CATALYTIC ACTIVITY OF NUCLEASE P1:
EXPERIMENT AND THEORY

DISCLAIMER

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Introduction

Nuclease P1 from *Penicillium citrinum* is a zinc dependent glyco-enzyme that recognizes single stranded DNA and RNA as substrates and hydrolyzes the phosphate ester bond. As a single chain of 270 amino acid residues, the enzyme has a molecular mass of about 36 kilodaltons (Maekawa et al. 1991). Although it exhibits 3’-phosphatase activity, P1 is primarily an endonuclease that is capable of hydrolyzing single stranded DNA completely to the level of mononucleosides. Nuclease P1 seems to recognize particular conformational states of the phosphodiester backbone and shows significant variation in the rate of hydrolytic activity depending upon which nucleosides are coupled by the phosphodiester bond. The efficiency of nuclease P1 in hydrolyzing the phosphodiester bonds of a substrate can be altered by modifications to one of the substrate bases induced by ionizing radiation or oxidative stress. Measurements have been made of the effect of several radiation induced lesions on the catalytic rate of nuclease P1. A model of the structure of the enzyme has been constructed in order to better understand the binding and activity of this enzyme on various ssDNA substrates.

Biochemical Materials and Methods

Normal dinucleoside monophosphates were purchased from Sigma and nuclease P1 was purchased from Boehringer Mannheim. Modified dinucleoside
monophosphates were isolated by reverse phase HPLC. Dinucleoside monophosphates (6 nmol) were solubilized in 100 µL of reaction buffer (60 mM Na acetate, pH 5.3, containing 42 µM Zn acetate). Hydrolysis was initiated by addition of 50 ng of P1 nuclease in 2 µL of buffer. The mixture was incubated at 37°C for the duration of the reaction which was terminated with the addition of 15 µL of 1 M TRIS. Decrease of the integrated area of absorption of reactants and increase of the integrated area of products were monitored by HPLC with detection at UV 254 nm.

**Kinetic Measurements**

Rates of hydrolysis by nuclease P1 for equimolar mixtures of the 16 possible dinucleoside monophosphates differ by more than two orders of magnitude. Box et al. (1993) showed that these differences correlated with the 5' nucleoside. Furthermore, the rate of hydrolysis is reduced if the 5' nucleoside is damaged by ionizing radiation or oxidative stress, (Box et al. 1993; Weinfeld et al. 1992). Results of more recent studies shown in Table I support the hypothesis that the 5' nucleoside is the principal binding locus between enzyme and substrate; however, they indicate that the 3' nucleoside also influences hydrolytic activity. Substrate d(TFpA), which

| d(TpG) d(TpC) d(TpA) d(TpT) d(TpTF) a d(TFpT) d(TGpA) b d(TpGOH) c |
|-----------------|---------|--------|--------|--------|--------|--------|--------|--------|
| 112             | 90      | 70     | 40     | 60     | 0      | 3      | 325    |

a) TF = Formamido remnant of Thymine
b) TG = R-5,6-Dihydroxy-5,6-Dihydrothymine (Thymine Glycol)
c) GOH = 8-OxoGuanine

Table I: Catalytic Constant (moles substrate hydrolyzed/mole enzyme) for P1 hydrolysis of the phosphodiester bond in dinucleoside monophosphates.
nuclease P1 was unable to cleave even after several hours of digestion, is a competitive inhibitor of the enzyme probably due to substrate binding at the 3' nucleoside only.

**Structural Data**

The crystal structure of nuclease P1 has been solved by Volbeda et al. (1991) at 2.8 Å resolution. Three water molecules and three zinc ions are present in the active site. One water molecule bridges Zn1 and Zn3. The Zn2 ion is coordinated by two water molecules, His126, His149 and Asp153. This configuration is structurally conserved in the active sites of several zinc enzymes (Valee and Auld 1990) and suggests that Zn2 is directly involved in catalysis. All three zinc ions are pentacoordinated with quasi-trigonal bipyramidal geometry.

The active site is characterized by two hydrophobic binding regions separated by about 20 Å, with the three zinc ions at the bottom of the binding cleft. One of these regions, which is characterized by nucleic-acid base stacking between Tyr144 and Tyr155, is believed to be the 5' nucleotide binding site. The other binding region, which is characterized by base stacking between Val132 and Phe61, has been identified as the binding site for the 3' nucleotides, which Volbeda et al. (1991) proposed as the most likely substrate binding site based on crystal soaking experiments with dAp(S)A, an uncleavable phosphorothioate substrate analog. We have undertaken modeling studies to gain more insight into the DNA binding domain and catalytic activity of nuclease P1.

