Substrate Binding and Turnover by the Highly Specific I-*Ppo*I Endonuclease†

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Received October 2, 1995; Revised Manuscript Received November 15, 1995⊗

ABSTRACT: Intron-encoded endonucleases are distinguished by their ability to catalyze the cleavage of double-stranded DNA with high specificity. I-*Ppo*I endonuclease, an intron-encoded endonuclease from the slime mold *Physarum polycephalum*, is a small enzyme (2 × 20 kDa) that catalyzes the cleavage of a large asymmetric DNA sequence (15 base pairs). Here, the interactions of I-*Ppo*I with its substrate were examined during both binding (in the absence of Mg^{2+}) and catalysis (in the presence of Mg^{2+}). Using circular permutation assays, I-*Ppo*I was shown to bend its substrate by 38 ± 4° upon binding. Two independent methods, gel mobility shift assays and fluorescence polarization assays, revealed that I-*Ppo*I binds tightly to its substrate. Values of K_d range from 3.3 to 112 nM, increasing with increasing NaCl concentration. Similar salt effects on the values of K_m were observed during steady-state catalysis. At low salt concentrations, the value of k_{cat}/K_m for the cleavage of an oligonucleotide duplex approaches 10^8 M^{-1} s^{-1}. Although other divalent cations can replace Mg^{2+}, catalysis by I-*Ppo*I is most efficient in the presence of an oxophilic metal ion that prefers an octahedral geometry: $Mg^{2+} > Mn^{2+} > Ca^{2+} = Co^{2+} > Ni^{2+} > Zn^{2+}$. Together, these results provide the first chemical insight into substrate binding and turnover by an intron-encoded endonuclease.

Cells use proteins to access and maintain the information encoded within their genome. Proteins can bind to double-stranded DNA with extraordinary specificity. After binding, enzymes can cleave or modify DNA with extreme efficiency. Understanding the molecular basis of such DNA recognition and manipulation is crucial for understanding many aspects of cellular function. Much of our current knowledge of protein−DNA interaction has been derived from investigations of prokaryotic transcriptional factors. Studies of these systems indicate that a primary determinant of specificity arises from direct contacts between the protein side chains and the DNA backbone and bases (Steitz, 1990). Additional elements affecting DNA recognition include conformational changes of the DNA (Travers, 1991), conformational changes of the protein (Spolar & Record, 1994), and the presence of ions (Woodhead et al., 1981; Record et al., 1991; Xu & Schildkraut, 1991a; Vipond et al., 1995).

Recently, a novel family of enzymes was added to the repertoire of proteins that bind to DNA with high specificity. These enzymes are the intron-encoded endonucleases. Similar to the more familiar bacterial restriction endonucleases, the intron-encoded endonucleases catalyze the sequence-specific cleavage of double-stranded DNA. The large size of their recognition sites, however, is more analogous to that of many transcriptional factors. Intron-encoded endonucleases have recognition sites that range from 15 to 39 base pairs (Perlman & Butow, 1989). This new class of enzymes provides an opportunity to examine both complex sequence recognition and catalysis in a single system.

Intron-encoded endonucleases have their coding sequences located either wholly or partially within a mobile group I intron. Their function is to initiate the homing of their encoding intron [for a review, see Dujon (1989)]. Briefly, intron homing is the site-specific, replicative transfer of mobile introns from an intron-containing allele to a cognate, intron-lacking allele. The endonuclease initiates this genetic event by catalyzing site-specific double-stranded cleavage in the intron-lacking allele. The sources of homing introns and their encoded endonucleases are numerous and diverse, having been isolated from various eukaryotic (Colleaux et al., 1986; Delahodde et al., 1989; Gauthier et al., 1991; Sargueil et al., 1991; Thompson et al., 1992; Perea et al., 1993; Schapira et al., 1993), prokaryotic (Bell-Pedersen et al., 1989), and archaeal organisms (Hodges et al., 1992; Dalgaard et al., 1993, 1994).

The intron-encoded endonuclease that is the focus of this study, I-*Ppo*I, was first isolated by Vogt and co-workers from the slime mold *Physarum polycephalum*. (Muscarella et al., 1990) [for nomenclature, see Dujon et al. (1989)]. I-*Ppo*I is encoded by the third intron located within the extrachromasomal nuclear DNA encoding the large ribosomal RNA subunit. Like the other intron-encoded endonucleases, I-*Ppo*I recognizes a large asymmetric sequence. In the presence of Mg^{2+}, I-*Ppo*I cleaves within the DNA sequence CTCTCT-TAA/GGTAGC, generating fragments with 4-nt, 3′-OH extensions (Muscarella et al., 1990; Ellison & Vogt, 1993).[1] The size of I-*Ppo*I is small relative to its 15-bp recognition site. The protein forms a 2 × 20 kDa dimer in solution (Ellison and Vogt, 1993). For comparison, the restriction endonuclease *Eco*RI is a 2 × 31 kDa protein with a cognate site of only 6 base pairs (Modrich & Zabel, 1976; Greene et al., 1981; Newman et al., 1981). Thus, the residues in I-*Ppo*I have much greater information content than do those in

† P.K.W. is a predoctoral trainee, Biotechnology Training Grant GMO8349 (NIH). R.T.R. is a Presidential Young Investigator (NSF), a Searle Scholar (Chicago Community Trust), and a Shaw Scientist (Milwaukee Foundation).

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⊗ Abstract published in *Advance ACS Abstracts,* January 1, 1996.

[1] Abbreviations: bp, base pairs; CN, coordination number; nt, nucleotides.

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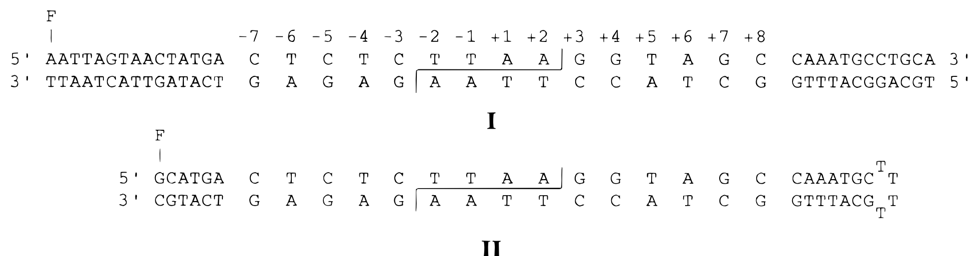

FIGURE 1: DNA used for assays of binding and catalysis by I-*Ppo*I: (top) **I**, a 42-bp duplex; and (bottom) **II**, a self-complementary 60-nt strand. The site recognized by I-*Ppo*I is numbered −7 to +8, and the site cleaved is indicated by a line. F indicates the location of the fluorescein tag used for the fluorescence polarization assays. No fluorescein is present in other assays.

