Mn^{2+}-dependent Catalysis by Restriction Enzymes: Pre-Steady-State Analysis of *Eco*RV Endonuclease Reveals Burst Kinetics and the Origins of Reduced Activity

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Abstract: The origins of divalent metal-dependent catalytic properties in phosphoryl transfer by *Eco*RV endonuclease have been investigated by transient kinetic methods. Pre-steady-state measurements on short oligodeoxynucleotide substrates reveal a burst of product formation for both Mg^{2+}- and Mn^{2+}-catalyzed DNA cleavage reactions, indicating that for each metal ion the product release step is partially or completely rate-limiting. However, the steepness of the burst is far greater for Mn^{2+} reactions, and analysis of the steady-state portions of the reaction profiles shows that the overall rate is 6-fold slower in the presence of this cofactor. The strongly rate-limiting product release step in Mn^{2+} reactions may arise from the higher intrinsic affinity of this metal ion for phosphates. Single-turnover experiments carried out with enzyme in molar excess over DNA were also used to isolate the chemical step of the reaction. In contrast to the slower steady-state rates, both these measurements and the pre-steady-state reaction bursts show that the bond-breaking and bond-making steps are significantly better catalyzed by Mn^{2+}. This supports models for catalysis deduced from X-ray crystal structures of the enzyme−substrate DNA complex, in which a divalent metal ion is directly ligated to the pro-S_P oxygen of the scissile phosphate group.

Divalent metal ion cofactors are essential for cleavage of dyad-symmetric duplex DNA target sites by homodimeric type II restriction endonucleases.[1] The reactions proceed via in-line attack of hydroxide ion on the scissile phosphate to generate a pentacovalent transition state, leaving products with 5′-phosphate and 3′-OH groups.[2] The metal ions may speed catalysis by providing a source of hydroxide ions, by neutralizing the incipient additional negative charge in the trigonal bipyramidal transition state, and/or by facilitating release of the 3′-O anion. Cocrystal structures of *Eco*RV, *Bam*HI, and *Bgl*I bound to DNA and divalent metal ions provide supporting evidence for each of these roles.[3]

The highest levels of activity in DNA cleavage are found with Mg^{2+} as the cation, and this metal is the likely cofactor *in vivo*. However, other divalent metal ions are also able to support activity to varying degrees. Among these, catalytic effects upon substitution of Mn^{2+} have been studied in some detail for several enzymes in the family. A number of distinct phenomena have been observed. First, DNA sequence discrimination is markedly decreased by the substitution of Mn^{2+} for Mg^{2+}.[4,5] For example, in *Eco*RV the preference for the cognate GATATC site relative to GTTATC is decreased by over 10^4-fold when Mn^{2+} is present.[4] This factor reflects both a 75-fold decrease in k_{cat}/K_m for cognate site cleavage, together with a remarkable 700-fold increase in k_{cat}/K_m for the noncognate site. Second, in *Eco*RV, *Eco*RI, and *Mun*I, Mn^{2+} can significantly reconstitute the deleterious effects on Mg^{2+}-dependent rates caused by enzyme mutations in the DNA binding cleft.[6,7] These effects are striking because they can be manifested for mutants located in numerous positions across the protein−DNA interface. In one case, an active-site mutation of Ile91 to leucine in *Eco*RV, Mn^{2+}-dependent catalysis shows both enhanced cleavage rates and improved sequence specificity, compared to Mg^{2+} reactions of the wild-type enzyme.[7] In general, however, overall rates for wild-type enzymes at cognate sites are slower in the presence of Mn^{2+},[4,8] even though this metal is more acidic and lowers the pK_a of an associated water molecule by a greater amount.[9]

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(9) pK_a values for water ligands in *aquo*−metal complexes vary in the literature. Values of 11.4 for Mg^{2+} and 10.6 for Mn^{2+} have been reported (Dahm, S. C.; Derrick, W. B.; Uhlenbeck, O. C. *Biochemistry* **1993**, *32*, 13040; Kragten, J. *Atlas of metal−ligand equilibria in aqueous solution*; Halsted Press: Chichester, England 1978; Westermann, K.; Naser. K.−H.; Brandes, G. *Inorganic Chemistry*, 12th ed.; VEB Deutscher Verlag fur Grundstoffindustrie: Liepzig, Germany, 1986; (p 53−55). However, a value of 10 for Mn^{2+}, with some uncertainty, is also reported (Wilkinson, G., ed. *Comprehensive Coordination Chemistry*; Pergamon Press: Oxford 1987; Vol. 2; p 310) and the early literature reports values of 10.6 for Mn^{2+} and 12.8 for Mg^{2+} (Chabarek, S.; Courtney, R. C.; Martell, A. E. *J. Am. Chem. Soc*. **1952**, *74*, 5057). However, it is always found that the pK_a of waters ligated to Mn^{2+} is lower than that of waters ligated to Mg^{2+}.

