Elucidation of the metal-binding properties of the Klenow fragment of *Escherichia coli* polymerase I and bacteriophage T4 DNA polymerase by lanthanide(III) luminescence spectroscopy

Michelle West Frey[†], Steven T Frey[‡], William DeW Horrocks Jr, Barbara F Kaboord and Stephen J Benkovic

Background: The exonuclease active site of the Klenow fragment (KF) of *Escherichia coli* DNA polymerase I has a double binding site for the two essential divalent metal ions in the presence of the nucleotide monophosphate dTMP.

Results: The luminescence spectroscopy observed upon binding of Eu^{3+} to the exonuclease active site of T4 DNA polymerase was interpreted relative to the binding of Eu^{3+} or Tb^{3+} observed with KF. Both wild-type enzymes tightly bind a single Ln^{3+} ion but in two isomeric forms. The single mutants of KF (D424A) and T4 (D219A) also bind a single Eu^{3+} ion tightly, but the alignment of the coordinating ligands is altered. The KF double mutant (D355A, E357A) exhibits a markedly altered and weakened binding site (K_d = 20–26 μM). Eu^{3+} serves as a competitive inhibitor of Mg^{2+}-induced polymerase and exonuclease activity, validating its use as a probe for these active sites.

Conclusions: Ln^{3+} luminescence spectroscopy is established as a sensitive way to determine the consequences of exonuclease binding-site mutations and to examine binding site similarities and differences among DNA polymerases from different sources. The binding sites of KF and T4 DNA polymerase are shown to be quite similar.

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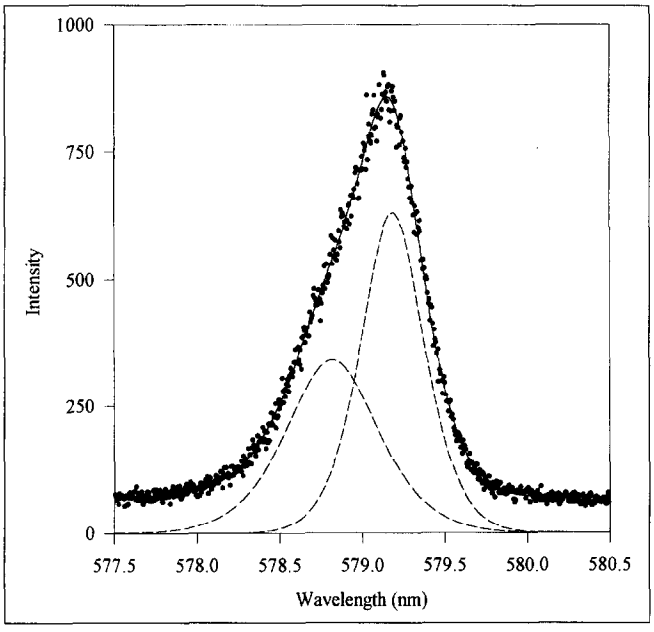
Introduction

The ability to quickly and accurately reproduce the information stored in the parental DNA strands is of paramount importance to the preservation and transmission of genetic material. Accuracy in DNA replication is achieved, in part, with the aid of a proofreading 3'→5' exonuclease. This activity, which may be present either on the same polypeptide chain as the polymerase or as a separate subunit [1], is responsible for the hydrolytic removal of misincorporated nucleotides from the 3' end of the growing chain [2]. Although the specific properties of the associated 3'→5' exonuclease activity differ from one polymerase to the next, certain characteristics appear to be universal. The exonuclease active site, normally located in the amino-terminal portion of the polypeptide chain, requires divalent metal ion binding for activity, and the cleavage reaction proceeds with stereochemical inversion at phosphorus [3]. Various metal ions, including Mg^{2+}, Mn^{2+}, Zn^{2+} and Co^{2+} [4–6], support hydrolytic activity, but the native metal has not been unambiguously identified for any polymerase enzyme. Recent crystallographic studies of the Klenow fragment of *Escherichia coli* polymerase I (pol I) and its mutants have provided great insight into several features of the 3'→5' exonuclease active site [7–13]. The polymerase and exonuclease activities are present on two distinct domains, separated by ~30 Å. The exonuclease active site possesses two metal-ion-binding sites, A and B, that are in close proximity to one another, and are both essential for catalysis. Although the polymerase activity is also known to require divalent metal ions, none were detected in the Klenow fragment (KF) crystal structure. Mutagenesis of the residues believed to be involved in metal ligation resulted in the loss of metal-ion binding by the KF mutants and the abolishment of exonuclease activity. Amino acid homology studies [14–18], based upon the primary sequence and crystal structure of KF, have demonstrated for several polymerases, including bacteriophage T4 DNA polymerase (T4 pol), that the residues critical for metal-ion binding by the KF exonuclease active site are highly conserved in three colinear regions. Mutation of these residues in T4 pol results in T4 proteins that are devoid of exonuclease activity [19,20].