*Eco*RI or other type II restriction endonucleases.

In this study, we examine the conformational changes of DNA upon binding and the effects of monovalent and divalent ions on DNA binding and turnover by the I-*Ppo*I endonuclease. We find that the I-*Ppo*I bends its substrate upon binding. The strength of the binding interaction is affected dramatically by NaCl, and this effect allows us to estimate the number of Coulombic interactions between I-*Ppo*I and DNA. In addition, we have determined the steady-state kinetic parameters for the I-*Ppo*I-catalyzed cleavage of its recognition site and begun to examine the role of the requisite divalent cation in effecting catalysis of DNA cleavage.

MATERIALS AND METHODS

Materials. I-*Ppo*I, purified from an *Escherichia coli* expression system, was a generous gift from Promega Corp. (Madison, WI). The endonuclease is the larger (185 amino acid residues), major form of the protein produced in *P. polycephalum*. Concentrations of the endonuclease were determined by using the method of Bradford (1976) with a kit from BioRad (Richmond, CA) and bovine serum albumin (BSA) as the standard. Stock solutions of endonuclease were stored in 25 mM Tris-HCl buffer (pH 7.4) containing NaCl (250 mM), ethylenediaminetetraacetic acid (EDTA) (10 mM), dithiothreitol (DTT) (1 mM), and glycerol (10% v/v) and were diluted into solutions of bovine serum albumin (200 μg/mL) and glycerol (10% v/v) prior to use.

Poly(dIdC)•poly(dIdC) was from Sigma Chemical (St. Louis, MO) and was used without further purification. [γ-^{32}P]ATP, 6000 Ci/mmol, was from NEN Research Products (Wilmington, DE). Sephadex G-50 NICK columns and fluorescein phosphoramidite were from Pharmacia (Uppsala, Sweden). Fluorescein antibody columns were made by Dr. John Hill (University of Wisconsin—Madison) using antibodies from Dr. Edward Voss (University of Illinois at Urbana). DNA was purified with a Mermaid kit from Bio100 (La Jolla, CA) after electrophoresis in agarose gels. T4 polynucleotide kinase and the restriction endonucleases were from either Promega (Madison, WI) or New England Biolabs (Beverly, MA). Shrimp alkaline phosphatase was from United States Biochemical (Cleveland, OH).

General Methods. DNA oligonucleotides were synthesized with β-cyanoethyl phosphoramidite chemistry (Sinha et al., 1984) on an Applied Biosystems Model 392 DNA-RNA synthesizer. Complementary oligonucleotides were annealed by heating them to 90 °C in a solution of 10 mM Tris-HCl buffer (pH 8.0) containing NaCl (200 mM) and then allowing them to cool slowly to room temperature. For binding and turnover assays, duplex DNA was purified from native polyacrylamide (15% w/v) gels. The band corresponding to double-stranded DNA was excised and soaked overnight in 0.30 M sodium acetate buffer (pH 5.0) containing EDTA (1 mM). After soaking, the supernatant was extracted with phenol/chloroform (1/1 v/v) and precipitated with ethanol. DNA substrates were radiolabeled on their 5′-OH group by treatment with [γ-^{32}P]ATP and T4 polynucleotide kinase. Salts and unincorporated nucleotides were removed by chromatography on Sephadex G-50 columns.

Gel Mobility Shift Assay for DNA Binding. Gel mobility shift assays were based on the retardation of the electrophoretic mobility of a ^{32}P-labeled DNA molecule upon the binding of a protein (Fried & Crothers, 1981; Garner & Revzin, 1981). These assays were performed essentially as described (Ausubel et al., 1989). The DNA was a 42-bp duplex (**I**) with the sequence indicated in Figure 1. [^{32}P]**I** (20 pM) was incubated at 25 °C with increasing amounts of I-*Ppo*I in a solution of 10 mM Tris-HCl buffer (pH 7.5) containing NaCl (50 mM), EDTA (1 mM), glycerol (10% v/v), and bovine serum albumin (50 μg/mL). Mg^{2+} was omitted to prevent DNA cleavage. After 1 h, the binding reaction mixtures were loaded directly onto a polyacrylamide (4% w/v) gel running at 30 V/cm. The gels were dried and counted using a Betascope 630 Blot Analyzer from Betagen (Waltham, MA). The disappearance of the band corresponding to free DNA was followed to determine the equilibrium dissociation constant. The data were fit with the program SIGMAPLOT 4.16 to eq 1, which describes binding to a single site.

$$B = \frac{B_{\max}F}{K_d + F} \tag{1}$$

In eq 1, B is the concentration of bound protein, $B_{\max}$ is the total concentration of binding sites, F is the concentration of free protein, and K_d is the dissociation constant for specific binding (Attie & Raines, 1995). Dissociation constants were calculated for the endonuclease dimer.