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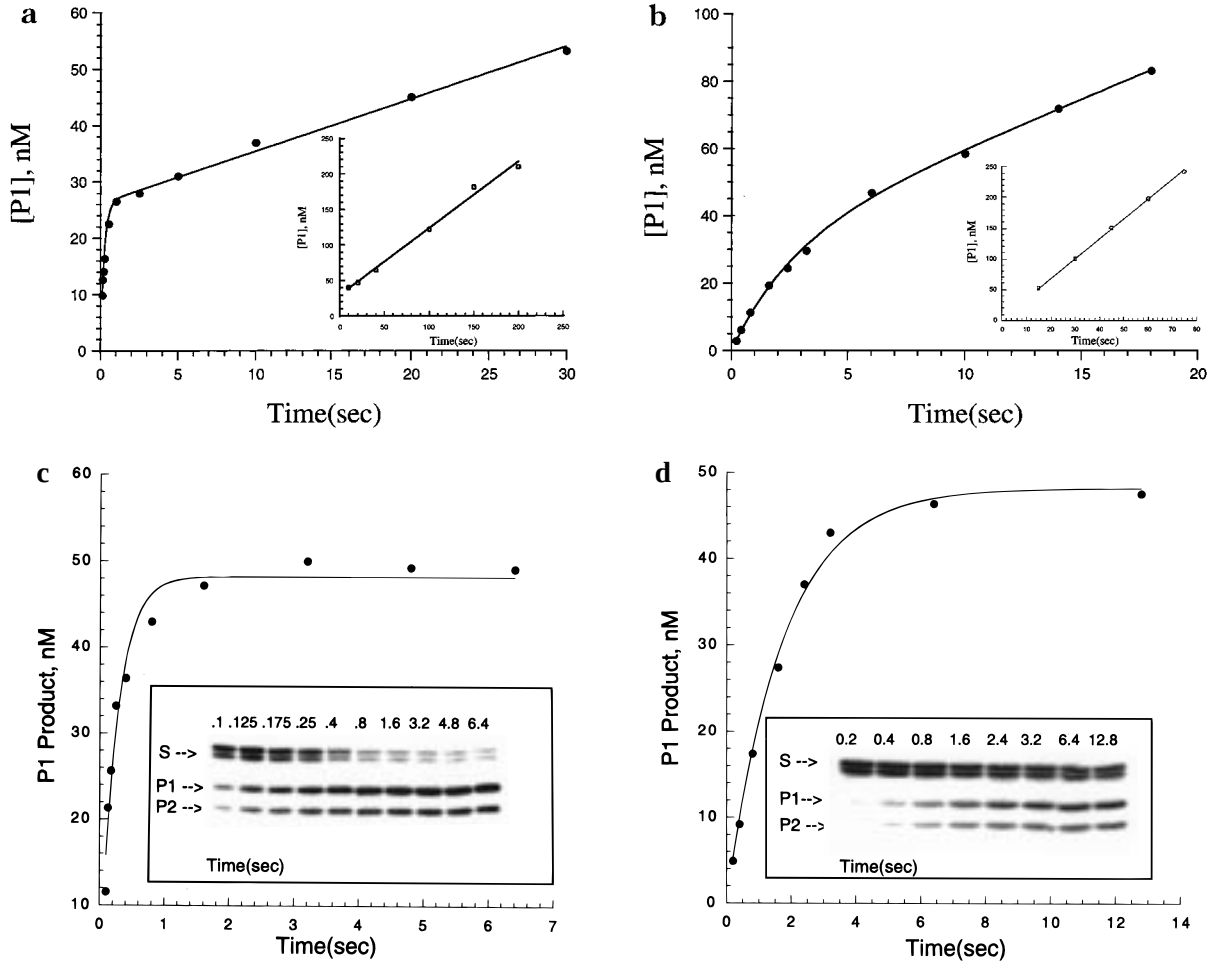