In the absence of crystallographic data for the T4 polymerase enzyme, we sought another method to examine

Figure 1



Curve-resolved $^7F_0 \rightarrow ^5D_0$ excitation spectrum of one equivalent Eu^{3+} ion bound to wild-type KF. Wild-type KF (10 μM) was equilibrated with one equivalent of Eu^{3+} and the excitation spectrum (577.5–581.5 nm) collected by monitoring emission at 614 nm. The spectrum was resolved into its component peaks, with maxima at 578.8 and 579.2 nm, using the program Peakfit (Jandel Scientific).

the metal-ion-binding properties of both the wild-type and mutant enzymes [19], to determine whether the loss of activity was indeed due to disruption of the proposed metal-ion-binding sites. The ability of certain lanthanide(III) (Ln^{3+}) ions to luminesce in aqueous solution at room temperature renders them effective probes of the metal-ion-binding sites in a number of proteins and enzymes. In particular, calcium(II) ion-binding proteins have lent themselves to study by this technique owing to the similarity of the size of Ca^{2+} and Ln^{3+} ions (the ionic radius of Ca^{2+} is 1.14–1.32 Å, that of Eu^{3+} is 1.09– 1.26 Å) [21]. The Ln^{3+} ions are less similar in ionic radius to the putative native metal ions of polymerases, Mg^{2+} or Zn^{2+} (the ionic radius of Mg^{2+} is 0.86–1.03 Å and that of Zn^{2+} is 0.88– 1.04 Å). Nonetheless, Ln^{3+} ions have been successfully substituted for native Mg^{2+} ions in several systems [22,23]. Indeed, Wong [24] has used tyrosine-sensitized Tb^{3+} luminescence to observe metal binding in T7 DNA polymerase. Furthermore, Sm^{3+} was observed to bind tightly to KF in an X-ray crystal structure study [7]. The resolution of this structure did not, however, permit the determination of the stoichiometry of metal ion binding, or the precise nature of the metal-binding pockets.

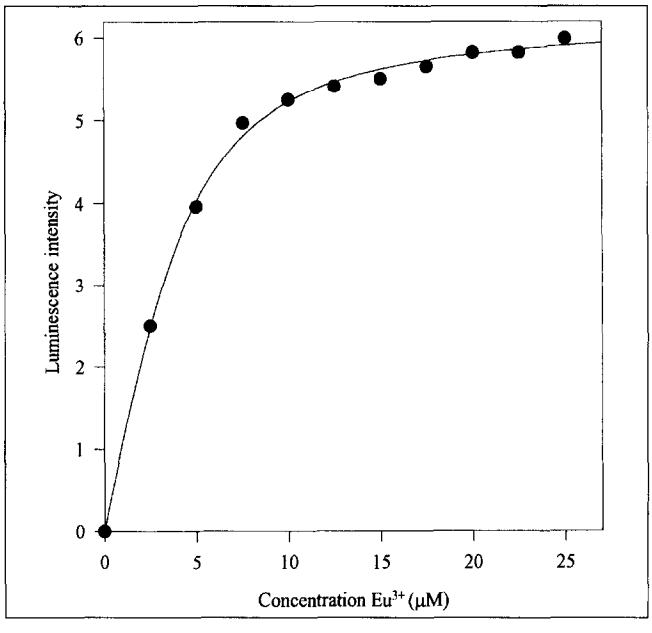
In this study, we have used laser-induced Eu^{3+} excitation spectroscopy and tyrosine-sensitized Tb^{3+} emission spectroscopy to investigate the metal-ion-binding properties of the KF and T4 polymerases, and several mutants of these enzymes. KF and its derivatives provide reference systems for the comparison with T4 DNA polymerase results. The stoichiometries, metal-ion-binding constants, and the number of metal-ion-coordinated water molecules are revealed in this work, as well as other, more subtle, characteristics of metal-ion binding.

