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Kinetic Analysis of Product Release and Metal Ions in a Metallonuclease

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Abstract

Most nucleases rely on divalent cations as cofactors to catalyze the hydrolysis of nucleic acid phosphodiester bonds. Here both equilibrium and kinetic experiments are used to test recently proposed models regarding the metal ion dependence of product release and the degree of cooperativity between metal ions bound in the active sites of the homodimeric PvuII endonuclease. Equilibrium fluorescence anisotropy studies indicate that product binding is dramatically weakened in the presence of metal ions. Pre steady state kinetics indicate that product release is at least partially rate limiting. Steady state and pre steady state data fit best to models in which metals remain bound to the enzyme after the release of product. Finally, analysis of cooperative and independent binding models for metal ions indicates that single turnover kinetic data are consistent with little to no positive cooperativity between the two metal ions binding each active site.

Keywords

metallonuclease; mechanism; kinetics; product release; metal ions; cooperativity

Nucleases are unique among hydrolytic enzymes in their challenge: To catalyze the cleavage of a phosphodiester bond, one of the most stable bonds in biochemistry [1], in a charge repulsive, polyanionic context. Due the high negative charge density and relatively high natural abundance [2], divalent cations like Mg(II) and Ca(II) are natural cofactors for this process. Since the early 1990's many metallonucleases have been crystallized, and a large fraction of these enzyme active sites feature more than one metal ion [3]. These structures have served as the inspiration for mechanisms involving two metal ions. Indeed, given the steps necessary to achieve the hydrolysis of phosphodiester bonds, it makes mechanistic sense to distribute the various steps between metal ions. In most proposed mechanisms, one metal ion is principally involved in nucleophile activation (metal A in Fig. 1), while the other (metal B) is often depicted as contributing to transition state stabilization but also involved in facilitating product release by coordinating a water molecule which donates a proton to the leaving group.

With regard to the roles of the metal ions in various mechanistic steps, most of the attention has been devoted to the early steps of substrate binding [4], nucleophile activation [5], and other aspects of the chemical step. Indeed, these have been analyzed in some detail for the restriction enzyme PvuII endonuclease [3,6]. Like many of its relatives, this metallonuclease

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has been crystallized with its target DNA with two Ca(II) ions per active site [7]. The metal ions are held by conserved acidic residues common to all protein metallonucleases (Fig. 1). Metal ion binding studies involving ITC and ^{25}Mg NMR spectroscopy established two metal ion binding sites per active site and provided mM equilibrium binding constants for Ca(II), Mn(II), and Mg(II) [8,9]. Studies of the metal dependence of cognate DNA binding yielded association and dissociation rate constants as a function of metal ion concentrations [10]. Substrate association is metal ion dependent. In the presence of Mg(II), DNA binding is 60-fold faster than in the absence of added metal ions. Finally, global analysis of single turnover cleavage data showed that cleavage can occur with the involvement of either one or two metal ions in the chemical step [6].

Two aspects of metallonuclease mechanism have received less attention. The first is metal ion involvement in product release, a step which is almost universally a slow step. Existing data take two forms. The first is x-ray crystallography. Product structures of a number of restriction enzymes have been reported with either Mn(II) or Mg(II) as the cofactor [11-13]. The structure of the EcoRV K38A product complex revealed positions for Mn(II) ions sufficiently different from those of the substrate complex to inspire the proposal that metal ions move during the reaction [14]. For the BamHI product structure [13], the authors note that the former scissile phosphate no longer serves as a bridging ligand, although this is preserved in other restriction enzyme-product structures. Interestingly, they also note that coordination spheres are less optimal, and that temperature factors for the metal ions in the product complex structure are notably high, giving an impression that the metal ions are less rigidly held and metal ion binding is possibly weaker than Ca(II) ions in the substrate complex. Differences in metal ions used notwithstanding, this would suggest that that metal ion affinity in the product complex is smaller than in other complexes.

More recently, a mutation was introduced into the active site of RNaseH and the product structure solved in the presence of Mg(II) [15]. In this structure, the phosphate moves away out of the metal ion coordination spheres. Metal B retains a perfect geometry, but metal A does not and has higher temperature factors. The authors argue that one metal leaves during product release to facilitate the process, and that the strict coordination requirements of Mg(II) could favor cleavage and product release [16].

Pre steady state kinetics has also been utilized to examine metallonuclease product release. As is the case for many enzymes, the few such studies indicate that this step is rate limiting [17-19], often orders of magnitude slower than the chemical step(s). The authors of a study of MunI endonuclease plasmid kinetics proposed that metal ions which remain in the active site retard product dissociation [19].

The other less developed aspect of metallonuclease mechanism is the interaction between the metal ion binding sites in each active site. This has mechanistic implications because the degree of cooperativity influences how much of the enzyme is bound to one vs. two metal ions per active site. From an electrostatic standpoint, the proximity of two positive charges would necessitate compensatory factors, perhaps in the form of the negatively charged metal ion ligands and/or the anionic substrate. Indeed, there are numerous examples of metal ions held closely in a number of metalloenzyme systems. Both fructose-1,6-bisphosphatase [20], and inositol polyphosphate 1-phosphatase [21] have two metal ions in their active sites about 4 Å apart, a distance typical for metallonucleases [3]. From a thermodynamic standpoint, cooperativity in binding in dinuclear sites is best characterized among the regulatory metalloproteins like calmodulin. Among the well known EF-hand proteins [22], in which Ca(II) binding sites are farther apart (several Å) than in metallonucleases, the cooperativity between sites is strong, 4-7 kcal/mol depending on the conditions [23,24]. While there are a

number of ITC studies of metal ion binding to metallonucleases [8,25-30], there is little mention of metal ion binding cooperativity.

In this study, we examine the metal ion dependence of product release and metal ion cooperativity and using a combination of equilibrium and kinetic analysis of the well characterized *PvuII* endonuclease. We capitalize on the insights obtained from previous work on *PvuII* endonuclease to examine the aforementioned issues. Equilibrium product binding studies are combined with global fitting of single turnover, steady state, and pre steady state cleavage data collected as a function of Mg(II) concentration to explore alternate pathways to metal complexation and product release. Although not widely applied in the study metallonucleases, this approach has the unique power to provide mechanistic information using the native cofactor Mg(II) in the context of the processes of substrate binding, cleavage, and product release, a clear advantage over crystallographic analyses.