Figure 1. (A−D) Pre-steady state and single-turnover reactions of *Eco*RV. The 16-mer substrate 5′-GGGAAAGATATCTTGG with an off-center target site yields 9-mer (P1) and 7-mer (P2) products after blunt-ended cleavage at the center TA-step (panels C and D). Time courses of product formation under pre-steady-state conditions are shown for Mn^{2+}-dependent reactions (panel A) and for Mg^{2+}-dependent reactions (panel B). The insets of panels A and B show steady-state data sampled by hand under conditions of higher substrate:enzyme molar ratios (see Experimental Section). Single-turnover reactions in the presence of Mn^{2+} and Mg^{2+} are shown in panels C and D, respectively.

The structural and mechanistic origins of the observed Mn^{2+} ion effects on catalysis by type II restriction enzymes have been obscure. A more detailed kinetic and structural characterization should provide better insight into the induced-fit conformational transitions occurring during complex formation, since DNA binding specificity is often greatly enhanced in the presence of metal ions.[10,11] Moreover, other enzymes of nucleic acid metabolism also exhibit altered catalytic properties depending on the type of metal ion present.[12] Insights derived from detailed analysis of a well-characterized restriction endonuclease, employed as a model, should therefore generalize to other more complex systems. Here, we address the basis for the reduced rates of Mn^{2+}-catalyzed restriction enzyme reactions at cognate sites by transient kinetic analyses of *Eco*RV endonuclease. These measurements show that the bond-making and bond-breaking steps of catalysis are faster in the presence of Mn^{2+} and that the reduced activity with this cofactor arises from the much slower rate of a physical step after cleavage.

Results and Discussion

Steady-state kinetic analysis using plasmid substrates has shown that the second-order rate constant k_{cat}/K_m for cognate-site DNA cleavage by *Eco*RV decreases by 75-fold when Mn^{2+} is substituted for the preferred Mg^{2+} cofactor.[4] We investigated the origins of this effect in more detail via transient kinetic experiments with short 16-mer oligonucleotide duplexes containing the cognate GATATC *Eco*RV site (Figure 1). To establish whether the rate-limiting step in catalysis differs between the two metal ions, rapid chemical quench experiments were performed under conditions of 8-fold substrate excess and saturating divalent metal ion concentrations. Metal-ion titration experiments have previously established that rates plateau at concentrations of 10 mM $MgCl_2$ and 2.5 mM $MnCl_2$.[13]

The pre-steady-state reactions showed a clear rapid burst of product formation for the Mn^{2+} reactions, followed by a much slower linear steady-state increase (Figure 1A). A small burst was also observed for reactions performed with Mg^{2+} as cofactor (Figure 1B). The observation of burst kinetics establishes that a physical step following DNA cleavage is at least partially rate-

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Table 1. Mg^{2+}- and Mn^{2+}-Dependent Catalytic Rates[a]

	Mg^{2+}	Mn^{2+}
k_2 (sec^{-1})		
single turnover	0.6 ± 0.06	4.1 ± 0.2
pre-steady state	0.55 ± 0.01	4.4 ± 0.1
k_{cat} (sec^{-1})	0.23 ± 0.02	0.04 ± 0.004

[a] Kinetic scheme for duplex DNA cleavage by *Eco*RV (E) is depicted below. S and P represent the intact and cleaved duplexes, and k_2 is the rate constant for cleavage of either single strand. For the dyad-symmetric substrate studied, the rates of cleavage for the two strands can be determined from the appearance of the 7-mer and 9-mer products (Figure 1), and are identical within experimental error. It is well-established that the number of metal ions required for catalysis (M_n) is at least two per enzyme subunit.[16,24]

$$E + S + Mn^{2+} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}S{\cdot}Mn^{2+} \xrightarrow{k_2} E{\cdot}P{\cdot}Mn^{2+} \xrightarrow{k_3} E + P + Mn^{2+}$$

limiting for both Mn^{2+} and Mg^{2+} reactions. Rates of product formation were fit to the equation

$$P = A(1 - e^{-k_1 t}) + k_2 t \quad (1)$$

where P is the amount of cleaved product normalized to the total enzyme concentration, k_2 is the steady-state rate of product appearance, A is the burst magnitude, and k_1 the rate constant for the early exponential burst phase. The rate constants k_1 are determined to be 4.4 ± 0.1 s^{-1} and 0.55 ± 0.01 s^{-1} for the Mn^{2+} and Mg^{2+} reactions, respectively (Table 1). For the Mn^{2+} reaction profile, extrapolation of the burst magnitude to the ordinate yields an estimate that $110 \pm 15\%$ of the enzyme active sites are capable of catalysis.[14]