Results

Eu^{3+} excitation spectroscopy – spectral analysis

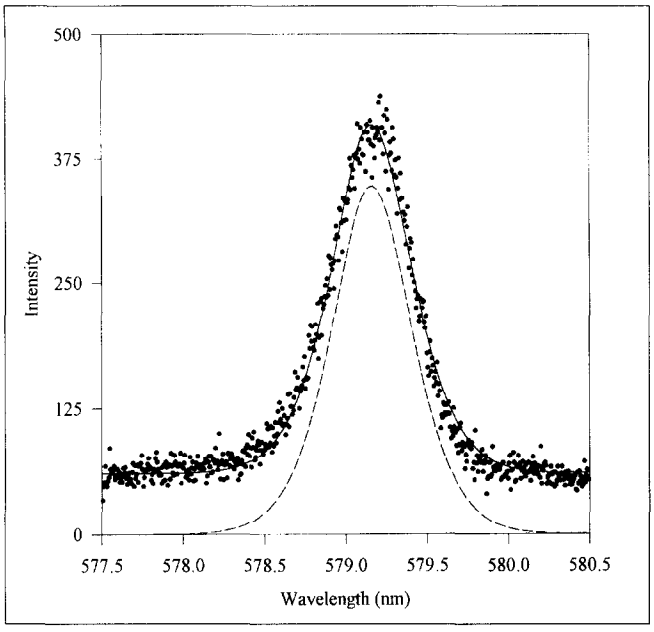
Since both the ground and excited states are non-degenerate, the number of peaks in the $^7F_0 \rightarrow ^5D_0$ excitation spectrum indicates the number of distinct Eu^{3+} environments. For wild-type KF, a broad asymmetric band is observed between the addition of zero and one equivalent of Eu^{3+}. This band has a peak maximum at 579.2 nm and what appears to be a shoulder at ~579 nm. The spectrum can be decomposed into two component bands, one centered at 578.8 nm and the other at 579.2 nm (Fig. 1). Beyond two added equivalents of Eu^{3+} the spectrum gradually becomes broader and more intense towards the shorter wavelengths. This is probably due to the contribution of $Eu(H_2O)_9^{3+}$, which has a peak maximum at 578.7 nm. Evidence for the presence of $Eu(H_2O)_9^{3+}$ is also provided in the excited-state lifetime analysis discussed below. Moreover, other experiments (*vide infra*) indicate the existence of a weaker site, whose occupation at higher concentrations of Eu^{3+} may further complicate the overall spectrum.

Figure 2



The Eu^{3+} binding curve for wild-type KF. The luminescence intensity for 579.5 nm excitation was measured on 5 μM wild-type KF with various concentrations of Eu^{3+} ion. The resulting curve was fit to a single binding site model (using the program EQUIL) yielding a Eu^{3+} K_d of 1.3 μM.

Figure 3



$^7F_0 \rightarrow {}^5D_0$ excitation spectrum of KF D424A single mutant (KF exo$^-$) with one equivalent of bound Eu^{3+} (10 μM). This spectrum is fit to a single Lorentzian–Gaussian function centered at 579.2 nm. This band corresponds closely to the higher wavelength band observed in the wild-type KF excitation spectrum shown in Figure 1.

Owing to the presence of multiple species, satisfactory curve-resolution of the spectrum cannot be achieved beyond one added equivalent of Eu^{3+}. Figure 2 shows the binding curve for wild-type KF, which can be fit to a single-binding-site model with a dissociation constant, K_d, of 1.3 μM. Beyond six equivalents of added Eu^{3+} (data not shown) the curve rises sharply again, an effect attributed to additional weak binding and/or the onset of precipitation. Overall, the plot is interpreted to show that KF wild-type enzyme binds a single Eu^{3+} ion tightly and perhaps a second ion much more weakly. This analysis is further substantiated by the excited state lifetime analysis and a Tb^{3+} titration of this enzyme (*vide infra*). The two bands that appear in the $^7F_0 \rightarrow {}^5D_0$ excitation spectrum below one equivalent of added Eu^{3+} ion are attributed to isomeric forms of the tight binding site, which are in fast exchange on the timescale of the luminescence. Previous model-system [25,26] and protein [27] studies provide precedence for the observation of fast-exchanging isomers.