MATERIALS AND METHODS

Materials

Chelex resin was purchased from Biorad (Hercules, CA). Puratronic MgCl₂ was purchased from Alfa Aesar (Ward Hill, MA). Concentrations of stock solutions were determined by flame atomic absorption spectroscopy using a Perkin Elmer AAnalyst 700 spectrophotometer. All buffers were applied to a Chelex column to remove adventitious metal ions. Subsequent pH adjustments were made with metal-free nitric acid. All solutions were determined by atomic absorption spectroscopy to be metal-free to the limits of detection [31].

Preparation of *PvuII* Endonuclease

Purification of *PvuII* endonuclease was accomplished using phosphocellulose chromatography and heparin sepharose affinity chromatography as previously described [32]. Adventitious metal ions were removed via exhaustive dialysis against metal-free buffer [33]. Enzyme was quantitated using $\epsilon_{280} = 36,900 \text{ M}^{-1}\text{cm}^{-1}$ for the monomer subunit and handled with metal-free sterile pipet tips and sterile plasticware.

Preparation of Oligonucleotides

The nonself complementary bottom 14mer strand 5'-CAGGCAGCTGCGGA-3' and its complement were purchased HPLC purified from IDT. DNA was quantitated using ϵ_{260} values provided by the vendor. All oligonucleotide concentrations are expressed with respect to the strand or duplex as indicated. DNAs were rendered metal-free through at least two exchanges of > 90% volume with deionized distilled water using Centricons. Samples were subsequently handled using metal-free pipet tips and sterile plasticware. Duplexes were annealed by heating a mixture of 1 eq. of one strand with 1 eq. of complementary strand to 95 °C and cooling the sample to room temperature overnight. Samples were stored in sterile water at 4°C for immediate use or lyophilized for storage.

Radiolabeling was accomplished with 17 pmol of duplex DNA and ³²P-γATP (33 pmols of a 6000 Ci/mmol stock) (Perkin Elmer, Boston, MA) and polynucleotide kinase (1 unit) as per manufacturer's instructions (New England Biolabs, Beverly, MA). Following incubation for 2 h. at 37 °C, the duplex was purified using Sephadex G-50 resin (Sigma, St. Louis, MO).

Assays of *PvuII* Endonuclease Activity

The hydrolysis activity of *PvuII* endonuclease was assessed discontinuously by denaturing PAGE analysis. ³²P endlabeled 14mer duplex DNA added to an appropriate concentration of unlabeled DNA was incubated with *PvuII* endonuclease in 50 mM Tris, 100 mM NaCl pH 7.5, 37°C. Mg(II) concentration was varied, and NaCl was adjusted uniquely at each Mg(II)

concentration to maintain constant ionic strength across the entire series. At the indicated time, the reaction was quenched with an equal volume of 250 mM EDTA in 50% glycerol. Product was separated from substrate using a 20% polyacrylamide/8M urea/0.5X TBE gel with 0.5X TBE as the running buffer. Relative amounts of substrate and product were visualized with a Storm phosphorimager, which converts radioactivity into a digital image. Using ImageQuant software, the fraction of product formed was converted to concentration, which is plotted vs. incubation time. Single turnover kinetic data were collected as previously described [6].

Steady State Kinetics

Reaction mixtures typically contained 2 nM enzyme and DNA concentrations in at least a 5-fold excess. The reaction was initiated by the addition of metal-free enzyme. At appropriate time points during the first 10% of product conversion (typically 5-8 per curve), aliquots were quenched with EDTA and analyzed as describe above. The reaction rates were determined from the linear region of the reaction progress curve and normalized to enzyme concentrations. K_m and k_{cat} were obtained from nonlinear regression of direct v_0 vs. $[S]$ plots using Kaleidagraph software.

Pre-Steady State Kinetics

Below 2.5 mM Mg(II), pre-steady state reaction samples were collected manually. At higher Mg(II) concentrations, the first 30 sec of the reaction was monitored with a Biologic SFM4/Q quenched-flow device (Claix, France). Reaction mixtures typically contained 15-20 nM enzyme and DNA concentrations in at least 5-fold excess. Equivalent volumes of solutions containing 2x DNA concentration and 2x enzyme concentration were loaded into the instrument. Most experiments were conducted with both solutions containing the required Mg (II) concentration in the reaction buffer. However, experiments in which metal-free enzyme was mixed with substrate and double the required Mg(II) concentration yielded the same binding constants within experimental error (10%) [6]. At appropriate time intervals (250 ms - 30 s), the reaction was quenched by mixing with 140 uL of 100 mM EDTA solution. The collected samples were analyzed via PAGE as described above. The pre steady state data set is comprised of 133 data points (time, [product]) across 12 curves over 6 Mg(II) concentrations ranging from 2 to 10 mM.

Using Kaleidagraph software, pre steady state data were fit to the equation

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

where k_1 is the burst rate constant, which corresponds to the single turnover rate constant k_{obs} ; the linear phase is the steady state rate (k_2), and A is the burst amplitude. To facilitate comparison to k_{cat} [18], steady state rate constants were converted to first order rate constants using the enzyme concentration.

Global Fitting of Kinetic Data Using DynaFit

The program DynaFit [34] was used to globally fit reaction progress data to various mechanistic models. Input includes arrays of progress curves (product vs. time) as a function of Mg(II) concentration, as well as enzyme, DNA and metal ion concentrations. DynaFit converts a reaction scheme, consisting of individual reversible and irreversible reactions involving metal ion binding, substrate binding and product conversion, into a series of differential equations. Starting parameters (equilibrium or rate constants for dissociation and association) are provided and either fixed or permitted to float during the fit. DynaFit returns values for floated parameters with errors, provides standards deviations for the global fits as well as for each

progress curve, and plots the experimental data with the curves generated by nonlinear regression. Percent errors refer to specific parameters. To simulate experimental k_{obs} vs. $[\text{Mg}(\text{II})]$ plots, data points generated from the fits were used to refit to the first order rate equation to yield k_{obs} .