Fluorescence Polarization Assay for DNA Binding. Fluorescence polarization assays were based on the increase of the rotational correlation time of a fluorophore-labeled DNA molecule upon the binding of a protein (LeTilly & Royer, 1993). The protein•DNA complex, due to its increased size, tumbles more slowly than does free DNA. The ensuing reduction in the rotational correlation time of the fluorophore causes an increase in polarization, which allows the binding to be monitored (LeTilly & Royer, 1993). As shown in eq 2, polarization (P) is defined as the ratio of the difference between the vertical ($I_{\|}$) and horizontal ($I_{\perp}$) emission components to their sum.

$$P = 10^3 mP = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \quad (2)$$

Both specific and nonspecific DNA substrates were used in the fluorescence polarization assays: a 42-bp binding-site oligonucleotide (**I**, Figure 1), a self-complementary 60-nt binding-site oligonucleotide (**II**), and a 25-bp dIdC oligonucleotide [$(dIdC)_{25}\cdot(dIdC)_{25}$]. The fluorescein tag was linked to oligonucleotides by a six-carbon spacer to the terminal 5′-OH group of one strand of each duplex. Fluorescein was incorporated from its phosphoramidite in the final coupling step of DNA synthesis. To increase the fluorescein-specific activity, the fluorescein-labeled strand was purified by chromatography on an immobilized anti-fluorescein antibody column. Briefly, the strand was loaded onto the column in 10 mM sodium phosphate buffer (pH 6.0) containing EDTA (0.10 mM) and NaCl (0.10 M). The loaded column was washed with the same buffer, and the strand was then eluted by denaturing the antibodies with aqueous HCl (0.2 M). The resulting solution was neutralized by addition of KOH (0.2 M). The purified strand was annealed to its complement, and the resulting duplex was purified by native gel electrophoresis.

Fluorescence polarization was measured at room temperature (25 ± 2 °C) on a Beacon Fluorescence Polarization System (PanVera, Madison, WI) with excitation at 488 nM and emission at 520 nM. Fluorescein-labeled DNA duplex (450−550 pM) was incubated in a solution (1.10 mL) of 25 mM Bis-Tris/Tris/Bis-Tris propane-HCl buffer (pH 7.5) containing EDTA (2 mM), DTT (1 mM), and the indicated concentration of NaCl (this buffer system was used to allow for future analyses of pH dependence). Aliquots (2−5 μL) of I-*Ppo*I were added successively to the solution, which was then allowed to equilibrate. In each assay, the I-*Ppo*I concentration varied from 10 pM to 5 μM but the total volume of the solution varied by less than 10%. Three to six polarization measurements were made at each protein concentration. The dissociation constants were determined by fitting the data to eq 1 or 3, which describes binding to a single specific site as well as nonspecific sites:

$$B = \frac{B_{max}F}{K_d + F} + K_{ns}F \quad (3)$$

In eq 3, K_{ns} is a constant for nonspecific binding (Attie & Raines, 1995).

Circular Permutation Assay for DNA Bending. The plasmid pBend3 and the poly(dA)·poly(dT)-tract standards were gifts from Dr. Rick Gourse (University of Wisconsin—Madison). pBend3 contains the backbone of pBluescript SK-, with the tandem repeat region of pBend2 (Kim et al., 1989) cloned into the *Eco*RI and *Hin*dIII sites. To insert the recognition site of I-*Ppo*I, synthetic oligonucleotides containing the 42-bp sequence of **I** were cloned into pBend3 that had been digested with *Sal*I/*Xba*I. Five different 162-bp fragments were generated from the resulting plasmid using the *Mlu*I, *Nhe*I, *Eco*RV, *Nru*I, and *Bam*HI restriction endonucleases. In these isomeric fragments, the I-*Ppo*I recognition site is inserted 126, 114, 78, 54, and 23 bp from the end, respectively. The 5′-phosphoryl group of each fragment was hydrolyzed by treatment with alkaline phosphatase. The dephosphorylated fragments were purified from an agarose gel and then labeled with ^{32}P.

Each ^{32}P-labeled fragment (5000 cpm, <500 pg) was incubated for 30 min at room temperature in a solution (50 μL) of 10 mM Tris-HCl buffer (pH 7.5) containing I-*Ppo*I (50 nM), poly(dIdC)·poly(dIdC) (0.2 μg/mL), EDTA (1 mM), NaCl (50 mM), bovine serum albumin (50 μg/mL), and glycerol (10% v/v). The samples were then loaded directly onto a native TBE polyacrylamide (8 or 10% w/v) gel. After electrophoresis, the gels were dried and visualized by using autoradiography.

A standard curve of DNA bending was obtained by using poly(dA)·poly(dT)-tract standards. Poly(dA)·poly(dT)-tracts, which each induce an 18° bend from linearity (Nelson et al., 1987; Zahn & Blattner, 1987; Koo & Crothers, 1988; Koo et al., 1990), were phased at 10-bp intervals such that the bends were cumulative. Fragments containing two, three, four, and five poly(dA)·poly(dT)-tracts were prepared from plasmids pJT170-2, pJT170-3, pJT170-4, and pJT170-5, respectively, as described previously (Thompson & Landy, 1988). Digesting each plasmid with *Nde*I or *Bam*HI created two fragments, one with the poly(dA)·poly(dT)-tract distortions near the end of the fragments and one with the distortions near the center, respectively. The two fragments from each plasmid were combined and subjected to electrophoresis alongside the I-*Ppo*I·DNA complex in a polyacrylamide (8% w/v) gel.

Steady-State Kinetic Analysis of Catalysis. The ability of I-*Ppo*I to catalyze the cleavage of its recognition site was assayed by monitoring the cleavage of ^{32}P-labeled **I**. Reactions were performed at 37 °C in solutions (100 μL) of 10 mM Tris-HCl buffer (pH 7.5) containing I-*Ppo*I (5−100 pM), [^{32}P]**I** (10 pM to 50 nM), $MgCl_2$ (10 mM), DTT (1 mM), NaCl (0, 50, 100, or 200 mM), and bovine serum albumin (50 μg/mL). The substrate concentration was at least 10-fold greater than the enzyme concentration in each reaction mixture, except for that of the lowest substrate concentration in the absence of NaCl (accordingly, only a lower limit could be determined for the value of K_m in the absence of NaCl). Reactions were quenched at various times (5 min to 4 h) by addition of aliquots (10 μL) to a solution (10 μL) of sodium dodecyl sulfate (SDS) (2% w/v), EDTA (100 mM), glycerol (20% v/v), and bromophenol blue (0.01% w/v). Products were separated from reactants on a polyacrylamide (15% w/v) gel and quantified as in the gel mobility shift assays. Initial velocities were measured at six or more different substrate concentrations at each NaCl concentration. To obtain steady-state kinetic parameters, the initial velocity data were fit to the Michaelis−Menten equation with the program HYPERO (Cleland, 1979). Parameters were calculated for the endonuclease dimer.

