Flap endonucleases (FENs) are divalent metal ion-dependent phosphodiesterases, essential in DNA replication. Although still debated, metallonucleases are generally assigned a “two metal ion mechanism” where both metals contact the scissile phosphate diester bond. The spacing of two ions in T5FEN structures appears to preclude this mechanism. However, as the overall reaction catalysed by wild type (WT) T5FEN requires three Mg$^{2+}$ implying a third ion must also be present, a “two metal ion mechanism” remains possible. To investigate positions of ions required for chemistry, a mutant T5FEN was studied, where metal 2 (M2) ligands are altered to eliminate this binding site. In contrast to WT T5FEN, the overall reaction catalysed by D201I/D204S required two ions, but within the range tested maximal rate data would fit to a single binding isotherm. Calcium ions do not support FEN catalysis and inhibit reactions supported by viable cofactors. To establish participation of metal ions in stabilisation of enzyme-substrate complexes, $K_D$s of WT and D201I/D204S-substrate complexes were studied as a function of $[Ca^{2+}]$. At pH 9.3 (maximal rate conditions), Ca$^{2+}$ ions substantially stabilised both complexes. Inhibition of viable cofactor-supported reactions of WT and D201I/D204S T5FENs was biphasic with respect to Ca$^{2+}$ and ultimately dependent on $1/[Ca^{2+}]^2$. For both the NEUTRALISING MUTATIONS OF CARBOXYLATES THAT BIND METAL 2 IN T5FEN RESULT IN AN ENZYME THAT STILL HAS A TWO METAL ION REQUIREMENT

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By varying the concentration of viable cofactor ions, Ca$^{2+}$ ions were shown to inhibit competitively displacing two viable cofactor ions. Combined analyses imply M2 is not involved in chemical catalysis, plays a role in substrate binding, and that a “two metal ion mechanism” is plausible.

Flap endonucleases (FENs), essential in all life forms, are members of the 5’-nuclease superfamily of structure-specific phosphate diesterases that carry out essential divalent metal ion dependent nucleic acid hydrolyses (1). FENs act upon 5’-bifurcated structures known as flaps formed during lagging strand DNA replication and long patch base excision repair. Hydrolysis predominantly occur one nucleotide (nt) into the 5’-duplex adjoining the site of bifurcation. An unusual feature of these enzymes is their high density of active site carboxylates that coordinate essential divalent metal ion cofactors. Bacterial and bacteriophage FENs possess eight active site acidic residues, seven of which are conserved in FENs from higher organisms and also in other members of the 5’-nuclease superfamily (1-4). In contrast, a typical metallonuclease active site consists of three or four carboxylates (5). However, a sub-class of FEN paralogues, typified by *E. coli* ExolX, exist in eubacteria and appear to lack three of the metal-binding carboxylates (6).
Whilst the most common mechanism suggested for metal-dependent phosphoryl transferases involves two metal ions in contact with the scissile phosphate diester, this is not universally accepted (5,7-10). Debate about the validity or otherwise of two metal ion catalysis has focussed on several enzymes including flap endonucleases (11-16). Crystallographic studies of substrate-free FENs have demonstrated two active-site-bound metal ions (14,17-19). Metal ion 1 (M1) occupies a similar position in all structures. However, there is some variation in the position of the site coordinating a second metal ion (M2), often observed in FEN crystal structures. The distance between the M1 and M2 sites varies and the separations observed are generally much greater than the 4 Å required for both metals to contact the same phosphate diester. For example in bacteriophage T5FEN the M1-M2 separation of two Mn$^{2+}$ ions is 8 Å (Figure 1A) (17). However, in a substrate-free structure of hFEN1 two magnesium ions are bound with a separation of 3.4 Å in one of three proteins bound to PCNA (Figure 1B) (18). A very recent structure of hFEN1 bound to product DNA has two Sm$^{3+}$ ions bound in equivalent positions and these contact the cleaved phosphate monester (4).

Despite the 8 Å distance between M1 and M2 in substrate-free T5FEN structures, magnesium ion stimulation data are consistent with a mechanism where the major rate acceleration is brought about by two metal ions, but that overall ($k_{cat}/K_{m}$) there is a three divalent ion requirement (3). Thus an additional ion (M3) is bound with higher affinity to the enzyme substrate complex. If two metal ions that participate in chemical catalysis are coordinated to the central carboxylate of the T5FEN active site (D130), positioned as those observed in hFEN1 structures (Figure 1) (4)(2), two metal ion catalysis mechanisms where both metals coordinate the scissile phosphate diester could take place. As only M1 is observed in this position in substrate-free structures of bacteriophage FENs, in this scenario the third ion implicated by functional studies would have to bind in this location and the catalytic ions would therefore be M1 and M3 (Fig 1A). However, alternative explanations of a two ion requirement for the major rate acceleration are possible. For example, M2, as positioned in T5FEN substrate-free structures, would not be able to directly interact with the scissile phosphate diester but instead could catalyse chemical catalysis by more remote interactions. On the other hand, consistent with a catalytic M1 and M3 providing the main rate acceleration, M2 could play a role in enzyme-substrate complex stability.

Here we test earlier proposals for the roles of metal ions in reactions catalysed by T5FEN. To investigate whether divalent metals ions alter the stability of enzyme-substrate complexes, dissociation constants were determined in the absence of catalysis at varying concentration of Ca$^{2+}$ ions and pH. Previous studies of the T5FEN catalysed reactions demonstrated that catalytically inert Ca$^{2+}$ ions inhibited reaction with a $1/([\text{Ca}^{2+}]^2$ dependence, which is also consistent with an absolute requirement for two viable cofactors that the Ca$^{2+}$ ions displace. Potentially this could be used to test for a requirement for two metal ions in mutated FENs and other metalonucleases. However, it is possible that decreases in the rate of reaction may involve Ca$^{2+}$ binding non-competitively to the enzyme, substrate or enzyme-substrate complex. To establish competitive inhibition of FEN catalysed reactions by Ca$^{2+}$ ions the inhibitory characteristics were studied with different supporting cofactors at varying concentration. To probe the role of T5FEN M2, both the stimulation by viable cofactors and inhibition by Ca$^{2+}$ ions were evaluated for a mutated T5FEN where two of the carboxylates that ligand M2 are altered. The combined analyses support a mechanism for T5FEN where the main rate acceleration is afforded by two ions but not by M2, and imply a role for M2 in stabilisation of enzyme-substrate interactions.