Fluorescence Anisotropy

Cleavage product was simulated using an 11 mer duplex composed of the strand 5'-phosphate-CTG CGG TCG CG-3' and its complement labeled at the 5' end with dansyl (Midland Certified Reagent Company, Midland, TX). The PvuII half site is underlined. The duplex was designed to be stable under assay conditions. DNA binding experiments were performed on a SPEX Fluorolog-3 spectrophotometer equipped with polarizers and a thermostatted cell holder with stirring capability. The dansyl labeled duplexes were excited at 350 nm and emission was monitored at 543 nm with slits set to 7-9 nm. A concentrated enzyme stock was titrated into 2 μM duplex in 50 mM Tris, and either 130 mM NaCl and 0 mM MgCl_2 or 100 mM NaCl and 10 mM MgCl_2 , pH 7.5, 25°C. Reported anisotropy is an average of at least three measurements collected with an integration time of 1 sec. For those titrations which resulted in an isotherm, anisotropy values were normalized and fit to the Langmuir isotherm as previously described to yield the K_d [35].

RESULTS

Assessing the Role of Metal Ions in Product Release

Several studies indicate that product release is rate-limiting among metallonucleases [19,36, 37]. The authors of a study of MunI endonuclease plasmid kinetics proposed that metal ions which remain in the active site retard product dissociation [19]. Indeed, metal ions are present in the product complexes of metallonucleases so characterized [11,15]. Based on crystallographic work with metallonuclease product complexes, it has more recently been proposed that at least one metal ion must dissociate from metallonucleases to facilitate product release [15].

Therefore to assess issues regarding product release, it is first necessary to establish the product is at least partially rate limiting for PvuII endonuclease. Previously, we obtained steady state parameters K_m and k_{cat} as a function of $\text{Mg}(\text{II})$ concentration [6] and these data are reproduced in Fig. 2A. At 10 mM MgCl_2 , k_{cat} and K_m are $4.5 \times 10^{-3} \text{ s}^{-1}$ and 27 nM, respectively, and quite similar to values reported elsewhere for PvuII endonuclease [38]. K_m has a shallow metal ion dependence which has been examined previously [6]. k_{cat} has a strong metal ion dependence with a shape similar to that of the metal ion dependence of DNA (K_a) and the single turnover rate constant k_{obs} . At 10 mM $\text{Mg}(\text{II})$, the k_{cat} is 75-fold smaller than the single turnover rate constant k_{obs} under the same buffer conditions (0.3 sec^{-1} ; [6]). This dramatic difference indicates that a physical step following hydrolysis is at least partially rate limiting [18]. This is confirmed in data collected under pre steady state conditions. Fig. 2B features sample data collected at 10 mM MgCl_2 in which a burst phase is visible. When data collected at 10 mM MgCl_2 are fit to equation 1, the resulting average burst phase rate constant k_1 compares very well with the aforementioned single turnover rate constant (k_{obs}) (0.29 sec^{-1} vs. 0.3 sec^{-1}). When the average rate constant for the linear phase (k_2) is corrected for the enzyme concentration, a first order rate constant of $4 \times 10^{-3} \text{ sec}^{-1}$ is obtained and can be compared to the aforementioned k_{cat} [18]. Thus product release is at least partially rate limiting and can be successfully probed using steady state and pre steady state kinetics. To characterize the mechanism of product release, we performed both equilibrium binding experiments and examined kinetic models which differ in the order of release of metal ions and product.

Product Equilibrium Binding Studies—To begin, it would be helpful to know how enzyme affinity for product is influenced by metal ions. We know of no product binding equilibrium studies with metallonucleases from which to design experiments. It would be ideal to assess the metal ion dependence of product complex stability and to determine the metal ion stoichiometry of an enzyme-product complex. To a first approximation, a product DNA duplex can be thought of as a nonspecific (noncognate) DNA duplex. Since PvuII endonuclease binds noncognate DNA with a K_d of about 1 μ M, regardless of whether or not metal ions are present [4], we reasoned that product affinity will not be dramatically different from this. In our experience, obtaining accurate DNA binding constants in this concentration range is more technically difficult than those obtained in the nM regime. However, we can begin to understand the involvement of metal ions in product complex through simple equilibrium binding and kinetic studies. Given our success with the use of fluorescence anisotropy in enzyme-substrate interactions [4,10,35,39,40], we reasoned that the same approach could be applied here.

For the product equilibrium binding studies, a duplex representing one product of cleavage by PvuII was prepared (see Materials and Methods). Briefly, this duplex featured the 5' half of the PvuII recognition sequence (5'-CAG-) at the 3' end of a stable 11 mer duplex. The bottom strand featured a terminal 5' phosphate, a feature of the PvuII cleavage product [41]. Finally, the duplex was labeled on the 5' end of the top strand with a dansyl group, a fluorophore with a relatively low extinction coefficient suitable for observing weak (μ M) interactions.

Complex formation was followed via anisotropy in both the presence and absence of Mg(II). As illustrated in Fig. 3A, no discernible binding trend (i.e., increase in anisotropy as a function of [E]) is observed when Mg(II) is present, even when the duplex concentration is very high (up to 5 μ M, data not shown). In the absence of metal ions, shallow but reproducible trends can be fit to a K_d of approximately 10 μ M. This behavior is unique to the product duplex: Regardless of whether or not Mg(II) is present, modest binding trends are visible in parallel experiments conducted with a duplex that does not feature the terminal phosphate (Fig. 3B). Although weaker in affinity, this metal ion-independent behavior is more typical of noncognate DNA binding [4]. It is clear from these experiments that enzyme affinity for product is weak in the presence of Mg(II), suggesting a mechanism in which metal ions facilitate product release.

Multiple Turnover Cleavage Kinetics—Our approach to examining various aspects of the reaction mechanism has been to globally fit reaction progress curves collected as a function of Mg(II) concentration to various mechanistic schemes. This process is simplified by supplying parameters (association and dissociation rate constants, and cleavage rate constants) obtained from previously published independent experiments (Ref. [6] and Table 1). By comparing the qualities of the fits, parameters and associated errors, favored pathways can be identified.

Data analysis for this study is greatly facilitated by the determination of all parameters which precede product release. Applying pre-existing metal ion and DNA binding equilibrium data and single turnover kinetic data to a number of alternate mechanistic models, we previously established that a mechanism in which metals bind the enzyme before the DNA is kinetically preferred [6] (Fig. 4). In addition, the data fit well to a mechanism in which both EM₂S and EM₄S are capable of converting to product, that is, both one and two metal ion mechanism can be operative (model B1). All rate and equilibrium constants either applied or determined are summarized in Table 1 and will be utilized in the experiments described below. For additional details on these parameters and the analysis, the reader is referred to Ref. [6].