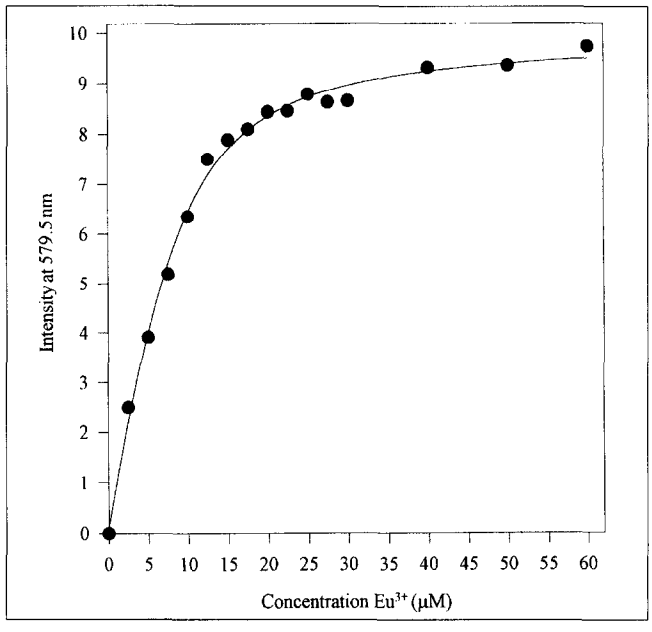
The D424A mutation in KF (KF exo$^-$) abolishes not only exonuclease activity, but also metal-ion binding at site B in the exonuclease active site as determined from crystallographic information [7–10,12]. The $^7F_0 \rightarrow {}^5D_0$ excitation spectrum of this mutant protein in the presence of one equivalent of Eu^{3+} ion consists of a single symmetric band centered at 579.2 nm (Fig. 3). This band corresponds closely to the higher wavelength band in the spectrum for wild-type KF, although it is somewhat broader (Table 1). Beyond one equivalent of added Eu^{3+}, a shoulder begins to appear which is attributable to the free $Eu(H_2O)_9^{3+}$ species, as corroborated by the excited state lifetime analysis (*vide infra*). Weaker Eu^{3+} binding, similar to that seen with the wild-type enzyme, is suspected to contribute to the excitation spectrum beyond one equivalent of added Eu^{3+}, although additional peaks cannot be satisfactorily resolved. The Eu^{3+} binding curve

Table 1

Eu^{3+} binding by wild-type and mutant polymerases.

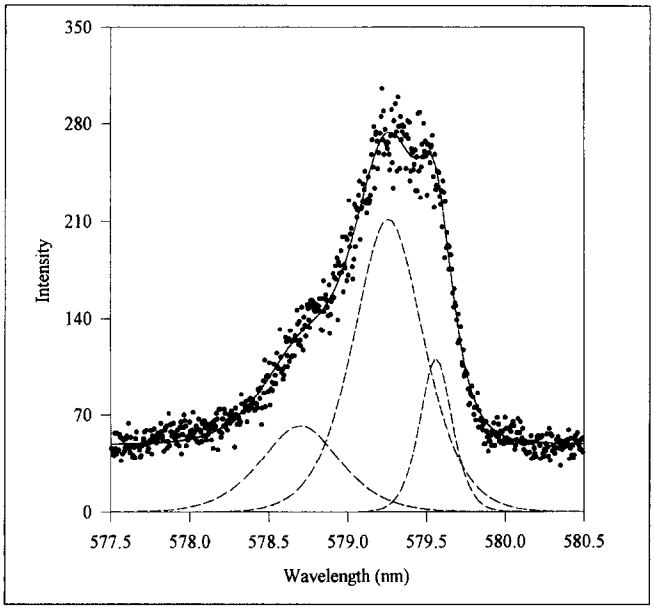
Enzyme	Excitation max. (full width at half max. nm)	Lifetime H_2O/D_2O μs	Number of coordinated H_2O molecules
KF wild-type	579.2 nm (0.45)	205/2100	4.6
	578.8 nm (0.71)	205/2100	4.6
KF single mutant (D424A) exo$^-$	579.2 nm (0.57)	180/1800	5.3
KF double mutant (D355A,E357A)exo$^-$	579.2 nm (0.53)	300/2200	2.7
	579.6 nm (0.23)		
T4 polymerase wild-type	579.3 nm (0.41)	210/2300	4.5
	579.0 nm (0.51)	210/2300	4.5
T4 polymerase (D219A) exo$^-$	578.9 nm (0.57)	206/2000	4.6
	579.3 nm (0.41)	206/2000	4.6

Figure 4



The Eu^{3+} binding curve for the KF D424A single mutant (5 μM). Emission intensity for 579.5 nm excitation is plotted as a function of Eu^{3+} concentration. The resulting curve was fit to a single-binding-site model with a dissociation constant, K_d, of 2.5 μM.