**Experimental Procedures**

**Materials**-Wild type (WT) and D201I/D204S T5FENs and HP5F substrate (5' FAM-pd(CGCTGTCAACACAGGCTTGCGTGTGTTC)) were prepared and purified to homogeneity as described (3,24,25). Divalent metal ion contaminants were removed by treatment with Chelex resin.

**Steady state kinetic analyses**- Steady-state kinetic parameters of WT and D201I/D204S T5FEN (E) were evaluated at 37°C using HP5F substrate (S) in 25 mM CHES or potassium glycinate pH 9.3, 0.1 mg/ml BSA and 1 mM DTT as described (3). MgCl$_2$ was added to the desired
concentration and the ionic strength (MgCl₂ + KCl) adjusted to 80 mM using KCl. Substrate concentrations were varied from 1/8 to 8 x Kₘ and reactions were sampled and quenched by addition of an equal volume of 25 mM EDTA at appropriate time intervals. Reactions were analysed by dHPLC equipped with a fluorescence detector and initial rates (v) were calculated as described (26). Steady state catalytic parameters were determined at each Mg²⁺ concentration by curve fitting to the Michaelis-Menten equation. Plots of v/[E] versus [S], where [S] < Kₘ were used to determine k₉/Kₘ.

**Calcium inhibition**—For magnesium supported T5FEN reactions, reaction mixtures containing 0.5 or 2 mM MgCl₂, 1 μM HP5F, 25 mM potassium glycinate, pH 9.3, 0.1 mg/mL BSA, varying [CaCl₂] with the appropriate amount of KCl to keep ionic strength constant as above were initiated by appropriate amounts of T5FEN (0.3-30 nM). For manganese supported T5FEN reactions, reaction mixtures containing 0.1, 0.2 or 0.4 mM MnCl₂, 0.2 μM HP5F, 25 mM MOPS, pH 7.5, 0.1 mg/mL BSA, CaCl₂ with the appropriate amount of KCl were initiated by addition of an appropriate amount of T5FEN (0.05-0.4 nM). For D201I/D204S-T5FEN reactions, reaction mixtures containing 0.5 or 2 mM MgCl₂, 0.5 μM HP5F, 25 mM CHES, pH 9.3, 0.1 mg/mL BSA, CaCl₂ with the appropriate amount of KCl were initiated by addition of the appropriate amount of D201I/D204S T5FEN (final concentration 5-40 nM). All reactions were sampled and quenched as above and used to determine k₉/Kₘ (v[E]) as a function of calcium ion concentration.

**Fluorescence anisotropy**—T5FEN DNA interactions were evaluated by fluorescence anisotropy using a Horiba Jobin Yvon FluoroMax-3® fluorimeter with automatic polarisers. The excitation wavelength was 490 nm (excitation slit width 8 nm) with emission detected at 509-511 nm through an 8 nm wide slit. Experiments contained 2 mM EDTA or varying concentrations of CaCl₂ (0.5-10 mM), with the appropriate amount of KCl as for kinetic analyses, 100 nM (WT T5FEN) or 10 nM HP5F (D201I/D204S), 25 mM HEPES, pH 7.5 or 25 mM potassium glycinate, pH 9.3, 0.1 mg/mL BSA, and 1 mM DTT. All experiments were carried out at 37°C. Anisotropy (r) was determined as described (27) initially prior to the addition of protein and then on the cumulative addition of enzyme.

Data were fitted, using Kaleidagraph, to the following equation with corrections made for dilution,

\[
r = r_{\text{min}} + (r_{\text{max}} - r_{\text{min}}) \left( \frac{(\[S]+[E]+K_D) - \sqrt{([S]+[E]+K_D)^2 - 4[S][E]}}{2[S]} \right)
\]

**Equation [1]**

where r is the measured anisotropy, [E] is the total protein concentration and [S] is the total HP5F concentration, r_{\text{min}} is the anisotropy of free DNA, r_{\text{max}} is the anisotropy of the DNA-protein complex and K₉ is the dissociation constant (27).

**Data analyses**—Curve fitting of Mg²⁺-dependent behaviour and Ca²⁺-dependent inhibitions were carried out by non-linear regression fitting using Kaleidagraph software weighted according to individual error values when required, using equations [2-7] (results section).

**RESULTS**

**The effect of divalent metal ions on WT FEN-substrate interactions.** Studies on the magnesium ion dependence of the WT T5FEN-catalysed reaction were carried out previously using the 5'-fluorescent overhanging hairpin substrate HP5F under pH-independent maximal-rate conditions (pH = 9.3) (3). These earlier kinetic studies revealed a requirement for two Mg²⁺ ions to maximise 1/Kₘ. However, the interpretation of this behaviour was complicated by the nature of this parameter, which in FEN-catalysed reactions that are rate limited by release of products reflects the stability of all forms of the enzyme-bound substrate and product. To examine binding of HP5F to T5FEN under similar conditions to catalytic experiments but in the absence of catalysis, fluorescence anisotropy was employed. In the presence of EDTA at pH 9.3 anisotropy (r) increased with addition of protein up to 10 μM but no maximal value was reached, implying K_D > 5 μM (Figure 2A). In contrast, in the presence of non-catalytically competent Ca²⁺ ions (1 mM) r reached a maximum value and could be fitted to a single binding isotherm (equation [1]) to yield a K_D = 202 ± 27 nM (Figure 2A). Thus, divalent metal ions substantially stabilise the T5FEN-HP5F complex at pH 9.3.
In contrast at pH 7.5 in the absence of divalent metal ions \( K_D = 231 \pm 13 \text{ nM} \) (Figure 2B). At this pH the presence of Ca\(^{2+}\) ions (1 mM) has a modest effect, with \( K_D = 120 \pm 13 \text{ nM} \). Thus either lowering the pH or adding divalent metal ions have large impacts on TSFEN-substrate interactions but the effects are not additive. Similar stimulation of binding of DNA substrates at lowered pH or by Ca\(^{2+}\) ions has been observed with other metallonucleases and been assigned to protonation or binding of Ca\(^{2+}\) ions at the active site carboxylates (28). Here, the protonation state of DNA binding resides could also be a factor (29,30).