To determine if there is a preferred pathway for product release, a number of modifications were made to the B1 model (Fig. 5). In models with a P1 designation, product is released with metal ions remaining bound to the enzyme. In models with a P2 designation, metals are released prior to product release. Initially, metal ion binding association constants (k_1 and k_3) are set equal for both substrate and product complexes to reduce the number of unknown parameters. In the global fits, the product dissociation rate constant (k_7) and the metal binding association constants (k_1 and k_3) are floated. Because the equilibrium product binding studies indicate that product affinity is not stimulated by metal ions, it is unlikely that the converse is true, that is, that product stimulates metal ion binding affinity. This is supported by the observation that substrate only modestly enhances metal ion binding [6]. To reduce the number of floating parameters, initially we have also constrained the k for metal binding to EM_2P to be equal to that for EM_2 (k_3).

Although equilibrium anisotropy experiments suggest that dissociation of product after metal ions is technically reversible (k_{-7} or k_{-8}), the binding is so weak (vide supra) that the step can be simplified as irreversible. As is described previously [6], k_2 is fixed at $3.7e4 \text{ M}^{-1}\text{s}^{-1}$. The results of fitting pre steady state and steady state data to these models and trials are summarized in Table 2. P1 has the lower standard deviation than P2 for steady state data; for pre steady state data, these values for both models are very similar. As for individual parameters, errors are very high for pre steady state model P2 and only slightly higher for steady state model P1. For both data sets, P2 yields high metal ion binding constants (above 10 mM). For the steady state P2 model, k_7 is exceptionally fast, indeed much faster than predicted as rate limiting to k_{cat} . Collectively, model P1 generally gives lower errors and more reasonable parameter values.

The difference in quality of the two fits is much easier to discern in plots of velocity vs. Mg (II) plots for steady state data (Fig. 6). Here P2 is clearly the poorer model. Thus it is clear that mechanisms in which product dissociates to reform EM_x complexes are kinetically preferred.

Finally, to determine if product influences metal ion binding equilibrium constants, model P3 was developed (Fig. 5). Here, metal ion dissociation from EM_xP is not constrained as equal to other metal ion equilibria but instead is given independent parameters. To compensate for the increase in the number of unknowns, a number of other parameters are fixed at values previously established (Table 1 and Ref. [6]). Namely, k_1 was set to $4.7e7 \text{ M}^{-1}\text{s}^{-1}$, k_3 was set to $1.1e8 \text{ M}^{-1}\text{s}^{-1}$, and k_{-9} and k_{-10} were set to 1000 s^{-1} (a common metal ion dissociation rate constant). The results of fitting pre steady state data to this model appear in Table 3. The rate constant for product dissociation when metal ions are present in the complex (EM_xP , k_7 , $6.6e-3 \text{ s}^{-1}$) is 3-fold faster than for the EP complex (k_8 , $2.3e-3 \text{ s}^{-1}$). However, these values are just outside of experimental error of one another and should therefore be regarded as similar. While K_{d10} is a bit high, K_{d9} is quite similar to K_{d1} and K_{d3} , as was assumed in the above analysis. This would suggest that while metal ion binding is enhanced by substrate, it does not appear to be so for product.

Assessing Cooperativity Between Individual Metal Ion Binding Sites

X-ray crystallography reveals two distinct metal ion binding sites in each PvuII active site (Fig. 1): Metal A ligates both substrate and the attacking water derived nucleophile; Metal B also ligates the scissile phosphate and is often implicated in assisting in protonation of the leaving group [3]. Given the proximity of the metal ions to one another, it is possible that these sites have at least a modest influence on one another. More specifically, that occupancy of one site influences the occupancy of the other. This possibility is examined through an analysis of single turnover kinetic data as described below.

The general models in Fig. 4 and 5 assume that the two metal ion equivalents bind sequentially and independently. That is, the first equivalent can bind in either site, and binding of the second

equivalent is not significantly influenced by occupancy of the first site. Although there is a slight difference between metal ion binding constants for E vs. EM_2 , these are within experimental error of one another.

In our previous calorimetric study of Ca(II) binding by PvuII endonuclease [8], we noted that the data fit comparably well to interacting and independent binding models, implying that any cooperativity for Ca(II) binding is weak at best. While these metal ions bind with similar binding constants, we have been interested to note that there is at least one important difference in Ca(II) and Mg(II) behavior in this system: They stimulate DNA binding to a differing degree, with Ca(II) promoting more avid DNA binding (100-fold; [6]). Thus it is prudent to examine metal ion cooperativity in the context of DNA cleavage using kinetic data. To our knowledge, this is the first time this approach has been applied to a metalloenzyme.

While kinetics alone cannot unequivocally assign metal ion equivalents to specific locations, it can be used to examine the relationship between two sites. Fig. 7 features three alternative models for metal ion binding. Models with a C1 designation refer to two equivalent and interacting metal ion binding sites. That is, either site fills (a or b), changing the binding constant for the second event regardless of the physical location. To reduce the number of rate constants and thus simplify the analysis, EM_{2a} is designated as capable of cleavage, and EM_{2b} is not. This simplification is supported by both theory [6] and experiment [5]. In models with a C2 designation, independent binding constants describe occupancy for each site, regardless of the order of occupancy. Models with a C3 designation differ from C2 models in that off rate constants for the second step(s) are floated instead of fixed, as is the case for the first two models. An interaction factor is also introduced, which is defined as K_{ba}/K_a or K_{ab}/K_b , where K_a is the metal ion binding dissociation constant describing site a, and K_{ba} describes the affinity for site a when site b is already occupied. While they are likely to be correlated, these studies cannot independently assign the a and b designations to specific metal ion locations in the crystal structure. For models with a C3 designation, the interaction factor is obtained by comparing the derived dissociation rate constants. Since affinities are expressed as dissociation constants, an interaction factor less than one reflects positive cooperativity; if it is greater than one, then the cooperativity is negative. These models are based on a model previously designated as B1 [6], in which EM_2S and EM_4S can convert to product.