Figure 5



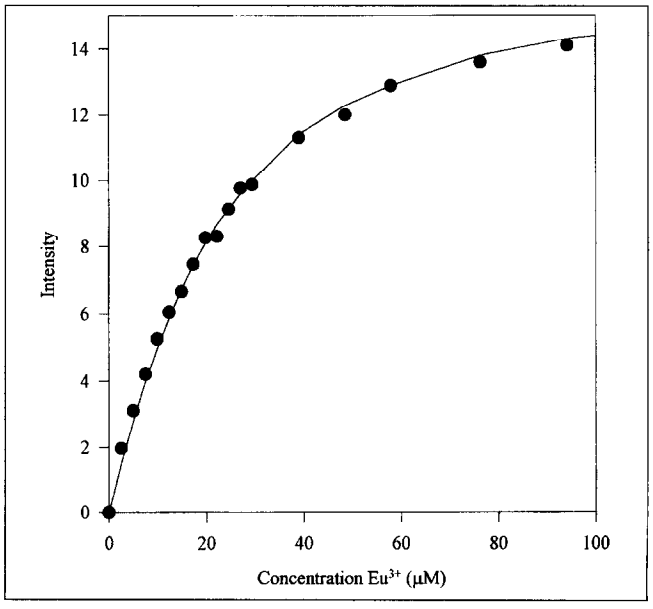
Curve-resolved $^7F_0 \rightarrow {}^5D_0$ excitation spectrum of one equivalent of Eu^{3+} ion bound to KF D355A, E357A double mutant enzyme (10 μM). The spectrum was resolved into three component peaks with maxima at 578.7, 579.2 and 579.6 nm. The peak at 578.7 nm corresponds to the $Eu(H_2O)_9{}^{3+}$ species. The presence of this species below one equivalent of added Eu^{3+} indicates that the other bands represent weakly bound Eu^{3+} ions.

for the KF single mutant D424A (Fig. 4) can be fit satisfactorily to a single-binding-site model with a K_d of 2.5 μM. The presence of only a single band in the $^7F_0 \rightarrow {}^5D_0$ excitation spectrum below one equivalent of added Eu^{3+} ion indicates that the mutation has altered Eu^{3+} ion binding, resulting in the loss of one of the isomeric forms of Eu^{3+} binding observed in wild-type KF.

The crystal structure of the KF exonuclease-deficient double mutant (D355A, E357A) reveals that both metal-binding sites (A and B) have been destroyed. The excitation spectrum of this mutant enzyme, below one equivalent of added Eu^{3+}, is rather broad with three apparent component bands. The spectrum can be resolved into three component bands, centered at 578.7, 579.2 and 579.6 nm, respectively (Fig. 5). The band at 578.7 nm corresponds to the $Eu(H_2O)_9{}^{3+}$ species. The presence of this complex below one equivalent of Eu^{3+} indicates that the other bands represent weakly bound species. Up to the addition of six equivalents of Eu^{3+}, the three bands are all present and can be easily resolved. The band at 579.6 nm is unusually narrow (see Table 1), indicative of a rigid environment. As in the case of wild-type KF, it is possible that the bands at 579.2 and 579.6 nm are due to isomeric forms of the same binding site which are in fast exchange on the time scale of the luminescence. In fact, an exceptionally good fit to a single-site binding model, yielding a dissociation constant of 20 μM, is obtained from luminescence intensity at 579.5 nm (from the unresolved spectra) as a function of Eu^{3+} concentration (Fig. 6). The notion that the 579.2 and 579.6 nm bands correspond to a single site is further supported by the excited state lifetime analysis and Tb^{3+} binding constant measurements (*vide infra*). Perhaps a fluxional process involving an equilibrium between two forms of the same site results in the two observed bands as is the case for wild-type KF. The metal-ion binding abilities of sites A and B have been, at the least, severely compromised, and most probably completely abolished in this mutant. It is likely that the binding observed corresponds to the weak binding species postulated for the wild-type and single-mutant enzymes. This idea is further supported by tyrosine-sensitized Tb^{3+} luminescence studies described in detail below.

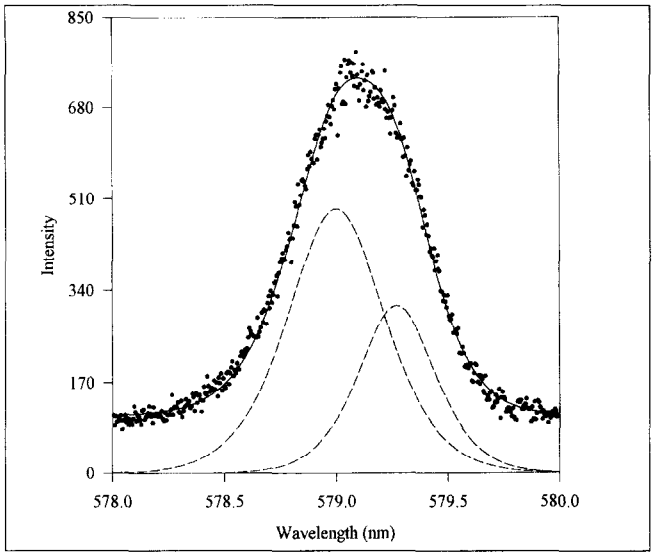
The $^7F_0 \rightarrow {}^5D_0$ excitation spectrum of wild-type T4 polymerase with less than one equivalent of Eu^{3+} consists of a broad band which can be resolved into two constituent peaks centered at 579.0 and 579.3 nm (Fig. 7). The band positions are quite similar to those of the KF model. Beyond one equivalent of Eu^{3+} a third peak arises corresponding to $Eu(H_2O)_9{}^{3+}$ at 578.7 nm. Peak resolution is possible throughout the titration until six equivalents of Eu^{3+} have been added. Beyond this point, metal-ion-induced precipitation begins to occur. The intensities of the resolved peaks grow proportionally as Eu^{3+} is added. Plotting either of these peak intensities against Eu^{3+} ion concentration generates a binding curve that can be fit to a