Assays for Metal-Ion Dependence of Catalysis. A variety of divalent cations were examined for their ability to substitute for Mg^{2+} and permit the sequence-specific cleavage of DNA by I-*Ppo*I. The reactions were performed in 10 mM Tris-HCl buffer (pH 7.5) containing [^{32}P]**I** (10 nM), I-*Ppo*I (2 nM), and the chloride salt of Mg^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+}, Ca^{2+}, or Zn^{2+} (10 mM of the puratronic grade from Johnson Mathey, Ward Hill, MA). The reaction mixtures were incubated at 37 °C and the reactions quenched after 1 h. In all manipulations, care was taken to avoid contamination of metal ions. Glassware was soaked overnight in 20% (v/v) nitric acid and rinsed thoroughly with 18-MΩ cm water, plasticware was soaked in a solution of EDTA (10 mM) overnight and rinsed thoroughly with 18-MΩ cm water, and

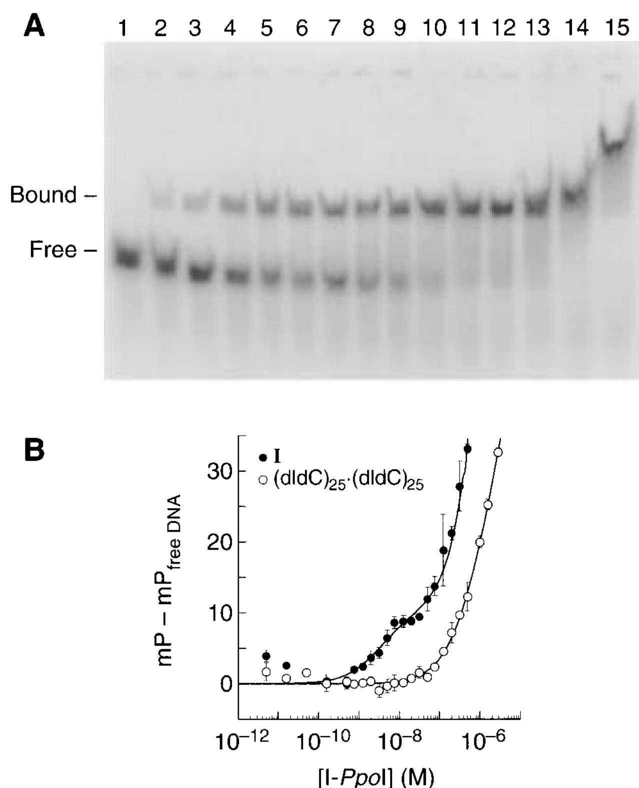

FIGURE 2: Two assays of the binding of I-*Ppo*I to **I**. (A) Gel mobility shift assay of binding of I-*Ppo*I to [^{32}P]**I** in 10 mM Tris-HCl buffer (pH 7.5) containing NaCl (50 mM), EDTA (1 mM), glycerol (10% v/v), and bovine serum albumin (50 μg/mL). The lanes, 1−15, have increasing concentrations of dimeric I-*Ppo*I: 0, 0.02, 0.05, 0.08, 0.13, 0.20, 0.32, 0.50, 0.79, 1.3, 2.0, 3.2, 5.0, 50, and 500 nM. The apparent K_d for specific binding is 0.7 nM. (B) Fluorescence polarization assay of binding of I-*Ppo*I to fluorescein-labeled **I** (●) or fluorescein-labeled $(dIdC)_{25}$·$(dIdC)_{25}$ (○). I-*Ppo*I was added to 25 mM Bis-Tris/Tris/Bis-Tris propane-HCl buffer (pH 7.5) containing fluorescein-labeled DNA (450−550 pM), NaCl (33 mM), EDTA (2 mM), and DTT (1 mM). Each data point is an average of 3−6 measurements. The apparent K_d for the specific site is 5 nM.

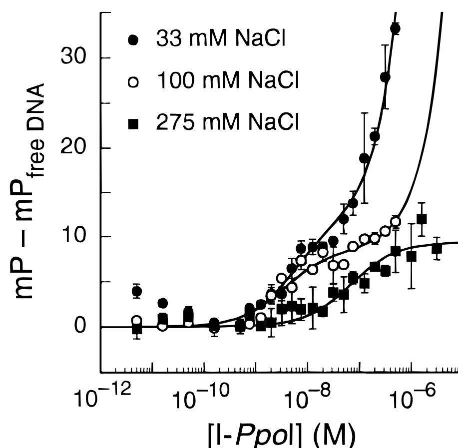

FIGURE 3: Binding of I-*Ppo*I to **I** at three free NaCl concentrations. I-*Ppo*I was added to 25 mM Bis-Tris/Tris/Bis-Tris propane-HCl buffer (pH 7.5) containing fluorescein-labeled **I** (450−550 pM), NaCl [33 mM (●, as in Figure 2), 100 mM (○), or 275 mM (■)], EDTA (2 mM), and DTT (1 mM). Each data point is an average of 3−6 measurements. The lines are best fits to either eq 1 (275 mM) or eq 3 (33 and 100 mM).

all buffers were passed through a column (10 mL) of Chelex-100 resin (Bio-Rad, Richmond, CA).

RESULTS

DNA Binding by I-PpoI. In the absence of Mg^{2+}, I-*Ppo*I binds to DNA but is unable to catalyze cleavage. The formation of an I-*Ppo*I·DNA complex can therefore be followed by elimination of Mg^{2+} from the reaction buffer. Two independent techniques were used to monitor DNA binding: gel mobility shift assays (Fried & Crothers, 1981; Garner & Revzin, 1981) and fluorescence polarization assays (LeTilly & Royer, 1993).

Gel mobility shift assays established that I-*Ppo*I retained specificity for its recognition site in the absence of Mg^{2+}. As shown in Figure 2A, an I-*Ppo*I·DNA complex was formed at low I-*Ppo*I concentrations. Unlabeled **I**, but not poly(dIdC)·poly(dIdC) (0.2 μg/mL), competed with [^{32}P]**I** for complex formation at low concentrations of I-*Ppo*I (data not shown). This complex is therefore a specific complex, and it has $K_d = 0.7$ nM. At high I-*Ppo*I concentrations, the I-*Ppo*I·DNA band was smeared, apparently due to the formation of less stable, higher-order complexes. These nonspecific complexes dissociated upon addition of poly-(dIdC)·poly(dIdC) (0.2 μg/mL) (data not shown).