The burst experiments were repeated under conditions of higher DNA:*Eco*RV molar ratios to obtain highly accurate values for the steady-state rates (Figure 1A and B, insets). Analysis of the linear portions of these reaction profiles shows that the steady-state rate (equivalent to k_{cat}) in the presence of Mg^{2+} exceeds that for the Mn^{2+}-dependent reaction by roughly 6-fold (Table 1). This is a 4-fold smaller effect as compared to the approximately 25-fold higher k_{cat} for Mg^{2+} reactions toward plasmid substrates.[4] The greater discrepancy between Mg^{2+} and Mn^{2+} overall cleavage rates on plasmid substrates may have its origins in a different mechanism for product dissociation, which could involve transfer of the enzyme to adjacent nonspecific sequences prior to release from the DNA.

DNA cleavage reactions were also carried out under single-turnover conditions. At 3-fold enzyme excess and saturating concentrations of DNA and divalent metal ions, the rate of the chemical step measured by rapid quench kinetics at pH 7.5 for Mn^{2+}-mediated cleavage exceeds that for Mg^{2+} by 7-fold (Table 1; Figure 1C and D).[15] The excellent agreement between the single-turnover and pre-steady-state measurements shows that the rate of the chemical step is being monitored in the burst. Identical values for the single-turnover rates were also obtained in reactions with 3-fold higher concentrations of both enzyme and DNA (data not shown), further confirming that an earlier binding step does not contribute to these rates. The thermodynamic dissociation constant K_d for the *Eco*RV−DNA complex in the absence of metal ions is approximately 1 nM under similar conditions of pH and ionic strength, much lower than the concentrations employed in these experiments.[10]

(14) The small burst amplitude observed for Mg^{2+} reactions makes estimation of active-site concentrations by extrapolation unreliable in this case.

(15) The rate of Mg^{2+}-dependent single-turnover reactions at 100 mM ionic strength under otherwise identical conditions is 1.1 ± 0.1 s^{-1} .[13]

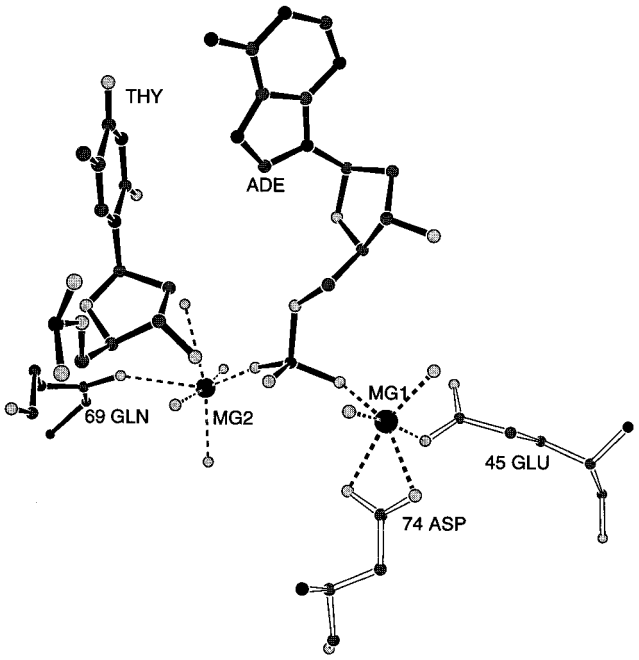

Figure 2. Structure of the *Eco*RV−DNA product complex determined in the presence of Mg^{2+} ions.[3c] The two magnesium ions are shown as larger black spheres; smaller isolated gray spheres represent the positions of water molecules. Thy and Ade represent the nucleotides of the center 5′-TA-3′ step, with the 5′-phosphorylated product evident. Dotted lines show inner-sphere metal−ligand contacts.