The variation in \( K_D \) as a function of \([\text{Ca}^{2+}]\) was determined at both pH 9.3 and 7.5. Calcium ion concentration was varied from 0.5-10 mM, maintaining the ionic strength by adjusting the concentration of KCl. Under these conditions, the slopes of log \( K_D \) versus log \([\text{Ca}^{2+}]\) were 0.15 ± 0.10 (pH 9.3) and -0.06 ± 0.06 (pH 7.5) indicating changes in enzyme substrate affinity were negligible (Figure 2D). Relative insensitivity of \( K_D \) to concentrations of divalent ions < 20 mM has been noted in other systems where divalent ions are cofactors (31). Importantly, at both pH values the changes in enzyme-substrate affinity on altering Ca\(^{2+}\) ion concentration are relatively small and so do not account for the inhibition characteristics of the enzyme-catalysed reaction by calcium ions described below.

**Inhibition of WTT5FEN catalysed reactions with Ca\(^{2+}\) ions.** Earlier studies have revealed that Ca\(^{2+}\) ions inhibit the TSFEN catalysed reaction and under some conditions this is dependent on \( 1/[\text{Ca}^{2+}]^2 \) (3). Assuming competitive inhibition with viable cofactor ions, a two Ca\(^{2+}\) ion inhibitory response could be a diagnostic test for a requirement for two viable metal ion cofactors. Previously, it was demonstrated that the concentration of Ca\(^{2+}\) ions required to reduce the rate of reaction by 50%, \( K_I \) according to equation [2], was a function of both the concentration and identity of the viable cofactor. This analysis assumes that replacement of a single viable cofactor with Ca\(^{2+}\) ion prevents reaction, where \( k_{\text{obs}} \) is the observed normalised initial rate of reaction (\( v/\text{[E]} \)) and \( k_0 \) is \( v/\text{[E]} \) in the absence of inhibitory ion. Thus at least one Ca\(^{2+}\) ion displaces one viable cofactor ion to inhibit reaction. However, the origins of the most informative feature of the inhibition, a two Ca\(^{2+}\) ion inhibitory form, required further investigation.

\[
\frac{k_{\text{obs}}}{k_0} = \frac{K_I}{K_I + [\text{Ca}^{2+}]}
\]

Equation [2]

Assuming two inhibitory binding sites and competitive inhibition, where calcium ions compete for the same sites as viable cofactors (M\(^{2+}\)), potentially eight enzyme-mono or -dimetal species can exist when Ca\(^{2+}\) and/or M\(^{2+}\) are present and so speciation could be very complex. This is particularly so when the inhibitory ions are bound with higher affinity by the protein than the M\(^{2+}\), and especially in the context of reactions where M\(^{2+}\) is not supplied at saturation. The simplest responses are predicted to arise when M\(^{2+}\) is bound with higher affinity than the inhibitory ion, a criteria fulfilled by the Mn\(^{2+}\)/Ca\(^{2+}\) pair. Hence, to reveal whether Mn\(^{2+}\)-supported FEN-catalysed reactions are inhibited by displacement of two viable cofactor ions by calcium ions, reaction was studied at three concentrations of viable cofactor ions (0.1 mM, 0.2 mM and 0.4 mM) with saturating HP5F substrate and varying concentrations of Ca\(^{2+}\). The reactions were studied at pH 7.5, where the Mn\(^{2+}\)-supported FEN reaction is optimal but where formation of manganese hydroxides are avoided. These three concentrations of Mn\(^{2+}\) span sub-saturating (0.1 mM) and maximal rate concentrations (0.2 mM and 0.4 mM) of viable cofactor. As the concentrations of Mn\(^{2+}\) and Ca\(^{2+}\) were varied, the ionic strength of reaction mixtures was kept constant by adjusting the amount of potassium chloride present. To avoid the concentration of ions being perturbed by binding to DNA the substrate was supplied so that the total concentration of phosphate diesters in HP5F was equal to or less than 5% of the concentration of Mn\(^{2+}\).

As observed previously, but shown here with a more extensive data set, at lower concentrations of Ca\(^{2+}\) (up to 80% inhibition, \( k_{\text{obs}}/k_0 = 0.2 \)) the data fitted acceptably to the simple competitive inhibition scheme (equation [2]). As the concentration of Mn\(^{2+}\) is increased \( K_I \) is also increased indicating inhibition is competitive so that \( K_I = 1.1 \pm 0.14 \text{ mM} \) at 0.1 mM Mn\(^{2+}\), \( K_I = 2.2 \pm 0.33 \text{ mM} \) at 0.2 mM Mn\(^{2+}\), \( K_I = 3.8 \pm 0.40 \text{ mM} \) at 0.4 mM Mn\(^{2+}\) (Figure 3A).
magnitude of \( K_I \) confirms that in analogy to dissociation constants determined for the substrate-free enzyme, Mn\(^{2+}\) ions must bind to the protein with higher affinity than Ca\(^{2+}\) ions.

In the case of the highest concentration of Mn\(^{2+}\) most of the data range fits acceptably to the simple inhibition model, although deviation is evident at the very highest concentrations of Ca\(^{2+}\). At lower concentrations of Mn\(^{2+}\), the data deviates from this simple inhibition scheme when inhibition is greater than 80\% (\( k_{\text{obs}}/k_0 < 0.2 \)). The slopes of log-log plots of normalised rates versus calcium ion concentration where \( k_{\text{obs}}/k_0 < 0.2 \) are -2.0 ± 0.1 (0.1 mM Mn\(^{4+}\)), -1.9 ± 0.1 (0.2 mM Mn\(^{2+}\)) rather than the slope of -1 predicted by the simple inhibition scheme. This indicates a two Ca\(^{2+}\) form of the enzyme is also catalytically inactive. Whilst a complete data fit to the complex overall inhibition scenario requires a knowledge of all eight interrelated dissociation constants, it is possible to generate a relatively simple description that assumes two Ca\(^{2+}\) ions bind competitively and independently and that all forms of the enzyme that contain Ca\(^{2+}\) are inactive. Equation [3] models the expected variation of rate \( k_{\text{obs}}/k_0 \) with [Ca\(^{2+}\)] defining two apparent \( K_I \)s for Ca\(^{2+}\) ions, where \( K_1 \) is an apparent dissociation of di-calcium forms of the enzyme and \( K_2 \) is an apparent dissociation constant of mono-calcium forms.