Figure 6



Intensity of the $^7F_0 \rightarrow {}^5D_0$ excitation band at 579.5 nm as a function of Eu^{3+} concentration for the KF double mutant (D355E, E357A) (5 μM). The theoretical fit is to a single binding site with a K_d of 20 μM.

Figure 7



$^7F_0 \rightarrow {}^5D_0$ excitation spectrum of wild-type T4 DNA polymerase (5 μM) with one equivalent of bound Eu^{3+} ion. Curve resolution reveals two component peaks centered at 579.0 and 579.3 nm. These band positions are quite similar to those found for wild-type KF.

single-binding-site model with a K_d of 0.80 μM (Fig. 8). Therefore, it appears that the two $^7F_0 \rightarrow {}^5D_0$ excitation bands observed below one equivalent of added Eu^{3+} ion are again attributable to isomeric forms of the same binding site. This is further corroborated by the analysis of excited state lifetimes (*vide infra*).

The D219A mutation that renders the T4 DNA polymerase exonuclease deficient (T4 exo$^-$) was proposed to be analogous to the D424A mutation of the KF single mutant [15,16]. Therefore similar results are expected. However, in contrast to the KF single mutant where only a single band is observed, the $^7F_0 \rightarrow {}^5D_0$ excitation spectrum of the T4 exo$^-$ protein consists of two resolvable peaks up to one equivalent of Eu^{3+} ion (Fig. 9). The peaks, centered at 578.9 and 579.3 nm, are close in wavelength to those of the wild-type T4 DNA polymerase although the relative intensities of the bands are reversed. The peak width of the lower wavelength band is somewhat broader than that observed for the wild-type enzyme. Above one equivalent of Eu^{3+} ion a third band gradually appears, which again corresponds to $Eu(H_2O)_9^{3+}$ at 578.7 nm. The three bands can be distinguished throughout the titration up to six equivalents of added Eu^{3+}. As in the case of the T4 wild-type polymerase, the relative intensities of the resolved peaks remain constant throughout the titration indicating that they represent isomeric forms of the same binding site. The binding curve generated by plotting the resolved peak intensity at 579.3 nm against Eu^{3+} ion concentration (data not shown) can be fit to a single-site model with a K_d of 0.17 μM. This mutation appears to have tightened the Eu^{3+}-binding interaction, but has not disrupted the fluxional isomeric nature of the site.

Lifetime analysis

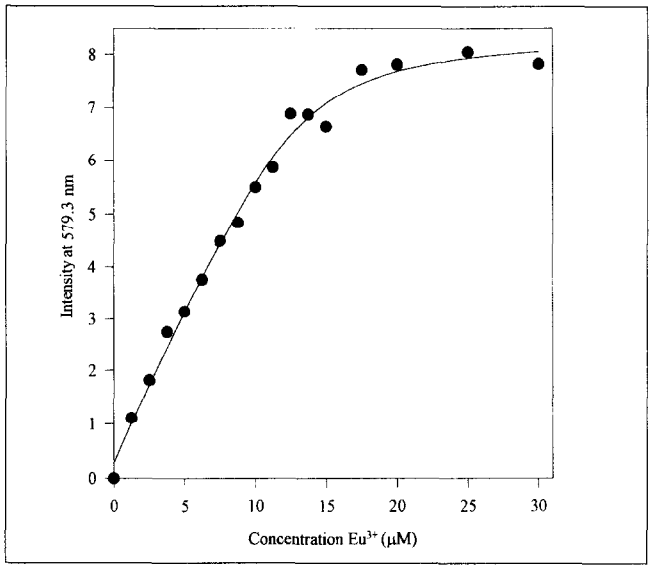
Individual binding sites may also be distinguished on the basis of Eu^{3+} $^5D_0$ excited state lifetime measurements. Once again, since this state is non-degenerate, barring coincidence of lifetimes, a single exponential decay is expected for each unique Eu^{3+} environment. Furthermore, owing to an isotope effect whereby O–H oscillators of Eu^{3+}-coordinated water molecules provide a non-radiative deexcitation pathway whereas their O–D counterparts do not, determination of the number of coordinated water molecules is possible from the equation [28]:

$$q = 1.05(\tau^{-1}_{H_2O} - \tau^{-1}_{D_2O})$$

where q is the number of coordinated water molecules and τ^{-1} is the reciprocal of the excited state lifetime in ms^{-1}. Since Eu^{3+} typically prefers coordination numbers of eight or nine, measurement of the number of coordinated water molecules allows one to infer the number of ligands provided by the binding pocket of the protein.