Examination of the data in Table 1 shows that the rate of phosphoryl transfer for Mg^{2+}-dependent reactions is 2- to 3-fold faster than the steady-state rate. This accounts for the observation of a small product burst under these experimental conditions. The measurements are in accord with stopped-flow fluorescence studies for Mg^{2+}-dependent reactions of *Eco*RV toward oligonucleotides, which showed that the rate of the chemical step is 2-fold faster than the k_{cat}.[16] Thus, for the preferred Mg^{2+} cofactor, the rate-limiting step is a combination of both cleavage and product release.[17] By contrast, the rate of phosphoryl transfer in Mn^{2+}-dependent reactions exceeds that of a physical step following bond-breakage by 2 orders of magnitude (Table 1). Therefore, in this case the rate-limiting step for oligonucleotide cleavage is entirely after the reaction chemistry.

In the *Eco*RV-product DNA−Mg^{2+} complex, two Mg^{2+} ions bind directly to the newly generated 5′-phosphate at the cleavage site (Figure 2).[3c] By making the assumption that Mn^{2+} ions are located in similar positions in the Mn^{2+}−DNA-*Eco*RV product complex, it would appear that conformational changes needed to release the products are inhibited to a greater degree in the presence of Mn^{2+}. This is consistent with the higher intrinsic affinity for phosphates exhibited by Mn^{2+} relative to Mg^{2+}.[18]

The finding that the phosphoryl transfer step is better catalyzed by Mn^{2+} than by Mg^{2+} is in accord with crystal structures of the *Eco*RV−DNA complex, which show a bound

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(17) In the case of plasmid substrates, product release is the slow step for Mg^{2+} reactions as well: Erskine, S. G.; Baldwin, G. S.; Halford, S. E. *Biochemistry* **1997**, *36*, 7567.

(18) The affinities of Mg^{2+} and Mn^{2+} ions for triphosphate anion, $P_3O_{10}^{5-}$, measured under identical experimental conditions, reveal a nearly 10-fold higher equilibrium constant for the binding of Mn^{2+}. (Hogfeldt, E. *Stability constants of metal-ion complexes*; Pergamon Press: Oxford, 1979; Part A, p 141−143).

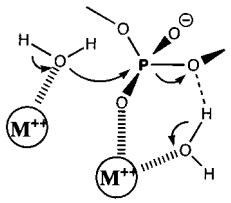

Figure 3. Essential elements of the proposed transition state for phosphoryl transfer by *Eco*RV.[20] Metal−ligand inner sphere contacts are shown as hatched lines. Dotted lines indicate hydrogen bonds.

divalent metal ion (Mg^{2+}, Mn^{2+}, or Ca^{2+}) ligated directly to the pro-S_P oxygen of the scissile phosphate.[3c,d] The increased rate of the chemical cleavage step with Mn^{2+} is readily explained by the greater acidity of this cation relative to Mg^{2+}: Mn^{2+} is better able to withdraw electrons from the P−O bond, rendering the phosphorus more electropositive and thus more susceptible to nucleophilic attack by hydroxide ion.[19] A recently proposed three-metal mechanism for *Eco*RV-catalyzed DNA cleavage also invokes the ionization of metal-ligated water molecules to both generate the attacking hydroxide ion and to protonate the leaving 3′-O anion (Figure 3).[20] However, the correlation of reaction rate with acidity of the metal ion, rather than with nucleophile strength [$Mg^{2+}(OH)^-$ is a more potent nucleophile than $Mn^{2+}(OH)^-$], suggests that the most important element of catalysis is neutralization of the additional negative charge accumulating in the transition state.