Both nonspecific and specific DNA binding by I-*Ppo*I were examined with fluorescence polarization assays. Three DNA fragments were used: **I**, **II**, and $(dIdC)_{25}$·$(dIdC)_{25}$. The fluorescein and linker in **I** and **II** did not interfere with binding by I-*Ppo*I as demonstrated by gel mobility shift assays (data not shown). The binding of **I** and $(dIdC)_{25}$·$(dIdC)_{25}$ in the presence of low ionic strength (33 mM added NaCl) is shown in Figure 2B. A small increase in polarization (10 mP) was observed upon binding to the specific recognition site of **I** [the results from **II** (data not shown) were similar to those from **I**, except that the change in polarization upon binding to **II** was approximately 60% larger]. This initial increase was followed by a second increase in polarization from nonspecific binding. The polarization did not reach a plateau at the highest protein concentration examined (1 μM), which indicates that the nonspecific sites were not saturated. The polarization was also observed to increase upon binding to $(dIdC)_{25}$·$(dIdC)_{25}$. Nonspecific binding of I-*Ppo*I to **I** and $(dIdC)_{25}$·$(dIdC)_{25}$ began at the same concentration of protein (50 nM). From the data in Figure 2, it is apparent that the change in fluorescence polarization of a fluorescein-labeled duplex provides an effective measure of the binding of I-*Ppo*I to DNA in homogeneous solution.

The effect of increasing NaCl concentration on the binding of I-*Ppo*I to its recognition site was examined by using fluorescence polarization to monitor complex formation. As shown in Figure 3, at high salt concentrations, specific binding is weakened slightly but nonspecific binding is impaired drastically. Dissociation constants were determined for binding to the specific site, and these results are reported in Table 1. Only lower limits are reported for nonspecific dissociation constants due to the inability to saturate the nonspecific sites, even at high protein concentrations.

DNA Bending upon Binding by I-PpoI. A circular permutation assay (Wu & Crothers, 1984) was used to detect protein-induced DNA bending by I-*Ppo*I. This assay is based on position-dependent effects of DNA distortions on the electrophoretic mobilities of DNA fragments. A fragment with a distortion located near the end migrates faster during electrophoresis than does an isomeric fragment with a

Table 1: Effect of NaCl on Binding of DNA by I-*Ppo*I[a]

[NaCl] (mM)	$K_d^{specific}$ (nM)	$K_d^{nonspecific}$ (nM)
33	5.4 ± 2.4	>175
50	3.3 ± 2.5	>350
100	3.4 ± 2.6	>550
150	3.7 ± 1.7	>1000
175	5.4 ± 3.1	>1000
200	36.0 ± 9.6	>1000
225	20.1 ± 11.0	>1000
250	111.7 ± 31.4	>1000
275	69.3 ± 19.0	>1000

[a] Determined by fluorescence polarization in 25 mM Bis-Tris/Tris/Bis-Tris propane buffer (pH 7.5) containing EDTA (2 mM), DTT (1 mM), BSA (50 μg/mL), and NaCl (as indicated).

distortion near the center. As shown in Figure 4A, the mobility of an I-*Ppo*I·DNA complex was dependent on the distance of the recognition site from the end of the fragment. The *Eco*RV fragment, which has the recognition site positioned in the center, migrated slowest when bound by I-*Ppo*I. The free DNA fragments did not display any variable mobility. The distortion of the recognition site is therefore not inherent to the DNA sequence itself but is induced upon binding of I-*Ppo*I.

The degree of the bend in DNA induced by I-*Ppo*I was determined from eq 4 (Thompson & Landy, 1988).

$$\mu_{rel} = \cos\left(\frac{\alpha}{2}\right) \qquad (4)$$

In eq 4, α is the degree to which linear DNA (α = 0) is bent. The relative mobilities of the complexes in which I-*Ppo*I was bound to the *Mlu*I or *Bam*HI fragments (μ_{end}) and the *Eco*RV fragment (μ_{middle}) were used to calculate $\mu_{rel} = \mu_{middle}/\mu_{end} = 0.946 \pm 0.011$, where the standard deviation was based on eight independent experiments. Substituting this value for μ_{rel} into eq 4 reveals that I-*Ppo*I bends DNA by α = 38 ± 4° upon binding to its recognition site. To verify this value, the relative mobility of the I-*Ppo*I·DNA complex was compared to that of poly(dA)·poly(dT)-tract standards. As shown in Figure 4B, the relative mobilities of the five fragments containing the poly(dA)·poly(dT)-tracts were within error of that predicted by eq 4. Thus, eq 4 accurately predicts the degree of bending for our system.

Steady-State Kinetic Parameters for Catalysis by I-PpoI. In the presence of Mg^{2+}, I-*Ppo*I catalyzes the hydrolysis of two phosphodiester bonds within its recognition site, generating 4-nt, 3′-OH extensions. The steady-state kinetic parameters for this reaction are reported in Table 2. The highest value of k_{cat}/K_m was observed at low concentrations of NaCl. As the NaCl concentration increased to 200 mM, the value of k_{cat}/K_m decreased by at least 200-fold. The decrease in k_{cat}/K_m (from >4.5 nM^{-1} min^{-1} to 0.023 nM^{-1} min^{-1}) was a result of a 400-fold increase in K_m and a 2-fold increase in k_{cat}. These results suggest that NaCl influences the binding rather than the turnover of substrate.

Metal-Ion Dependence of Catalysis by I-PpoI. A divalent cation, often Mg^{2+}, is required for most reactions catalyzed by DNA-modifying enzymes (Roberts & Halford, 1993). The specificity of the enzymes for the cofactor is, however, variable. Seven divalent cations, including Mg^{2+}, were tested for their ability to act as cofactors in the I-*Ppo*I-catalyzed reaction. All cations tested except for Cu^{2+} supported catalysis with varied success. The order of activity was Mg^{2+} > Mn^{2+} > Ca^{2+} = Co^{2+} > Ni^{2+} > Zn^{2+}. Catalysis with each of these cofactors gave the expected cleavage products, indicating that substrate specificity was not entirely metal-ion-dependent (Figure 5). Thus, other divalent cations can substitute for Mg^{2+}, but with a decrease in catalytic efficiency.

