).
away (24, 25). As a consequence, we propose that substrate and product bind differently to AP endo. Because the abasic site is already prone to deformation, the enzyme could induce a bend in the DNA, enabling the abasic site to become accessible to catalytic residues found in the nuclease fold. One-half a helical turn of DNA could lie along the backside of the enzyme to catalytic residues found in the nuclease fold. One-half a bend in the DNA, enabling the abasic site to become accessible site is already prone to deformation, the enzyme could induce a conformation such that other residues on the surface of AP endo. Because the abasic endo can act as a reductant for Jun protein.

These results leave several questions unanswered. In particular, AP endo has now been shown to interact with DNA polymerase β both by yeast two-hybrid analysis and electrophoretic mobility shift assays (26). Presumably the portion of the nuclease that interfaces with the polymerase must face the exterior of the molecule. However, one cannot presume that the amino-terminal domain fulfills this function, since many portions of the molecule distributed throughout the primary structure are solvent-accessible (3). Finally, Cys-65 and Cys-93, the residues previously thought to be responsible for AP endo activity as a redox factor in terms of binding Jun protein (11, 12), are clearly located internally and are not exposed to solvent even after extended incubation under reducing conditions. Although we cannot rule out a major conformational change occurring in the presence of Jun protein, one possible explanation for this observation is that Cys-65 and Cys-93 are not themselves involved in the redox activity. Rather, the two cysteines are responsible for maintaining the molecule in a conformation such that other residues on the surface of AP endo can act as a reductant for Jun protein.

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