\[
\frac{k_{\text{obs}}}{k_0} = \frac{K_1K_2}{K_1K_2 + K_1[\text{Ca}^{2+}] + [\text{Ca}^{2+}]^2}
\]

**Equation [3]**

Although good non-linear regression curve fits can be obtained using equation [3], the similar magnitudes of the apparent inhibition constants result in the same value for each with large errors. Similar non-linear regression curve fitting problems have been noted previously (32). When \( K_2 \) is fixed according to the single site inhibition model resulting values of \( K_1 = 5.3 \) mM (0.1 mM Mn\(^{4+}\)) and \( K_1 = 8.4 \) mM (0.2 mM Mn\(^{2+}\)) are observed. At higher Mn\(^{2+}\), the data fit to a simple one site inhibition model implying that the value of \( K_1 \) is outside the data range (Figure 3B). Hence, the magnitude of this parameter is dependent on the concentration of viable cofactor present, implying that two Ca\(^{2+}\) ions compete for the same sites as the two Mn\(^{2+}\) ions.

Further evidence for a requirement for two catalytic metals can be derived by an alternative analysis of the data. Assuming that the only catalytically competent species has two viable metal ion cofactors bound, it follows that when the dominant species contains two inhibitory ions the dependence of the normalised rate of reaction on viable cofactor ions will approach the square of its concentration. This will occur in situations where the viable cofactor has higher affinity than the inhibitory ion. Analysing the data in the region \( k_{\text{obs}}/k_0 < 0.2 \), a plot of log \( k_{\text{obs}}/k_0 \) versus log [Mn\(^{2+}\)] at fixed Ca\(^{2+}\) concentration does indeed display the expected slope close to 2 (Figure 3C).

Similar analyses have also been conducted for Mg\(^{2+}\) supported T5FEN reactions, which also display a biphasic response to Ca\(^{2+}\) inhibition. These studies were carried out at pH 9.3, which is optimal for the Mg\(^{2+}\)-supported reaction, and at saturating substrate. Two subsaturating concentrations of Mg\(^{2+}\) were studied (0.5 mM and 2 mM), a criteria imposed to keep total divalent ion concentration below a value that could potentially alter enzyme-substrate affinity. Analysis according to the one site inhibition model (equation [2]) reveals \( K_I = 0.11 \pm 0.01 \) mM at 0.5 mM Mg\(^{2+}\) and \( K_I = 0.22 \pm 0.01 \) mM at 2 mM Mg\(^{2+}\) (Figure 3D). The apparent \( K_I \) values are smaller than the concentration of Mg\(^{2+}\), indicating that Mg\(^{2+}\) ions are bound more weakly than Ca\(^{2+}\). Applying the two inhibitory ion model (equation [3]) yields apparent inhibition constants that are similar, \( K_1 = 8.7 \pm 0.1 \) mM (0.5 mM Mg\(^{2+}\)) and \( K_1 = 9.0 \pm 1.3 \) mM (2 mM Mg\(^{2+}\)), presumably reflecting the relative affinity of Ca\(^{2+}\) versus Mg\(^{2+}\) and the subsaturating conditions with respect to viable cofactor (Figure 3E).

The magnesium ion requirements of a metal site 2 mutant (D201I/D204S). Previous analyses, including the observation that D201I/D204S does not drastically impair reaction, suggest that T5FEN’s M2 may not play a direct role in catalysis (12, 24). In the designed D201I/D204S mutant two of the carboxylates that ligand M2 are altered (Figure 1A) as in E. coli ExoIX to abolish the binding site for this ion. With HP5F as substrate, the D201I/D204S mutation slowed the maximal single turnover rate of reaction by a factor of 40 at saturating Mg\(^{2+}\), a modest factor in the context of the entire T5FEN rate acceleration of \( 10^{16} \) to \( 10^{17} \) (33). To elucidate the role and minimal number of metal ions involved in the D201I/D204S mutant catalysed reaction,
kinetic parameters were determined as a function of magnesium ion concentration in an analogous fashion to experiments previously conducted on the WT enzyme using HP5F substrate (pH 9.3) and at constant ionic strength. Like WT T5FEN, the major site of reaction of D201I/D204S with HP5F is one nucleotide into the double stranded region of the 5'-overhanging hairpin substrate, although slightly increased amounts of reaction elsewhere are observed with the mutant protein. Total concentrations of products were evaluated during all kinetic analyses (25).

The variations of the individual kinetic parameters of D201I/D204S mutant with magnesium ion concentration are shown in Figure 4A-C. The turnover number ($k_{cat}$) is dependent upon increasing magnesium ion concentration until it reaches 10 mM Mg$^{2+}$. Data could not be collected below 0.05 mM Mg$^{2+}$ as the end point of the slowed reactions were <100% at these concentrations. A similar effect was previously noted for the WT enzyme, albeit at lower [Mg$^{2+}$] (3).

A previous study of the $k_{cat}$ or $k_{CT}$ response of WT T5FEN displayed a clear biphasic response to Mg$^{2+}$ ions, where a second order dependence on Mg$^{2+}$ at low concentrations of ions becomes first order at higher concentrations before approaching saturation (3). This situation can only be explained by the participation of two ions in chemical catalysis, and was best fit to a model where reaction required the presence of two ions that were bound independently and with differing affinity (equation [4]).

$$
(k_{cat})_{obs} = \frac{(k_{cat})_{max} [Mg^{2+}]^2}{(K_{DMgES1} + [Mg^{2+}]) (K_{DMgES2} + [Mg^{2+}])}
$$

Equation [4]

However, the $k_{cat}$ data for D201I/D204S fits acceptably to a single binding isotherm (equation [5]), where $(k_{cat})_{obs}$ is the observed turnover number at a given concentration of magnesium ions, $K_{DMgES}$ is the magnesium ion dissociation constant from ES complex, and $(k_{cat})_{max}$ is the maximal turnover rate at infinite magnesium ions, giving $K_{DMgES} = 2.5 \pm 0.3$ mM. This value is similar to that obtained for the lower affinity magnesium ion associated with the $k_{cat}$ response of the WT protein ($K_{DMgWTES} = 2.1$ mM (3)).

$$
(k_{cat})_{obs} = \frac{(k_{cat})_{max} [Mg^{2+}]^2}{K_{DMgES} + [Mg^{2+}]}
$$

Equation [5]

Applying a model where reaction requires the presence of two ions that are bound independently as used to fit WT data previously (equation [4]) places a higher-affinity dissociation constant outside the data range, although this number can only be regarded as a potential upper limit (Figure 4A).