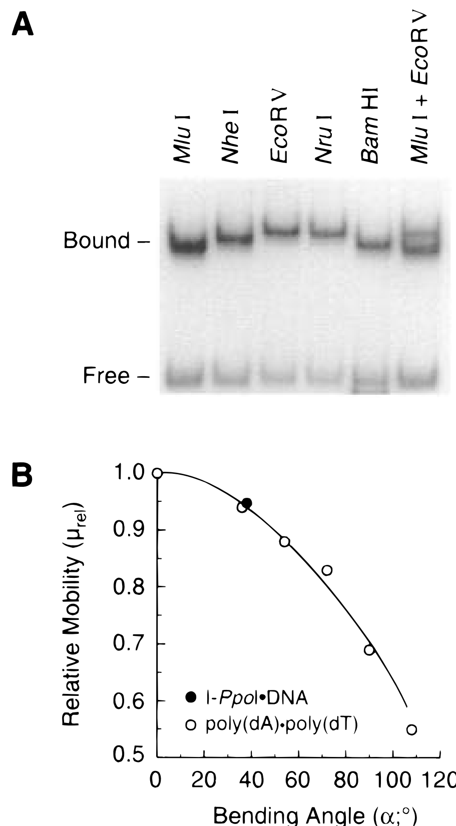

FIGURE 4: Bending of DNA upon binding of I-*Ppo*I. (A) The first five lanes contain circularly permuted DNA fragments generated with the restriction endonucleases *Mlu*I, *Nhe*I, *Eco*RV, *Nru*I, and *Bam*HI. The last lane contains the fragments from both *Mlu*I and *Eco*RV. The I-*Ppo*I recognition site is located near the end and middle of the *Mlu*I and *Eco*RV fragments, respectively. (B) Relative mobilities of the *Mlu*I and *Eco*RV fragments bound to I-*Ppo*I (○) and fragments containing different numbers of poly(dA)·poly(dT)-tracts (●). Equation 4 was used to draw the curve and to calculate that I-*Ppo*I bends DNA by α = 38 ± 4° upon binding to its recognition site.

Table 2: Effect of NaCl on Steady-State Kinetic Parameters for DNA Cleavage by I-*Ppo*I[a]

[NaCl] (mM)	k_{cat} (min^{-1})	K_m (nM)	k_{cat}/K_m (nM^{-1} min^{-1})
0	0.046 ± 0.003	≤0.01	≥4.5
50	0.060 ± 0.010	0.05 ± 0.02	1.2 ± 0.4
100	0.065 ± 0.005	0.3 ± 0.1	0.22 ± 0.03
200	0.090 ± 0.005	4.0 ± 0.7	0.023 ± 0.002

[a] Determined by gel electrophoresis in 10 mM Tris-HCl buffer (pH 7.5) containing $MgCl_2$ (10 mM), DTT (1 mM), BSA (50 μg/mL), and NaCl (as indicated).

DISCUSSION

Comparison of Catalysis by I-PpoI and Restriction Endonucleases. Catalysis by both I-*Ppo*I (Table 2) and many restriction endonucleases depends greatly on reaction conditions. A comparison of steady-state kinetic parameters is therefore a challenge (Bennett & Halford, 1989). Nonetheless, considering only the optimal conditions tested here for

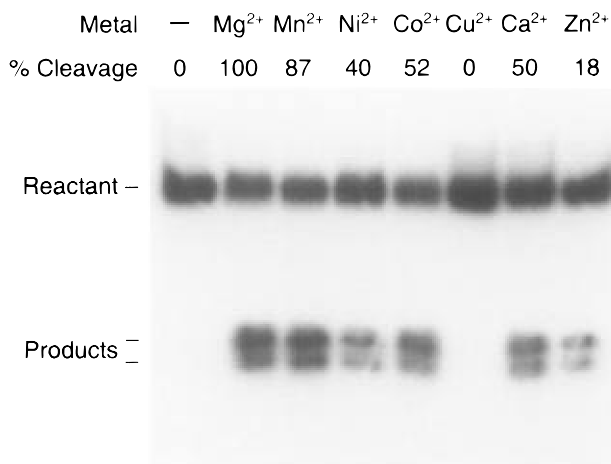

FIGURE 5: Cleavage of **I** by I-*Ppo*I in the presence of various divalent cations. Each reaction yielded products of 22 and 20 bp.

I-*Ppo*I (that is, low concentrations of NaCl at pH 7.5) and optimal conditions for the respective restriction endonucleases, the value of k_{cat}/K_m for I-*Ppo*I is similar of that of many restriction endonucleases. The value of k_{cat}/K_m for I-*Ppo*I is ≥4.5 nM^{-1} min^{-1} or ≥7.5 × 10^7 M^{-1} s^{-1} (Table 2). The values of k_{cat}/K_m for the cleavage of plasmid DNA by *Eco*RI, *Eco*RV, and *Bam*HI have been reported to be 5 × 10^7, 3 × 10^7, and 7 × 10^6 M^{-1} s^{-1}, respectively (Halford & Johnson, 1981; Nardone & Chirikjian, 1987; Halford & Goodall, 1988). These values of k_{cat}/K_m approach that expected for diffusion, which limits the value of all second-order rate constants (Blacklow et al., 1988).

Although the value of k_{cat}/K_m for catalysis by I-*Ppo*I is similar to those for various restriction endonucleases, the values of k_{cat} and K_m differ. I-*Ppo*I has a lower Michaelis constant accompanied by a lower turnover number. Under the optimal conditions used here, I-*Ppo*I has a K_m of ≤0.01 nM. In contrast, *Eco*RI, *Eco*RV, and *Bam*HI have K_m's of 2.5, 0.5, and 3.6 nM, respectively. The higher affinity of I-*Ppo*I for its substrate could reflect the larger size of its recognition site. The value of k_{cat} for I-*Ppo*I is 0.046 min^{-1}, while the values of k_{cat} for *Eco*RI, *Eco*RV, and *Bam*HI are 7.7, 0.9, and 1.5 min^{-1}, respectively. Under other reaction conditions, however, the values of the Michaelis constant and turnover number for catalysis by I-*Ppo*I are similar to those for restriction endonucleases. For example, at 200 mM NaCl and pH 7.5, the K_m of I-*Ppo*I increases to 4 nM (Table 2). At pH 10, the k_{cat} of I-*Ppo*I increases to 2.6 min^{-1} (Lowery et al., 1992).