Previously obtained single turnover cleavage progress curves obtained as a function of [Mg(II)] [6] were globally fit to the above models as described in Materials and Methods. The values for individual floated constants, their respective errors, and the resulting interaction factors appear in Table 4. Among the models, model C3 has larger errors on a couple of parameters (k_3 , k_{off}), and for models C1 and C3, at least one metal binding equilibrium constant is weak ($> 5mM$). Interestingly, the interaction factors, which are calculated from floated parameters, are no smaller than 0.34, which indicates the data fit well to a mechanism with no more than very weak positive cooperativity. The abilities of these models to simulate experimental k_{obs} vs. Mg(II) plots is featured in three panels of Fig. 8. The simulations are all fairly similar, with small deviations from experimental data at differing Mg(II) concentrations. This figure indicates that the data are as well represented by an independent binding model as weakly positively cooperative models.

To investigate how well the data are represented by a negative cooperativity model, another fit was performed. The metal ion binding cooperativity in this model depends on dissociation rate constants k_{-1} and k_{-3} . These were fixed at $3000 s^{-1}$, making the interaction factor 1.7 and thus negatively cooperative. Because model C2 contains two independent sites (i.e., noncooperative), and for model 1 k_1 cannot be fixed, this experiment was performed with model C3 (model C3*). As shown in Table 2, while many of the fitted parameters have values similar to the other models, the standard deviation is high. More insightful is Fig. 8 (lower right

panel), which illustrates that the [Mg(II)] dependence of k_{obs} is clearly not as well represented at low Mg(II) concentrations by an obligatory negative cooperativity model. Collectively, the interaction factors indicate that the negative cooperativity model can be ruled out and that there is little to no positive cooperativity between the metal ion binding sites, generally consistent with Ca(II) binding studies of the apo enzyme [8].

DISCUSSION

Metal Ion Binding to Substrate and Product Complexes

While it is by no means a universal observation, metal ions stimulate DNA binding in a number of metallonucleases [4]. The degree to which this occurs can depend on the metal ion used [39]. In our previous work, we noted that Mg(II) affinity is modestly enhanced (less than 3-fold) in enzyme-substrate complexes relative to the free enzyme [6]. It is interesting to note that the effect is not the same for the product complex, that is, metal ion binding is not stimulated in the product complex. Given that enzyme affinity for product is generally weak, there is no real advantage for this to be the case.

More interesting are the structural differences which are necessary for these thermodynamics. Clearly in an metallonuclease-product complex, the coordination environments for metal ion (s) are far less attractive. The scissile phosphate, which is a bridging ligand in enzyme-metal-DNA crystal structures, has been degraded and can no longer provide critical scaffolding for a dinuclear center. This points to the subtleties in the active site that are required to assemble the moieties needed for catalysis, and supports recent discussions about the changes in active site structure that occur during the reaction [14,16]. Based on crystallographic work, Yang proposed that in RNase H, one metal ion leaves after catalysis and one remains in the product complex [16]. It would be insightful to determine metal ion stoichiometry in a product complex. However, given the weak binding of both metal ions and product to the enzyme, it is unlikely that one could obtain data of sufficient quality to determine this.

Regarding the role of metal ions in the release of product, our equilibrium product binding studies indicate that EM_xP complexes are even less stable than EP complexes. Consequently, there is less EM_xP and more EM_x after cleavage. This is an efficient mechanism for multiple turnovers, as EM_x complexes can readily return to bind substrate.

Product Release and the Rate-Limiting Step

The data presented here indicate that product release for PvuII endonuclease is at least partially rate limiting ($1\text{e-}3\text{ s}^{-1}$). This rate is an order of magnitude slower than cleavage conducted with EM_2S and 1000-fold slower than cleavage with EM_4S . There is some support for the possibility that product release, rather than an enzyme conformational change, is solely responsible for the slow turnover number of PvuII endonuclease and other restriction enzymes. First, the enzyme turns over and dissociates DNA [10] at the same very slow rate (k_{cat} and k_{off} are both about $1\text{e-}3\text{ sec}^{-1}$). If product DNA dissociates from the enzyme at a rate similar to substrate dissociation, then there would be no reason to suspect the existence of a slower step. Second, the burst phase in pre steady state measurements is only visible at higher metal ion concentrations, where cleavage is conducted by the faster EM_4S species ($k\ 1.1\text{ sec}^{-1}$). At lower Mg(II) concentrations, cleavage is conducted by a much slower species (EM_2S ; $k\ 0.01\text{ sec}^{-1}$) and product release is no longer rate limiting. In order for a conformational change to mimic this behavior, it would have to have nearly identical metal ion dependence. If these are not a concomitant processes, this possibility seems unlikely. Finally, in a stopped-flow spectroscopic study of the EcoRV enzyme, Baldwin et al. [17] detected two conformational changes using Trp fluorescence: The first one was too fast to obtain a rate constant and thought to be associated with substrate binding. The later one exhibited a k_{obs} of 0.26 s^{-1} , which is twice as fast as

k_{cat} under these conditions. They speculate that this conformational process could be concomitant with either hydrolysis or product release, but is not sufficiently slow to be rate limiting.

Given the similarities between EcoRV and PvuII and the small difference in rate between the slow conformational change in EcoRV and its k_{cat} , it is possible that a PvuII conformational change could be, like product release, at least partially rate limiting. To investigate this convincingly, it would be necessary to collect some useful stopped flow data and use rate constants obtained this way to test kinetic models to which such a step has been added [42].

Metal Ion Cooperativity

In contrast to the regulatory metalloproteins, little information regarding the energetic interaction between metal ions in metallonucleases has been reported. ITC studies of metal ion binding to metallonucleases have rarely included details regarding the results of fitting to alternate binding models. For all metallonucleases for which two metal ion binding sites have been studied in this manner, the data fit well to an (independent) sequential binding model. These systems include flap endonucleases [26,27], CceI endonuclease [29], exonuclease III [30], and T7 endonuclease I [25]. Xylose isomerase, another binuclear enzyme, also binds two Mg(II) ions without cooperativity [43]. These results are markedly different from the Ca(II) binding regulatory proteins, which have interaction factors of 10^3 - 10^5 (4-7 kcal/mol) [23,24].