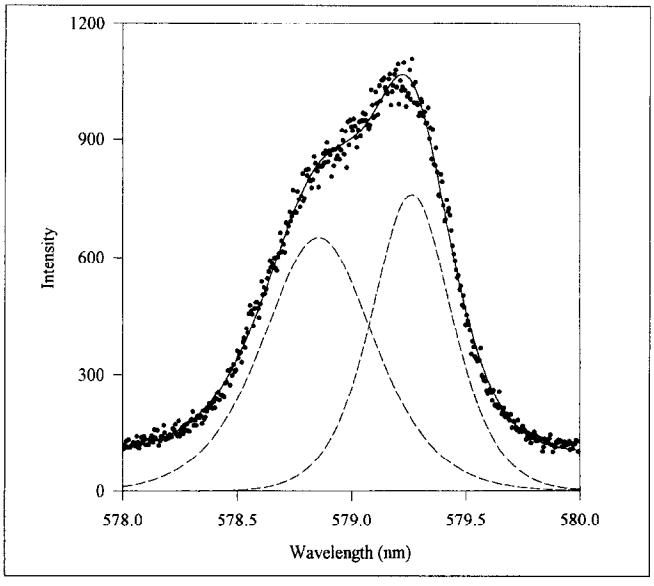
Although two bands are observed in the excitation spectrum of KF wild-type enzyme below one equivalent of Eu^{3+}, examination of the luminescence decay of the excited state at several excitation wavelengths reveals only a single lifetime of 205 μs (see Table 1). Observation of a single lifetime is further evidence that the two observed bands are due to isomeric forms of a single

Figure 8



Binding curve for Eu^{3+} and wild-type T4 DNA polymerase. Plotting the intensity of either resolved band from the excitation spectrum as a function of Eu^{3+} concentration yields a binding curve that fits to a single-binding-site model with a K_d of 0.80 μM.

Figure 9



The $^7F_0 \rightarrow {}^5D_0$ excitation spectrum of the T4 DNA polymerase (D219A) mutant in the presence of one equivalent of added Eu^{3+}. The excitation spectrum of the T4 exo$^-$ mutant is resolved into two constituent bands centered at 578.9 and 579.3 nm using the program Peakfit as described in the methods. The peak positions are similar to that seen for the wild-type T4 polymerase except that the relative intensities of the bands have been reversed. The presence of two bands in the excitation spectrum of the T4 exo$^-$ is in contrast to what is seen for the analogous mutation in KF, where only a single band was observed.

binding site which are in fast exchange on the timescale of the luminescence. Similarly, in D_2O a single 2100 μs lifetime is observed. According to the above equation, Eu^{3+} bound to this site is predicted to coordinate four to five water molecules. Therefore the protein is expected to contribute four to five coordinating atoms per binding pocket. Above two equivalents of Eu^{3+}, the lifetime data are best described by a double exponential decay with one lifetime corresponding to the $Eu(H_2O)_9{}^{3+}$ species (110 μs in H_2O) and the other equivalent to that observed at subsaturating concentrations of Eu^{3+} ion.

For the KF single mutant (D424A), a single lifetime of 180 μs is detected below one equivalent of Eu^{3+}, with a second lifetime growing in at higher concentrations corresponding again to the $Eu(H_2O)_9{}^{3+}$ species. In D_2O a lifetime of 1800 μs is measured for the enzyme-bound Eu^{3+}, resulting in the prediction of five coordinated water molecules and three to four ligands supplied by the protein.