I-PpoI·DNA in the Absence of Mg^{2+}. The Mg^{2+} cofactor is not required for specific binding of I-*Ppo*I to its recognition site. In the absence of Mg^{2+}, I-*Ppo*I is unable to catalyze the cleavage of DNA. Still, the enzyme maintains enough contacts with DNA to favor its cognate sequence over noncognate sequences. Although many restriction endonucleases form stable complexes with DNA in the absence of Mg^{2+}, they do not necessarily bind specifically. For example, *Eco*RV, *Taq*I, *Cfr*9I, and *Bcg*I bind equally well to DNA containing or lacking their recognition sites (Taylor et al., 1991; Zebala et al., 1992; Siksnys & Pleckaityte, 1993; Kong et al., 1994). In this respect, I-*Ppo*I is similar to the restriction endonucleases *Eco*RI, *Bam*HI, *Rsr*I, *Fok*I, and *Sma*I (Halford & Johnson, 1980; Terry et al., 1983; Aiken et al., 1991; Xu & Schildkraut, 1991b; Waugh & Sauer, 1993; Withers & Dunbar, 1995), which bind to their recognition sites specifically in the absence of a divalent metal ion.

The topology of the DNA in the I-*Ppo*I·DNA complex formed in the absence of Mg^{2+} deviates from that of typical B-form DNA. I-*Ppo*I induces a bend of 38 ± 4° upon binding (Figure 4). The distortion of DNA observed here is consistent with the results of previous footprinting studies, which revealed two regions of increased sensitivity to methylation by dimethylsulfate, a small chemical probe (Ellison & Vogt, 1993). These hypersensitive regions are located on both sides [A(+5) and G(−3), A(−4)] of the central six base pairs and may be the origins for the observed bend of the recognition site by I-*Ppo*I. The ease with which a DNA sequence adopts this requisite conformation may provide a level of discrimination for the enzyme between cognate and noncognate sites. In addition, this distortion may enable the small I-*Ppo*I protein to make contact with its large recognition site, which could span nearly 1.5 helical turns of DNA.

Some restriction endonucleases are also known to distort the double helix. For example, X-ray diffraction analysis of the *Eco*RI·DNA complex shows that this enzyme unwinds three base pairs, opening up the helical grooves and creating three separate kinks (Frederick et al., 1984). *Eco*RV creates a central 50° kink and compresses the major groove of its cognate DNA but does not distort noncognate DNA (Winkler et al., 1993). In contrast, *Pvu*II and *Bam*HI do not distort their recognition sites (Cheng et al., 1994; Newman et al., 1995). The shape, direction, and groove adjustments of the bent I-*Ppo*I recognition site await direct structural analysis.

Gel mobility shift assays are valuable tools for visualization of individual species and extraction of information on the binding and bending of DNA by I-*Ppo*I. These assays suffer, however, from an inability to mimic true solution conditions. To complement the binding data that are obtained from mobility shift assays, we used a second technique to measure binding.[2] Fluorescence polarization assays can be used to measure binding in solution without separating bound from free species. For I-*Ppo*I·DNA, the results obtained from the two independent methods are in gratifying agreement. In both assays, specific binding by I-*Ppo*I is observed when the concentration of endonuclease is <10 nM, and nonspecific binding is observed when the concentration is >50 nM (Figure 2). In the presence of 50 mM NaCl (which is the NaCl concentration in the incubation buffer used for the gel mobility shift assays), the dissociation constant measured with fluorescence polarization (K_d = 3.3 nM) is within 5-fold of that determined from the gel mobility shift assay (K_d = 0.7 nM).

Effect of NaCl on I-PpoI·DNA. The affinity of I-*Ppo*I for its recognition site is influenced dramatically by the concentration of NaCl (Figure 3). This change in affinity is likely to be a result of the polyanionic nature of the DNA helix. The binding of DNA by a protein is accompanied by the release of cations from the DNA. The accompanying increase in entropy is a driving force for binding (Record et al., 1976). Theory predicts that a protein·nucleic acid complex will dissociate with increasing cation concentration as in eq 5.

[2] Filter-binding assays were unsuccessful because the I-*Ppo*I·DNA complex was not retained by nitrocellulose filters.

$$\frac{\partial \log K_d}{\partial \log[\text{cation}]} = k + m'\psi \qquad (5)$$

In eq 5, k is the number of anions displaced upon binding, m' is the number of cations displaced upon binding, and Ψ is a measure of counterion binding to the nucleic acid, which is 0.88 for double-helical native DNA (Record et al., 1991). Our values of K_d and cation concentration from Table 1 are plotted in Figure 6. This plot shows two regions of linearity: a segment at higher salt concentrations for which the affinity of I-*Ppo*I declines with increasing salt and a segment at lower salt concentrations for which there is little change in affinity. Using the values of K_d at higher salt concentrations, $m' = 6.3 \pm 1.4$, which suggests that approximately six cations are displaced from DNA upon binding of I-*Ppo*I at pH 7.5.

At salt concentrations <150 mM, our data do not follow eq 5. Similar deviations have been observed for the binding of other proteins to DNA at low salt concentrations (Jen-Jacobson et al., 1983; Murdoch et al., 1991). These deviations may result from aggregation of the protein or from exceeding the detection limits of the technique used to measure binding. For example, at low ionic strength, the precipitation of *Eco*RI gives an artificially high value of K_d (Jen-Jacobson et al., 1983). We have observed no evidence that I-*Ppo*I precipitates or aggregates in solutions of low ionic strength. Alternatively, the true dissociation constant of I-*Ppo*I at low salt concentrations may be beyond the detection limits of our assays. If virtually all added protein binds to DNA, then an accurate measure of dissociation constants is difficult to obtain because so little protein remains free in solution. Our analysis of catalysis by I-*Ppo*I indicates that the affinity of I-*Ppo*I for DNA does indeed become even tighter at salt concentrations <150 mM (Table 2 and Figure 6).