Unlike the more commonly used ITC approach, global fitting of single turnover kinetic cleavage data applied here provides information about the native cofactor Mg(II), the binding of which cannot be observed in this system using ITC [8]. Fitting of kinetic data described above is inconsistent with negative cooperativity: In this scenario, the formation of E and EM_2 is favored. However, the cleavage rate constants for EM_2S (0.01 sec^{-1}) is 100 fold smaller than that of EM_4S . So the population of EM_2S cannot dominate over a wide Mg(II) concentration range. The independent or weakly positive cooperativity models favor the formation of EM_4 , and thus are more consistent with the behavior of single turnover rate constants over a wide range of Mg(II) concentrations (Fig. 8). This lack of strong positive cooperativity is consistent with ITC metal ion binding studies of the aforementioned metallonucleases, some of which were performed with nonnative metal ions and in the absence of substrate.

While it might initially seem counterintuitive for two metal ions held about 4 Å apart to bind without negative cooperativity, it is helpful to note that metallonuclease active sites feature a number of mutually repulsive, negatively charged groups which serve as metal ion ligands. Metal ions binding to these ligands helps neutralize charge in an active site designed to bind polyanionic substrates, clearly a favorable process.

There are mechanistic consequences to metal ion cooperativity. If the sites are strongly positively cooperative, then very little of the enzyme will be populated with a single metal ion, and EM_4 (i.e., two metal ions per active site) dominates in the absence of substrate. If there is negative or no cooperativity, then EM_2 (i.e., one metal ion per active site) can be more abundant. Regarding metal ion binding in the presence of substrate, we have previously explored a mechanism in which EM_2S can bind additional metal ions to form EM_4S and proceed to cleavage. Single turnover data fit this model ($\text{A1}'$) as well as to the aforementioned B1 model [6]. This was also the case when adding product release and metal ion cooperativity models to this same basic scheme (data not shown). While it is unlikely that additional insights about cooperativity of metal ion binding in EM_xS complexes are available from adding in this alternative step, we speculate that metal ion cooperativity in substrate complexes is probably not strong, either. Metal ion binding behavior in the presence of DNA has been consistently similar to that in its absence, with binding constants differing by no more than a few fold.

Collectively, the data all support a pathway in which cleavage can occur, albeit slowly, with only one metal ion.

Conclusion

This work completes a mechanistic pathway for a metallonuclease in which metal ions are pivotal in both physical and chemical steps. In addition to providing critical support to the hydrolytic steps, they facilitate substrate binding and tend to remain bound to the enzyme following product release. The advantage to this strategy is clear, and it is expected that these results extend to other related systems. Whether or not this is the case and how the mechanisms of other metallonuclease systems are similar to or different from this one is the next experimental challenge.

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References

- Williams N, Takasaki B, Wall M, Chin J. *Acc Chem Res* 1999;32:485–493.
- Cowan, JA. *The Biological Chemistry of Magnesium*. VCH; NY: 1995.
- Dupureur CM. *Curr Chem Biol* 2008;2:159–173.
- Conlan LH, Dupureur CM. *Biochem* 2002;41:1335–1342. [PubMed: 11802735]
- Mones L, Kulhanek P, Florian J, Simon I, Fuxreiter M. *Biochem* 2007;46:14514–14523. [PubMed: 18020376]
- Xie F, Qureshi S, Papadakis GA, Dupureur CM. *Biochem* 2008;47:12540. [PubMed: 18975919]
- Horton JR, Cheng X. *J Mol Biol* 2000;300:1049–1056. [PubMed: 10903853]
- José TJ, Conlan LH, Dupureur CM. *J Biol Inorg Chem* 1999;4:814–823. [PubMed: 10631614]
- Dupureur CM, Conlan LH. *Biochem* 2000;39:10921–10927. [PubMed: 10978180]
- Conlan LH, Dupureur CM. *Biochem* 2002;41:14848–14855. [PubMed: 12475233]
- Kostrewa D, Winkler FK. *Biochem* 1995;34:683–696. [PubMed: 7819264]
- Grigorescu, A.; Horvath, M.; Wilkosz, P.; Chandrasekaran, K.; Rosenberg, J. *Nucleic Acids and Molecular Biology*. Pingoud, A., editor. Springer; 2004. p. 137-177.
- Scheuring Vanamee, E.; Viadiu, H.; Lukacs, CM.; Aggarwal, AK. *Nucleic Acids and Molecular Biology*. Pingoud, A., editor. Springer; 2004. p. 215-236.
- Horton NC, Perona JJ. *Biochem* 2004;43:6841–6857. [PubMed: 15170321]
- Nowotny M, Yang W. *EMBO J* 2006;25:1924–1933. [PubMed: 16601679]
- Yang W, Lee JY, Nowotny M. *Mol Cell* 2006;22:5–13. [PubMed: 16600865]
- Baldwin GS, Vipond IB, Halford SE. *Biochem* 1995;34:705–714. [PubMed: 7819266]
- Sam MD, Perona JJ. *J Am Chem Soc* 1999;121:1444–1447.
- Sasnauskas G, Jeltsch A, Pingoud A, Siksnys V. *Biochem* 1999;38:4028–4036. [PubMed: 10194315]
- Villeret V, Huang S, Zhang Y, Lipscomb WN. *Biochem* 1995;34:4307–4315. [PubMed: 7703244]
- York JD, Ponder JW, Chen Z-W, Mathews FS, Majerus PW. *Biochem* 1994;33:13164–13171. [PubMed: 7947723]
- Voet, D.; Voet, J.; Pratt, C. *Fundamentals of Biochemistry*. Vol. 3. Wiley; Hoboken: 2008.
- Linse S, Johansson C, Brodin P, Grundstrom T, Drakenberg T, Forsen S. *Biochem* 1991;30:154–162. [PubMed: 1988017]
- Waltersson Y, Linse S, Brodin P, Grundstrom T. *Biochem* 1993;32:7866–7871. [PubMed: 8347591]
- Hadden JM, Declais AC, Phillips SE, Lilley DM. *EMBO J* 2002;21:3505–3515. [PubMed: 12093751]
- Zheng L, Li M, Shan J, Krishnamoorthi R, Shen B. *Biochem* 2002;41:10323–10331. [PubMed: 12162748]