Earlier studies of the WT protein revealed that both 1/K$_M$ and $k_{cat}/K_{M}$ are Mg$^{2+}$ dependent. Below 0.25 mM Mg$^{2+}$ 1/K$_M$ has a [Mg$^{2+}$]$^2$ dependence (slope of 2 log log plot). This required a two ion model for the WT enzyme, where two ions were bound independently with similar affinity or cooperatively. The magnesium ion response of 1/K$_M$ for D201I/D204S differs from that of the WT protein and fits a single-binding isotherm (equation [6]) yielding $K_{DMgE} = 0.5 \pm 0.2$ mM (Figure 4B).

$$
\left(\frac{1}{K_M}\right)_{obs} = \frac{1}{(K_{DMgE1} + [Mg^{2+}]) (K_{DMgE2} + [Mg^{2+}])}
$$

Equation [6]

Below 0.25 mM $k_{cat}/K_{M}$ for the WT enzyme was dependent on [Mg$^{2+}$]$^3$, requiring a three ion model. In contrast, a plot of log ($k_{cat}/K_{M}$) versus log [Mg$^{2+}$] for D201I/D204S (Figure 4C) reveals a slope of 1.8 $\pm$ 0.2 implying the overall involvement of two ions in the reaction catalysed by the mutated protein, rather than the three ion requirement of the WT enzyme. Fitting the data to equation [7], assuming the mutated FEN is only capable of reaction with two ions present that are bound independently, with $K_{DMgE} = 0.5$ mM (as defined by the 1/K$_M$ data) yields $K_{DMgE} = 3.3 \pm 0.2$ mM.

$$
\left(\frac{k_{cat}}{K_M}\right)_{obs} = \frac{(k_{cat})_{max} [Mg^{2+}]^3}{(K_{DMgE1} + [Mg^{2+}]) (K_{DMgE2} + [Mg^{2+}])}
$$

Equation [7]

Thus, combined magnesium stimulation data for D201I/D204S provide evidence that the overall reaction of the mutated protein requires at least two Mg$^{2+}$ ions and differs from the WT protein, but the restricted data range makes it impossible to
ascertain whether chemical catalysis ($k_{cat}$) has a one or two ion requirement.

**Substrate binding and calcium ion inhibition of D201I/D204S.** Binding of HP5F and D201I/D204S were studied in an analogous fashion to the WT protein at pH 9.3 to ascertain whether divalent metal ions were required to bind substrate and whether potentially binding of Ca$^{2+}$ ions by DNA substrate could be inhibitory. In contrast to the WT protein, in the absence of divalent metals ions a dissociation constant could be determined ($K_D = 2300 \pm 200$ nM), presumably due to the substitution of two active site carboxylates with neutral residues (Figure 2C). This was decreased by two orders of magnitude upon addition of 0.5 mM Ca$^{2+}$ ions ($K_D = 28 \pm 3$ nM). The effect of varying [Ca$^{2+}$] on $K_D$ was modest; the slope of a log-log plot of $K_D$ versus [Ca$^{2+}$] is $0.09 \pm 0.04$.

To determine the number of viable cofactors required by D201I/D204S, Ca$^{2+}$ inhibition of the Mg$^{2+}$-supported reactions was investigated in an identical manner to the WT protein. Applying the single-site inhibition model (equation [2]) yielded $K_i = 2.1 \pm 0.2$ mM (2 mM Mg$^{2+}$) and $K_i = 1.1 \pm 0.1$ mM (0.5 mM Mg$^{2+}$) (Figure 4D). Importantly, data obtained at 0.5 mM Mg$^{2+}$ clearly displays 1/[Ca]$^2$ dependence at Ca$^{2+}$ ion concentrations above 6 mM, whereas increasing the concentration of Mg$^{2+}$ leads to deviation from the single site inhibition model only at the very highest [Ca$^{2+}$] employed (>15 mM) (Figure 4E). Respective values of $K_{II} = 7.7 \pm 0.9$ mM (0.5 mM Mg$^{2+}$) and 40 \pm 18 mM (2 mM Mg$^{2+}$) were obtained by fitting the data to equation [3]. As addition of Ca$^{2+}$ ions at low concentration stimulates substrate binding and the stability of complexes is similar at inhibitory concentrations, the data imply that when a substantive part of T5FEN’s M2 binding site is abolished the FEN catalysed reaction still requires at least two catalytically viable ions.

**DISCUSSION**

The work described in this manuscript supports the hypothesis that the major rate acceleration in the reactions catalysed by flap endonucleases requires the presence of two metal ions. Stimulation of chemical catalysis ($k_{cat}$ or $k_{ST}$) by WT T5FEN has revealed previously that the reaction has a requirement for two magnesium ions that are bound independently with differing affinity (3). Confirmation of this requirement is demonstrated by the characteristics of inhibition of the WT reaction by catalytically inert Ca$^{2+}$ ions, shown here to be competitive with viable cofactors. The response of the FEN reaction to added Ca$^{2+}$ is biphasic and ultimately dependent on 1/[Ca]$^2$, indicative of a two viable cofactor requirement. The possibility that higher order inhibition is the result of altered T5FEN-substrate interactions as a result of added Ca$^{2+}$ is ruled out. Moreover, stimulation of reactions under higher Ca$^{2+}$inhibitory conditions by Mn$^{2+}$ is a second order process with respect to stimulatory cofactor. Thus the conclusion of more than one experimental approach is that the chemical process catalysed by T5FEN involves at least two catalytic metal ions.