In conclusion, we have shown that the reduced overall activity of Mn^{2+}-catalyzed cleavage by *Eco*RV arises from either slow product release or a slow conformational change following catalysis and that the rate of phosphoryl transfer is faster with this metal than with Mg^{2+}. These findings enhance our understanding of the catalytic mechanism and also emphasize the importance of a transient kinetic analysis in which individual microscopic rate constants are measured.[21] The steady-state measurements, taken alone, would be misleading in this case since they could be interpreted to indicate no direct metal ion interaction with the scissile phosphates. The detailed analysis of Mn^{2+}- versus Mg^{2+}-mediated cleavage is also pertinent to understanding function in other classes of metal-dependent enzymes carrying out phosphoryl transfer reactions. In several known cases, Mn^{2+} not only affects a canonical activity in a quantitative manner, but also introduces an entirely new function. For example, Mn^{2+} allows phosphorylase kinase to catalyze phosphorylation of certain substrates on tyrosine rather than on serine residues.[22] The RNase H activity of HIV-1 reverse transcriptase is altered by Mn^{2+} such that hydrolysis of double-stranded RNA (which is not an Mg^{2+}-mediated activity) becomes possible.[23] Mn^{2+}-catalyzed reactions by this enzyme are also more sensitive to inhibition by azidothymidylate.

Differences in steric and electronic properties of Mn^{2+} and Mg^{2+} must ultimately underlie these effects. As suggested for *Eco*RV, differences in catalytic efficiency at the level of the direct chemical step may often be reasonably interpretable in terms of altered capacities to function as Lewis acids (as reflected in the pK_a values of associated water molecules) or in altered intrinsic affinities for phosphates. However, we envision that Mn^{2+} effects on specificity, on the reconstitution of mutant activities, and on the introduction of new activities may be more difficult to rationalize because they could well depend on metal-specific conformational changes. Explanations for these phenomena must ultimately be sought in detailed structural terms and will require crystallographic analyses of wild-type and modified enzyme−substrate complexes in the presence of Mg^{2+} and Mn^{2+}. Because of the large database of modified complexes exhibiting metal-dependent functional differences[1b] and the relative ease of crystallization, *Eco*RV should be a very useful general model for exploring these questions.

Experimental Section

Preparation of Enzyme and DNA Substrates. *Eco*RV endonuclease was expressed in *Escherichia coli* and purified by column chromatography, as described.[3d] Purified enzyme preparations were dialyzed into 10% (v/v) glycerol, 0.4 M NaCl, 20 mM potassium phosphate (pH 7.3), and 1 mM DTT, followed by concentration to approximately 1.0 mg/mL in an Amicon ultrafiltration cell. Small aliquots were then flash-frozen and stored at −70 °C. The 16-mer DNA substrate 5′-GGGAAAGATATCTTGG and its complement were synthesized, 5′-end-labeled with [γ-^{32}P]ATP, annealed together, and purified as described.[13] The melting temperature of this duplex substrate is 51 °C.

Single-Turnover Cleavage Assays. Single-turnover reactions in the presence of Mn^{2+} or Mg^{2+} were carried out with 150 nM purified enzyme, 50 nM DNA at 37 °C in an assay buffer containing 50 mM Hepes (pH 7.5), 200 mg/mL BSA, and 1 mM DTT. Saturating concentrations of metal ion were used in all experiments (10 mM $MgCl_2$; 2.5 mM $MnCl_2$), and the ionic strength was adjusted with NaCl to a constant value of 140 mM. Reactions were carried out in a rapid-quench instrument (Kintek RQF-3), with the enzyme and DNA kept in separate syringes and divalent metal ions present in each syringe, ensuring the fastest cleavage rates.[13] Mixing the enzyme−DNA complex without divalent metal ions in one syringe, with $MgCl_2$ in the second syringe, gave slower rates as also observed in stopped-flow fluorescence experiments.[16] Ten time points were taken per reaction. Reactions were quenched with a solution containing 4 M urea and 75 mM EDTA. Aliquots of 10 μL from each timepoint were mixed with an additional 8−10 μL of quench solution containing bromphenol blue dye and separated on 8 M urea, 20% polyacrylamide gels, followed by visualization via autoradiography on the Molecular Dynamics Storm840 PhosphorImager. Rate constants were determined by fitting the data to a first-order exponential. Data were plotted using the program *Scientist*.

Pre-Steady-State Measurements. Reactions under pre-steady-state conditions performed in the rapid-quench instrument were carried out as described for the single-turnover experiments, except that concentrations of 200 nM DNA and 25 nM *Eco*RV were used. For longer time courses in which the steady-state data were sampled by hand, smaller molar ratios of enzyme to DNA were employed. For Mn^{2+} reactions, these longer time-courses were carried out with 25 nM enzyme and 800 nM DNA. For Mg^{2+} reactions, the longer time-courses were carried out with 15 nM enzyme and 900 nM DNA.

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