27. Feng M, Patel D, Dervan JJ, Ceska T, Suck D, Haq I, Sayers JR. *Nat Struct Mol Biol* 2004;11:450–456. [PubMed: 15077103]
28. Black CB, Cowan JA. *J Biol Inorg Chem* 1998;3:292–299.
29. Kvaratskhelia M, George SJ, Cooper A, White MF. *Biochem* 1999;38:16613–16619. [PubMed: 10600123]
30. Casareno RL, Cowan JA. *Chem Commun* 1996;15:1813–1814.
31. Holmquist B. *Methods Enzymol* 1988;158:6–12. [PubMed: 3374394]
32. Dupureur CM, Hallman LM. *Eur J Biochem* 1999;261:261–268. [PubMed: 10103058]
33. Wagner FW. *Methods Enzymol* 1988;158:21–32. [PubMed: 3374375]
34. Kuzmic P. *Anal Biochem* 1996;237:260–273. [PubMed: 8660575]
35. King J, Bowen L, Dupureur CM. *Biochem* 2004;43:8551–8559. [PubMed: 15222766]
36. Taylor JD, Halford SE. *Biochem* 1989;28:6198–6207. [PubMed: 2675966]
37. Nobbs TJ, Szczelkun MD, Wentzell LM, Halford SE. *J Mol Biol* 1998;281:419–432. [PubMed: 9698558]
38. Simoncsits A, Tjornhammar M-L, Rasko T, Kiss A, Pongor S. *J Mol Biol* 2001;309:89–97. [PubMed: 11491304]
39. Bowen LM, Dupureur CM. *Biochem* 2003;42:12643–12653. [PubMed: 14580211]
40. Papadakos GA, Nastri H, Riggs P, Dupureur CM. *J Biol Inorg Chem* 2007;12:557–569. [PubMed: 17308914]
41. Gingeras TR, Greenough L, Schildkraut I, Roberts RJ. *Nucleic Acids Res* 1981;9:4525–4536. [PubMed: 6272209]
42. Beebe JA, Fierke CA. *Biochem* 1994;33:10294–10304. [PubMed: 7520753]
43. Van Bastelaere PBM, Callens M, Vangrype WAE, Kersters-Hilderson HLM. *Biochem J* 1992;286:729–735. [PubMed: 1417732]

Fig. 1. General two metal ion mechanism for the hydrolysis of nucleic acids by a protein catalyst
 In most current two metal ion mechanism models, metal ion A coordinates the attacking water molecule and ligates the scissile phosphate. X⁻ refers to the uncertain means by which the attacking water molecule is activated. Metal ion B also interacts with the DNA and at least one acidic group, and is proposed to be involved in coordinating the water molecule which donates a proton to the leaving group. Adapted from Ref. [3].

Fig. 2. Steady State and Pre Steady State Kinetic Cleavage Data for PvuII endonuclease
 (A) $[\text{Mg(II)}]$ dependence of k_{cat} and K_m adapted from Ref. [6]. (B) Sample progress curve for DNA cleavage by PvuII endonuclease. Conditions: 15 nM enzyme, 100 nM duplex, 10 mM Mg(II) , 50 mM Tris, 100 mM NaCl pH 7.5, 37°C. Multiple data sets were fit to equation 1 and the constants averaged to yield a burst phase rate constant of 0.29 sec^{-1} and a linear phase first order rate constant of $4 \times 10^{-3} \text{ sec}^{-1}$. See text for further details.

Fig. 3. Product Binding as Monitored by Fluorescence Anisotropy

(A) Product binding (terminal phosphate). (B) Duplex without the terminal phosphate. Closed circles, without Mg(II); closed squares, with Mg(II). Lines are drawn to highlight trends and do not represent fits to the data. See text for other details. Conditions: 50 mM Tris, pH 7.5, 37°C in either 130 mM NaCl and 0 mM MgCl₂ or 100 mM NaCl and 10 mM MgCl₂.

Fig. 4. PvuII Reaction Scheme Resulting from Fits of Single Turnover Data to Various Models
 Basic features of the B1 model from Ref. [6]. E refers to the homodimeric enzyme; S represents the DNA duplex. M, metal ion. Only those pathways which are kinetically preferred pathway are included. A pathway in which metals bind prior to substrate is strongly preferred, and both EM_2S and EM_4S are capable of cleavage.

Fig. 5. Product Release Models

All are based on the B1 models described in Ref. [6] and summarized in Fig. 4 in which both species lead to product but they do not interconvert. E refers to the homodimeric enzyme; S represents the DNA duplex. M, metal ion. In models with a P1 designation, product dissociates prior to metal ions. In models with a P2 designation, metal ions dissociate prior to product. Model P3 is designed to evaluate metal ion binding to enzyme-product complexes.

Fig. 6. Fits of Steady State Data to Various Product Release Models

Experimental data are indicated with closed circles. Models are as described in Fig. 5.

Conditions: 75 nM duplex, enzyme at least 5-fold below substrate concentration, 50 mM Tris, pH 7.5, 37°C with NaCl adjusted to constant ionic strength.

Fig. 7. Alternate Models for Metal Ion Binding

All models are based on the B1 model in which both EM_2S and EM_4S can convert to product (lower panel). E refers to the homodimeric enzyme; S represents the DNA duplex. M, metal ion. Models with a C1 designation refer to two equivalent and interacting metal ion binding sites. Models with a C2 designation are independent binding models. Finally, models with a C3 designation differ from C2 models in that off rate constants for the second step(s) are floated instead of fixed, as is the case for the first two models. The remainder of the mechanism is as in Fig. 4, except that $EM_{2a}S$ is capable of cleavage while $EM_{2b}S$ is not. See text for other details.

Fig. 8. Simulation of k_{obs} vs. $[\text{Mg(II)}]$ Plots by Kinetic Models Which Discriminate Metal Ion Binding Pathways

Models are based on the model B1 (Fig. 4) from Ref. [6] and are illustrated in Fig. 7; parameters appear in Table 4. Model C1, equivalent sites with positive cooperativity. Model C2, two independent sites. Model C3, two nonequivalent sites with positive cooperativity. Model C3*, enforced negative cooperativity (k_{-1}' and k_{-3}' are fixed at 3000 s^{-1}). Experimental data are indicated with closed circles. Simulated data indicated with open squares. k_2 was fixed to $3.7 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$.