A designed metal site 2 mutant catalyses phosphate diester hydrolysis with a decrease in the rate of reaction of only 40-fold, implying that T5FEN’s M2 is not responsible for major rate accelerations. Previous experiments have indicated that the overall reaction of the WT enzyme ($k_{cat}/K_M$) requires at least three divalent ions. In contrast, magnesium ion stimulation data demonstrate that the overall reaction catalysed by D201I/D204S requires at least two ions. However, within the experimental data range tested chemical catalysis requirements for the mutated protein ($k_{cat}$) can be assigned to a single-ion dependence. In theory these data could indicate that a single metal ion is required for catalysis by the mutant. However, inhibition of the mutated protein by Ca$^{2+}$ ions clearly demonstrates a requirement for two viable cofactors, in an analogous fashion to the WT protein. The combined data therefore imply that another ion (referred to here as M3), not present in substrate-free T5FEN structures, is bound by both the WT and mutated enzymes. Together with M1 this provides the major rate enhancement of phosphate diester hydrolysis.

Whilst the data presented here implicate two ions in catalysis they do not provide information on the location of T5FEN’s M3. For a two-metal-ion mechanism where both ions contact the scissile phosphate diester, M3 would have to be located within < 4 Å of M1 where both could be liganded by the central carboxylate of the T5FEN active site D130. As this is the
arrangement of two Mg\textsuperscript{2+} ions shown in a substrate-free structure of hFEN (Figure 1B) (18) and the position of two Sm\textsuperscript{3+} ions in an hFEN-1 product complex (4) this positioning of ions for chemical catalysis appears highly plausible. Thus we suggest that T5FEN’s M3, not visible in substrate-free structures of bacteriophage enzymes, is equivalent to the ion designated M2 in hFEN-1 structures (Figure 1A&B).

However, despite the necessity of two ions for chemical catalysis, the WT T5FEN protein does have a three metal ion requirement to maximise the rate of its overall ($k_{cat}/K_M$) reaction (3), which is reduced to two ions for D201I/D204S. In the absence of catalysis at pH 9.3 the dissociation constant of substrate HP5F is > 5 µM, but addition of Ca\textsuperscript{2+} ions substantially lowers this value. Enhancement of substrate affinity it also observed with the T5FEN M2 site mutant upon addition of Ca\textsuperscript{2+}, although overall dissociation constants in the presence and absence of divalent ions are lowered as the result of the neutralising mutations. This, together with the $1/K_M$ divalent metal dependencies of both the WT and D201I/D204S mutant proteins, implies a role for metal ions in FEN-substrate complex stability.

Unlike the WT protein, which requires two ions to maximise $1/K_M$, data for the mutated FEN fits to single binding isotherm. Therefore, the combined data suggest that T5 FEN’s M2 stabilises protein-substrate interaction in WT T5FEN, but it is not an absolute requirement when neutralising mutations are present in D201I/D204S.

Consideration of functional and structural analyses earlier led us to suggest that for FENs and related superfamily members to achieve a state where the scissile phosphate diester contacted metals ions involved in chemical catalysis the terminus of the duplex where reaction occurs would have to be unpaired (3). Structural analyses of mutant T4FEN bound to a pseudo-Y substrate in the absence of divalent ions (15) demonstrate that the scissile phosphate diester is located within a duplex bound in front of and parallel to the carboxylate rich active site (Figure 5A-B). Modelling indicated that the unpairing of two nucleotides would be required to place the scissile phosphate in the vicinity of M1 (and presumably T5FEN M3) (Figure 5C). Recent structural analyses of DNA complexes of hFEN-1 and hEXO-1 lend support to this unpairing mechanism (2,4). When 5’-phosphate monester contacts active site metals ions, one nucleotide of the duplex is unpaired implying that for the scissile bond to occupy the same site two nucleotides must unpair.

To achieve contact between scissile phosphate and metals the unpaired DNA has to traverse the active site. It is interesting to note that the phosphate diester adjacent to the scissile bond (+1) would also have to move further towards the carboxylate rich region, closer to T5FEN’s M2 which would presumably act to stabilise this unpaired state. We suggest that in accord with the functional analyses of WT T5FEN, two metal ions are required to position the substrate in reactive conformation, M1 which later plays a direct role in chemical catalysis and “non-catalytic” M2. It is tempting to speculate that an M2 that plays a substrate positioning role can be replaced with Ca\textsuperscript{2+} explaining why WT T5FEN has an overall three metal ion requirement but is inhibited by replacement of only two viable cofactors.

In unpaired hFEN-1 and hEXO-1 DNA complexes the +1 phosphate interacts with the protein N-terminus (2,4), which occupies a similar position to T5FEN’s M2 (Figure 1A&B). The N-terminal region of the protein is disordered in bacterial and bacteriophage FEN structures and though to be a site of protein-protein interaction (17,21,22). In T5FEN, the first 19 residues can be removed whilst retaining full enzymatic activity and so the N-terminus is unlikely to play a substrate-positioning role (12,23). Thus apparent discrepancies between metal ion binding sites in FEN structures could be explained by a functional equivalence of a divalent metal ion and the N-terminus.

The designed neutralisation of D201/D204 to I201/S204, based on the sequence of another 5’-nuclease family member E. coli ExoIX (6), presumably facilitates passage of the substrate across the same region of the protein without a requirement for a M2, yet still with the participation of M3. Individual mutations of the equivalent carboxylates (D185 and D188) in the FEN domain of E. coli DNA polymerase to alanine, which could be viewed as more conservative than D201/D204S mutant, were both reported to have a much larger deleterious effect on enzyme activity than the designed double mutation introduced here (34). This is presumably in part because a greater active site charge remains
in the individual Ala mutants, but stabilising substrate interactions with the side chains of Ile and Ser are also a possibility in D201I/D204S. A mutated T5FEN where the same two carboxylates are changed to arginine (D201R/D204R) retains activity in line with a non-catalytic substrate positioning/stabilising role for T5 FEN’s M2, perhaps compensating for the lack of a bound metal ion with the cationic arginine (12). The decrease in the reaction rate observed with our designed M2 mutant may reflect a change in the partitioning of substrate between bound duplex and unpaired active site positioned form. Such a situation would be consistent with the properties of exonucleolytic reactions catalysed by the mutated protein (24). Although not a feature of the T5FEN catalysed reactions of 5’-flap substrates (Sengerová and Grasby, unpublished observation), the exonucleolytic reactions of T5FEN are diffusion controlled indicating that forward reaction steps are faster than substrate dissociation. Despite more stable enzyme-DNA complexes with D201I/D204S, both in the presence and absence of divalent ions, reactions are no-longer diffusion controlled implying a slowed later step in the reaction sequence. Furthermore this rate-limiting later step is insensitive to the nature of the leaving group, consistent with a physical rather than chemical process, such as substrate unpairing.