**Protein Model**

Currently, the only structural information available for nuclease P1 comes
from the published stereodiagrams of the Cα trace and the active-site residues (Volbeda et al. 1991). These diagrams were digitized and 3-dimensional cartesian coordinates derived from them were used to create a coordinate file in the standard Protein Data Base (PDB) format. Given the Cα coordinates and the amino acid sequence, MAX-SPROUT (Holm and Sander 1991) was used to generate coordinates for the whole protein. MAX-SPROUT utilizes an algorithm that scans a database of known crystal structures for fragments that locally fit the Cα coordinates according to distance criteria. The fragments are joined to create the full backbone. Sidechains are added from a database of frequently occurring rotamers. The reconstructed coordinates for P1 nuclease were further refined using the SYBYL program (TRIPOS Associates, Inc.). Peptide bonds were added at 12 gaps in the protein backbone that could not be sealed by MAX-SPROUT and the disulfide bridges reported in the crystal structure between Cys80-Cys85, and Cys72-Cys217 (Volbeda et al. 1991) were also added. Finally, the Simplex algorithm in the energy minimization utility of SYBYL was used to optimize bond lengths within the structure.

**Active-Site Charge Distribution**

Table II shows calculated geometries and atomic charges for zinc ions coordinated to a single water molecule or an amino-acid residue analog. Histidine and aspartic acid residues were abbreviated to methylimidizole and acetate, respectively. Quantum chemical calculations were carried out in the Modified Intermediate Neglect of Differential Overlap (MINDO) approximation on each zinc complex and residual atomic charges were determined by fitting the electrostatic potential at the optimal geometry. Molecular mechanics optimization was then
performed with the AMBER force field using the default charges (Weiner et al. 1984) or the charges derived from our MINDO calculations. In the latter case, residual positive charge on Zn is reduced and the residual negative charge on the coordinating atom increases relative to the default AMBER charges. If the fragment containing the atom binding to Zn is large (i.e. an amino acid rather than a water molecule) the net electrostatic interaction is reduced and the complex expands.

<table>
<thead>
<tr>
<th>Source</th>
<th>Zn Charge</th>
<th>WAT(O) Charge</th>
<th>Zn-WAT(O) Bond</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMBER</td>
<td>2.0000</td>
<td>-0.8340</td>
<td>2.143</td>
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<tr>
<td>MINDO</td>
<td>1.7649</td>
<td>-1.1120</td>
<td>1.896</td>
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<table>
<thead>
<tr>
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<th>Zn-Charge</th>
<th>ASP(Oe1) Charge</th>
<th>Zn-ASP(Oe1) Bond</th>
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</thead>
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<tr>
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<tr>
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<th>HIS(N) Charge</th>
<th>Zn-HIS(N) Bond</th>
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<tr>
<td>AMBER</td>
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<td>MINDO</td>
<td>1.3784</td>
<td>-0.3024</td>
<td>2.065</td>
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</tbody>
</table>

Table II: Charges (esu) and Bond Lengths (Å) in Simple Zinc Ligands

Our results for Zn coordinated to simple ligands (Table II) suggested that the default AMBER charges would not be adequate to describe electrostatic interactions in the active site of P1 nuclease. For this reason, the catalytic site was separated into the two molecular areas for quantum investigation of residual atomic charges. Area 1 contained Zn2, His126, His149, Asp153, Wat1 and Wat2 while Area 2 contained Zn1, Zn3, Trp1, His6, Asp45, His116, Asp120 and Wat3. Coordinates of atoms in the two areas were extracted from the reconstructed coordinate set of the entire protein. Some bond lengths and angles were adjusted based on similar work for the active site of carboxypeptidase A (Banci et al. 1992). MINDO calculations were carried out
on the two areas and residual atomic charges were calculated by fitting the
electrostatic potential of each area at its optimal geometry, which did not differ
greatly from that extracted from the reconstructed coordinate set. The geometry and
residual charges of Area 1 derived by this semi-empirical quantum-chemical
approach are shown in Figure 1.

Figure 1: Semi-empirical quantum results for the geometry and atomic charges of
the portion of the active site of P1 nuclease most important to its catalytic activity.
Future Work

All of the components have been prepared for refinement of our model of P1 nuclease by molecular dynamics (MD) simulation. The reconstructed atomic coordinates, with small modifications to accommodate the MINDO optimum geometries of areas 1 and 2, provide a starting structure for these simulations. The AMBER all-atom force field (Weiner et al. 1984) augmented by the MINDO residual charges of the catalytic site and parameters for bonded interactions with Zn (Merz et.al,1991) provide the information needed to approximate the multi-dimensional Born-Oppenheimer potential-energy surface. For the enzyme in the absence of substrate, Na+ and Cl- counterions are added to neutralize any residues with net charge. Finally, a layer of water will be added to the protein/counterion solute and several hundred picoseconds of MD simulations will be carried out in the constant-temperature mode of the AMBER software package (Pearlman et al. 1991). Eventually, we expect to compare the results of this simulation with experimental atomic-level structural data. In the mean time, we will develop models for complexes of P1 nuclease with native and modified dinucleotide monophosphates in an effort to gain more insight from our kinetic data.

Acknowledgements

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