Table 1Summary of Kinetic Parameters Determined from Previous Studies^a

Parameter	Value	Source
K_1	$4.7e7 \text{ M}^{-2}\text{s}^{-1}$	[6]
k_{-1}	1000 s^{-1}	[6]
K_1	4.6 mM	[6]
K_2	$3.7e4 \text{ M}^{-1}\text{s}^{-1}$	[6]
k_{-2}	0.001 s^{-1}	[10]
K_2	27 nM	[6]
K_3	$1.1e8 \text{ M}^{-2}\text{s}^{-1}$	[6]
k_{-3}	1000 s^{-1}	[6]
K_3	3.1 mM	[6]
K_4	$2e5 \text{ M}^{-1}\text{s}^{-1}$	[6]
k_{-4}	0.001 s^{-1}	[10]
K_4	5 nM	[6]
K_5	1.1 s^{-1}	[6]
k_6	0.011 s^{-1}	[6]

^a See Fig. 4 for definitions of parameters. k indicates a rate constant, with positive subscripts indicating association processes and negative subscripts indicating dissociation processes. K indicates a dissociation equilibrium constant.

Table 2Summary of Fit Results with to Product Models^a

Pre-steady State Kinetics		
Model	P1	P2
Standard deviation $\times 10^{-9}$	2.6	2.7
k_1 ($M^{-2}s^{-1}$)	3.4e7 (43)	3.9e6 (610)
k_3 ($M^{-2}s^{-1}$)	5.6e7 (36)	9.4e8 (610)
k_7 (s^{-1})	0.0061 (33)	0.010 (34)
K_{dl} (mM)	5.4	16
K_{d3} (mM)	4.2	1.0
Steady-State Kinetics		
Model	P1	P2
Standard deviation $\times 10^{-4}$	1.7	3.4
k_1 ($M^{-2}s^{-1}$)	2.7e7 (120)	1.4e8 (34)
k_3 ($M^{-2}s^{-1}$)	2.4e8 (120)	8.0e6 (30)
k_7 (s^{-1})	4.7e-3 (6.4)	0.15 (43)
K_{dl} (mM)	6.1	2.7
K_{d3} (mM)	2.0	11

^a Models appear in Fig. 5 and are based on the B1 model from Ref. [6]. Scales for the standard deviations for the two data sets are different because of the scales of the data: $[P]$ 10^{-8} M vs. time for pre steady state data and velocity vs. $[Mg(II)]$ 10^{-3} M for steady state data. Percent error appears in parentheses. k_2 and k_4 are set to $3.7e4$ and $2e5$ $M^{-1}s^{-1}$, respectively. k_{-2} and k_{-4} are fixed at 0.001 s^{-1} . Metal ion dissociation rate constants k_{-1} and k_{-3} are fixed to 1000 s^{-1} . k_5 is set to 1.1 s^{-1} and k_6 is set to 0.01 s^{-1} . Steps 5, 6, and 7 are assumed to be irreversible.

Table 3Results of Fit of Pre Steady State Data to Product Model P3^a

Standard deviation $\times 10^{-9}$	2.5
k_9 ($M^{-2}s^{-1}$)	6.4e7 (75)
k_{10} ($M^{-2}s^{-1}$)	1.6e7 (280)
K_7 (s^{-1})	6.8e-3 (39)
k_8 (s^{-1})	2.3e-3 (160)
K_{d1} (mM)	4.6
K_{d3} (mM)	3.1
K_{d9} (mM)	3.9
K_{d10} (mM)	7.8

^aModel appears in Fig. 5 and is based on model B1 from Ref. [6]. Percent errors are given in parentheses. k_2 and k_4 are set to $3.7e4$ and $2e5 M^{-1}s^{-1}$, respectively. k_1 was fixed to $4.7e7 M^{-2}s^{-2}$, k_3 $1.1e8 M^{-2}s^{-1}$, k_5 $1.1 s^{-1}$, k_6 $0.01 s^{-1}$ as determined from the fit to model 1. DNA dissociation constants k_{-2} and k_{-4} are fixed at $0.001 s^{-1}$, and metal ion dissociation rate constants k_{-1} , k_{-3} , k_{-9} and k_{-10} are fixed to $1000 s^{-1}$. Steps 5, 6, 7, and 8 are assumed to be irreversible.

Table 4Parameters with from Global Fits to Interacting Metal Ion Models^a

Model	C1	C2	C3	C3*
	Equiv. Interacting	Nonequiv. Independent	Nonequiv. Interacting	Negative Cooperativity
Derived Kinetic Parameters (% Error)				
Standard deviation	2.43	2.77	2.12	3.22
$k_1(\text{M}^{-2}\text{s}^{-1})$	2.7e7 (16)	1.6e8 (14)	4.1e7 (11)	4.7e8 (25)
$k_3(\text{M}^{-2}\text{s}^{-1})$	2.4e8 (12)	8.5e7 (10)	4.8e6 (60)	2.5e8 (14)
$k_5(\text{s}^{-1})$	0.97 (14)	1.5 (21)	1.5 (16)	1.7 (25)
$k_6(\text{s}^{-1})$	0.031 (75)	0.0125 (31)	0.022 (31)	0.007 (37)
$k_{\text{off}}(\text{s}^{-1})$	n/a	n/a	43 (59)	3000*
Derived metal binding affinities for site A and B, mM				
$K_{\text{d,a}}$	6.1	2.5	5.0	1.5
$K_{\text{d,b}}$	6.1	3.4	11.5	2.0
$K_{\text{d,ba}}$	2.1	2.5	1.2	2.5
$K_{\text{d,ab}}$	2.1	3.4	3.4	3.4
Interaction factor ^b	0.34	1	0.34	1.73

^a All models are based on the B1 model from Ref. [6] and were applied to single turnover data obtained as a function of Mg(II) concentration. Models are as defined in Fig. 7. k_2 is fixed at $3.7\text{e}4 \text{ M}^{-1}\text{s}^{-1}$, k_4 is fixed at $2\text{e}5 \text{ M}^{-1}\text{s}^{-1}$, and k_{-2} and k_{-4} are fixed at 0.001 s^{-1} . k_{-1} and k_{-3} are fixed at 1000 s^{-1} , and $k_{-3'}$ is fixed at 190 s^{-1} . "Off" refers to $k_{-1'}$ and $k_{-3'}$.

* Obligatory negative cooperativity model in which dissociation rates for steps $k_{-1'}$ and $k_{-3'}$ are fixed at 3000 s^{-1} .

^b The interaction factor is defined as $K_{\text{bA}}/K_{\text{a}}$ or $K_{\text{Ab}}/K_{\text{b}}$. K_{a} is the metal binding affinity of site a in apo enzyme and K_{ba} is the site a metal binding affinity when site b is occupied, as defined in Fig. 7.