In contrast to the ExoIX sub group, the carboxylate rich active site is conserved in the majority of the DNA 5’-nuclease superfamily (1). These include enzymes that catalyse structure specific reactions of an apparently disparate group of nucleic acid substrates including DNA bubbles (XPG) (35), four way junctions (GEN-1) (36) as well as flapped, nicked and 3’-overhang DNAs (FEN and EXO-1) (2,37,38). Since all of these enzymes carry out the same chemical transformation, like T5FEN, they too will require two ions to effect phosphate diester hydrolysis. Notably, the reaction specificities of the superfamily do have a common feature; the major site of reaction occurs one nucleotide into a double stranded region of the substrate, located at a junction. It seems highly likely that the unpairing mechanism is universally conserved in the seven/eight carboxylate enzymes to give rise to this reaction specificity. If this is the case, FEN superfamily members may also require either a further active site metal or a functionality of the protein such as the N-terminus for substrate unpairing in addition to two catalytic ions. Analyses of the nuclear RNA degradative 5’-exoribonuclease Rat1 (41) demonstrate similar secondary structure to FENs (5). In addition, we also note that two recent structures of the corresponding cystosolic enzyme Xrn1 (39,40), demonstrate these enzymes conserve the DNA 5’-nuclease superfamily active site and that RNA 5’-nucleases may in turn also utilise a similar catalytic mechanisms.

REFERENCES


FOOTNOTES
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Abbreviations used: nt nucleotide; T5FEN bacteriophage T5 flap endonuclease (in early literature referred to as T5 5'-3'-exonuclease and T5 5'-nuclease); T4FEN T4 flap endonuclease (referred to also as T4 RNase H); hFEN-1 human flap endonuclease; ExoIX E. coli exonuclease IX; EXO-1 exonuclease-1; XPG xeroderma pigmentosum complementation group G; GEN-1 XPG-like gap endonuclease (putative human Holliday junction resolvase); WT wild type

**FIGURE LEGENDS**

**Figure 1** The varying positions of metal ions in FEN structures. (A) The active site structure of T5FEN (1UT5) illustrating the seven active site carboxylates present in similar positions in all FENs and the eighth carboxylate (D201) present in the active sites of bacteriophage and bacterial enzymes. Two metal ions, M1 and M2 are bound with a separation of 8 Å. A metal site 2 mutant was created by alteration of the two aspartic acid residues (D210I/D204S). (B) The active site structure of hFEN-1 (1UL1x), illustrating the seven active site carboxylates present in similar positions in all FENs. Two metal ions, M1 and M2 are bound with a separation of 3.4 Å. Although all FENs conserve seven active site carboxylates, the position of M2 observed in each structure is variable. The N-terminus (blue) occupies a similar position to M2 in the bacteriophage enzyme (A).

**Figure 2** The effects of calcium ions on WT and D210I/D204S FEN-substrate dissociation constants evaluated by fluorescence anisotropy (r). Experimental details are contained within the materials and methods section and all experiments were carried out in triplicate. Constant ionic strength of potassium and calcium ions was maintained by varying the amount of KCl present. (A) Addition of WT T5FEN to 100 nM HP5F at pH 9.3, in the presence of 2 mM EDTA (squares) and 1 mM Ca$^{2+}$ ions (circles). Curve fitting to equation [1] yields $K_D = 202 \pm 27$ nM (1 mM Ca$^{2+}$). Without divalent metal ion present the binding curve did not reach saturation. (B) Addition of WT T5FEN to 100 nM HP5F at pH 7.5, in the presence of 2 mM EDTA (squares) and 1 mM Ca$^{2+}$ ions (circles). Curve fitting to equation [1] yields $K_D = 231 \pm 13$ nM (no divalent ion) and $K_D = 120 \pm 13$ nM (1 mM Ca$^{2+}$). (C) Addition of D210I/D204S T5FEN to 10 nM HP5F at pH 9.3, in the presence of 2 mM EDTA (squares) and 0.5 mM Ca$^{2+}$ ions (circles). Curve fitting to equation [1] yields $K_D = 2880 \pm 160$ nM (no divalent ion) and $K_D = 28 \pm 3$ nM (0.5 mM Ca$^{2+}$). (D) Variation of log $K_D$ as a function of log [Ca$^{2+}$] for WT T5FEN at pH 9.3 (circles), pH 7.5 (squares) and D210I/D204S T5FEN at pH 9.3 (diamonds). The slopes of the resultant plots are 0.15 ± 0.10, -0.06 ± 0.06 and 0.09 ± 0.04 respectively.