Effect of NaCl on Catalysis by I-PpoI. Catalysis by restriction endonucleases is usually sensitive to ionic strength, and that by I-*Ppo*I is not an exception. The value of k_{cat}/K_m decreases by 200-fold upon addition of 200 mM NaCl. This decrease comes primarily from the diminished affinity of the enzyme for its recognition site; K_m decreases by 400-fold. Concomitantly, k_{cat} increases by 2-fold. Although the origin of this nominal increase is not clear, Na^+ may serve to facilitate product release, which has been postulated to be the rate-limiting step in catalysis by the intron-encoded endonuclease I-*Sce*I (Perrin et al., 1993).

Effect of Metal Ions on Catalysis by I-PpoI. Although a divalent cation is required for catalysis by I-*Ppo*I, the enzyme does not have a strict preference for a particular metal ion. All metal ions tested, excluding Cu^{2+}, support catalysis (Figure 5). This result is in contrast to catalysis by *Eco*RI and *Eco*RV. The activities of these two enzymes are relatively metal-ion-dependent, and Ca^{2+} does not support catalysis despite binding to the same site as do other metal ions (Vipond et al., 1995; Vipond & Halford, 1995).

The metal-ion binding site of I-*Ppo*I is plastic. The enzyme binds productively to divalent cations with radii as small as Ni^{2+} (0.69 Å, CN 6; Shannon, 1974) and as large as Ca^{2+} (1.00 Å, CN 6). Preferences in the metal-ion binding site are, however, apparent. The cations best capable of supporting catalysis, Mg^{2+}, Mn^{2+}, Co^{2+}, and Ca^{2+}, are oxophilic and can adopt an octahedral coordination geometry.

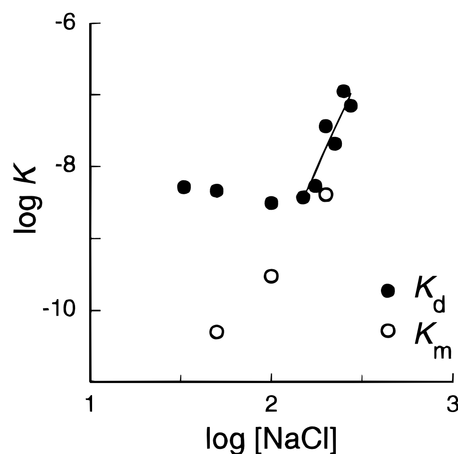

FIGURE 6: Binding of **I** to I-*Ppo*I in the presence of various concentrations of NaCl. The slope of the linear portion of the data suggests that approximately six cations are displaced from DNA upon binding of I-*Ppo*I.

Table 3: Sequence Alignment of I-PpoI with Type II Restriction Endonucleases

endonuclease	putative active-site motif[a]
I-*Ppo*I	$P_{108}D_{109}$ ... $D_{140}N_{141}K_{142}$
*Eco*RI	$P_{90}D_{91}$ ... $E_{111}V_{112}K_{113}$
*Eco*RV	$P_{73}D_{74}$ ... $D_{90}I_{91}K_{92}$
*Eco*571	$P_{77}D_{78}$ ... $D_{92}A_{93}K_{94}$
	$P_{811}D_{812}$ ... $D_{833}Q_{834}K_{835}$
*Fok*I	$P_{453}D_{454}$ ... $D_{471}T_{472}K_{473}$
*Hha*II	$P_{98}D_{99}$ ... $E_{115}L_{116}K_{117}$
*Kpn*I	$P_{147}D_{148}$ ... $D_{163}P_{164}K_{165}$
*Ngo*PII	$P_{72}D_{73}$ ... $E_{88}S_{89}K_{90}$
*Pst*I	$P_{304}D_{305}$ ... $D_{320}K_{321}K_{322}$
*Rsr*I	$P_{95}D_{96}$ ... $E_{116}S_{117}K_{118}$

[a] Based on Thielking et al. (1991) and Anderson (1993).

In contrast, the other divalent ions tested are thiophilic or prefer other coordination geometries. Thus, I-*Ppo*I may be similar to *Eco*RV, which binds Mg^{2+} in octahedral coordination with the oxygens from the scissile phosphoryl group, the side chains of Asp74 and Asp90, and three water molecules as its ligands (Kostrewa & Winkler, 1995). Asp74 and Asp90 of *Eco*RV are part of the active site motif, P(D/E)...(D/E)XK, which also appears in other type II restriction endonucleases (Thielking et al., 1991; Anderson, 1993), and in I-*Ppo*I. The sequence alignment in Table 3 suggests that Asp109 and Asp140 of I-*Ppo*I may coordinate to Mg^{2+} or another divalent metal ion.

Conclusions. This study represents the first detailed biochemical analysis of binding and catalysis by an intron-encoded endonuclease. We have found that tight, specific binding by I-*Ppo*I induces a conformational change in DNA in the absence of Mg^{2+}. In addition, the interaction of I-*Ppo*I with DNA, both in equilibrium and during steady-state catalysis, weakens with increasing concentrations of monovalent cations in a manner consistent with the entropy gained from the release of cations from DNA being a driving force for binding. Catalysis by I-*Ppo*I is efficient, with values of k_{cat}/K_m at low concentrations of NaCl approaching the diffusion limit. Finally, the metal-ion binding site of I-*Ppo*I prefers oxophilic, octahedral divalent cations but is otherwise nonspecific. Our work demonstrates that the recent discovery of intron-encoded endonucleases such as I-*Ppo*I provides a unique opportunity for dissection of both enzymatic

catalysis and protein−DNA interactions in a single system.

ACKNOWLEDGMENT

We are grateful to Dr. Catherine A. Royer for invaluable advice and assistance in the use of fluorescence polarization and to Dr. Robert Lowery, Dr. George S. Golumbeski, Dr. Kimberly Knoche, Suzanne Selman, and others at Promega Corp. for their generous support of this work.

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BI952363V