**Figure 3** Inhibition of WT T5FEN catalysed reactions supported by Mn$^{2+}$ and Mg$^{2+}$ viable cofactors by the addition of calcium ions. All data points represent the average of three experiments, but error bars are omitted for clarity. Data at 0.1 mM Mn$^{2+}$ and 2 mM Mg$^{2+}$ are from (3). (A) Inhibition of 0.1 mM (black circles), 0.2 mM (red squares) and 0.4 mM (blue diamonds) Mn$^{2+}$ supported reactions by calcium ions. Experiments were carried out as described in materials and methods. Data is fit to a simple single site inhibition model (equation [2]) to yield $K_I = 1.1 \pm 0.11$ mM, 2.2 ± 0.32 mM and 3.8 ± 0.41 mM respectively. Although data fits well in the range $k_{obs}/k_0 > 0.2$, the deviation from this behaviour at higher concentrations of Ca$^{2+}$ ions is evident. (B) The same data as in (A) fit to a more complex inhibition model where Ca$^{2+}$ displaces either one or two Mn$^{2+}$ ions (equation [3]) yielding $K_I = 5.3$ mM (0.1 mM Mn$^{2+}$) and 8.4 mM (0.2 mM Mn$^{2+}$). In the case of data obtained at 0.4 mM Mn$^{2+}$, where there is limited deviation from the single inhibition model (equation [2]) the value of $K_I$ is outside the data range. (C) The dependence of log $k_{obs}/k_0$ (where $k_{obs}/k_0 < 0.2$) on log [Mn$^{2+}$] at 20 mM Ca$^{2+}$ circles, 17.5 mM Ca$^{2+}$ squares, 15 mM Ca$^{2+}$ diamonds, 12.5 mM Ca$^{2+}$ crosses and 10 mM Ca$^{2+}$ triangles. The slopes of the straight line
fits are 1.8 ± 0.1 at 20 mM Ca\textsuperscript{2+}, 1.8 ± 0.1 at 17.5 mM Ca\textsuperscript{2+} and 1.8 ± 0.1 at 15 mM Ca\textsuperscript{2+}. Although there are only two available data points at 12.5-10 mM Ca\textsuperscript{2+} the same trend is observed in this data. The intercept in each case is a function of 1/[Ca\textsuperscript{2+}];\textsuperscript{2}. (D) Inhibition of 0.5 mM (black circles), 2 mM (red squares) Mg\textsuperscript{2+} supported reactions by calcium ions. Experiments were carried out as described in materials and methods. Data is fit to a simple single site inhibition model (equation [2]) to yield \( K_I = 0.1 ± 0.01 \text{mM} \) (0.5 mM Mg\textsuperscript{2+}) and 0.2 ± 0.02 mM (2 mM Mg\textsuperscript{2+}). Although data fits well in the range \( k_{\text{obs}}/k_0 > 0.2 \), the deviation from this behaviour at higher concentrations of Ca\textsuperscript{2+} ions is evident. (E) The same data as in (D) fit to a more complex inhibition model where Ca\textsuperscript{2+} displaces either one or two Mg\textsuperscript{2+} ions (equation [3]) yielding \( K_{I1} = 8.7 ± 0.1 \text{mM} \) (0.5 mM Mg\textsuperscript{2+}) and 9.0 ± 1.3 mM (2 mM Mg\textsuperscript{2+}).

**Figure 4** The variation of the catalytic parameters of D210I/D204S mutant T5FEN with concentration of magnesium ions and the inhibition of this reaction by calcium ions. Catalytic parameters and rates of reaction were determined at pH 9.3 with varying concentrations of Mg\textsuperscript{2+} ions and when required Ca\textsuperscript{2+} ions as described in materials and methods. Experiments were carried out in triplicate and in A-C standard errors are shown. In D-E standard errors are omitted for clarity. (A) The variation in \( k_{\text{cat}} \) as a function of magnesium ion concentration for D210I/D204S. Data have been fit to equation [4] (black curve, \( K_{DMgES} = 2.5 ± 0.3 \text{mM} \) and \( k_{\text{cat}} \text{max} = 9.6 ± 0.3 \text{min}^{-1} \)) and equation [5] (red curve, \( K_{DMgES1} = 0.02 ± 0.004 \text{mM} \) and \( K_{DMgES2} = 2.7 ± 0.2 \text{mM} \) and \( k_{\text{cat}} \text{max} = 10.1 ± 0.5 \text{min}^{-1} \)). (B) The variation in \( 1/K_M \) for D210I/D204S as a function of magnesium ion concentration fitted to equation [6], to yield \( K_{DMgE1} = 0.5 ± 0.2 \text{mM} \) and \( (1/K_M) \text{max} = 0.05 ± 0.002 \text{nM}^{-1} \). (C) The variation in \( k_{\text{cat}}/K_M \) for D210I/D204S as a function of magnesium ion concentration fit to equation [7] with \( K_{DMgE1} = 0.5 \text{mM} \) to yield \( K_{DMgE2} = 3.3 ± 0.2 \text{mM} \) and \( (k_{\text{cat}}/K_M) \text{max} = 0.18 ± 0.01 \text{min} \text{nM}^{-1} \). (D) Inhibition of 0.5 mM (black circles), 2 mM (red squares) Mg\textsuperscript{2+} supported reactions catalysed by D210I/D204S by calcium ions. Experiments were carried out as described in materials and methods. Data is fit to a simple single site inhibition model (equation [2]) to yield \( K_I = 1.1 ± 0.1 \text{mM} \) (0.5 mM Mg\textsuperscript{2+}) and 2.2 ± 0.2 mM (2 mM Mg\textsuperscript{2+}). Although data fits well in the range \( k_{\text{obs}}/k_0 > 0.2 \), the deviation from this behaviour at higher concentrations of Ca\textsuperscript{2+} ions is evident. (E) The same data as in (D) fit to a more complex inhibition model where Ca\textsuperscript{2+} displaces either one or two Mg\textsuperscript{2+} ions (equation [3]) yielding \( K_{I1} = 7.7 ± 0.9 \text{mM} \) (0.5 mM Mg\textsuperscript{2+}) and 40 ± 18 mM (2 mM Mg\textsuperscript{2+}).

**Figure 5** (A) Model of T5FEN (1UT5) bound to a pseudo-Y DNA created by overlay with the structure of T4RNaseH (2IHN, T4FEN) bound to DNA (T4 protein not shown). Active site carboxylates are shown as sticks with red oxygens and T5 metal ions (M2 foreground) are shown in grey. The duplex where reaction occurs is bound in front of and parallel to the active site. The protein-DNA structure 2IHN was solved in the absence of metal ions, hence the substrate arches away and does not occupy the carboxylate rich active site. The scissile phosphate diester bond positioned 7Å from M1 is coloured yellow and the adjacent duplex phosphate+1 lilac. The two 5’-terminal nucleobases of the duplex are depicted in blue. (B) Close up of the active site in this model with colouring as in 5A. (C) Proposed unpairing of the two terminal base pairs of the duplex to allow the scissile phosphate diester (yellow) to move towards M1. This relocation requires that the adjacent phosphate diester -1 moves further towards the carboxylate active site and closer to M2. In other FENs and related superfamily members the N-terminus plays a similar role to M2 (2,4).
Figure 2
Figure 3
Figure 4
Figure 5