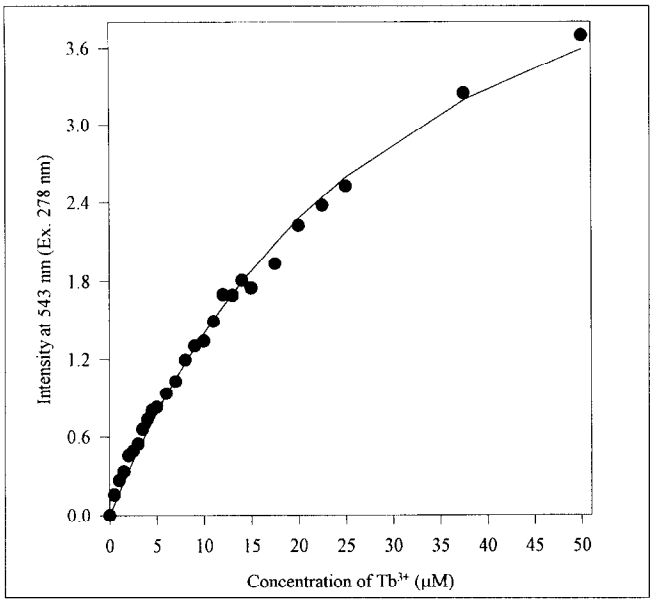
Two lifetimes are derived from the exponential decay monitored at 579.2 nm for the KF double mutant (D355A, E357A). One of these lifetimes is equivalent to that observed for the $Eu(H_2O)_9{}^{3+}$ species, whereas the second (300 μs) is due to enzyme-bound Eu^{3+}. A single lifetime of 300 μs is observed at 579.5 nm where free Eu^{3+} does not contribute. The single lifetime suggests, as does the binding curve, that the two bands seen in the excitation spectrum indeed represent a single binding site with two isomeric forms in fast exchange. Measurement of the protein-bound Eu^{3+} lifetime in D_2O (2200 μs) suggests three bound water molecules, and hence five to six protein-donated liganding atoms.

Analysis of the lifetimes for the wild-type and mutant T4 DNA polymerases is very similar. Below one equivalent of added metal ion, a single lifetime is detected for both proteins; 206 μs for the T4 exo$^-$ and 210 μs for the wild-type. As in the case of wild-type KF, the single lifetime indicates that the two observed bands are due to the isomeric forms in fast exchange. It is particularly noteworthy that the lifetimes for wild-type KF and single mutant as well as the T4 wild-type and T4 exo$^-$ proteins are all quite similar (~200 μs) suggesting that the binding pockets for these enzymes are nearly identical. At concentrations of Eu^{3+} in excess of the protein concentration, the free Eu^{3+} lifetime of 110 μs is again noted, confirming the existence of this species in the spectral data. Calculation of the number of coordinated water molecules (four to five per Eu^{3+} ion) indicates that four to five coordinating atoms are supplied by the protein binding sites.

Tyrosine-sensitized Tb^{3+} luminescence

Another valuable technique afforded by the room-temperature luminescence of lanthanide ions is tyrosine-sensitized terbium(III) emission spectroscopy. This method has been used extensively to probe the metal-binding properties of several proteins [21]. In this experiment, intrinsic protein tyrosine residues absorb light of 278 nm wavelength and sensitize the luminescent emission of Tb^{3+} ions that are bound in close proximity to these residues. Although Tb^{3+} may itself be directly excited by light of this wavelength, the resulting emission is quite feeble and does not contribute significantly to sensitized spectra. Therefore, only Tb^{3+} ions that are bound to the protein are observed, unlike the situation for Eu^{3+} excitation spectroscopy. The absence of any contribution from the metal-aquo species simplifies the analysis of the KF data in the case of the Tb^{3+} experiments. Additionally, the signal from weakly bound Tb^{3+} becomes more apparent and may be examined quantitatively. The emission spectra of the Tb^{3+}-bound protein was monitored from 400 to 600 nm with excitation at 278 nm. Tb^{3+} emission peaks at 485 and 543 nm are readily apparent on the tail of the intrinsic protein fluorescence at shorter wavelengths. Tb^{3+} emission at 543 nm is sufficiently removed from the tyrosine emission that its intensity can be monitored at this position during the course of a titration. A plot of the intensity at 543 nm as a function of Tb^{3+} concentration for the KF double mutant is shown in Figure 10. The data describe a loose

Figure 10



Tb^{3+} binding curve for the KF D335A, E357A double mutant. Emission intensity at 543 nm (excitation at 278 nm) as a function of the concentration of added Tb^{3+} ion to 5 μM KF double mutant enzyme. The solid line represents the theoretical fit to a single-binding-site model, yielding a Tb^{3+} dissociation constant of 26 μM.

binding curve that is best modeled to a single site with a K_d of 26 μM. This value is in close agreement with that obtained from the Eu^{3+} excitation data (K_d = 20 μM).

Titration of the KF single mutant (D424A) with Tb^{3+} results in a binding curve (data not shown) that fits reasonably well to a single-site model with a K_d of 7.7 μM. This value is somewhat greater than the K_d determined for Eu^{3+} (2.5 μM). This is probably the result of the presence of a second weaker site, or precipitation, which contributes to the intensity at higher concentrations of Tb^{3+} ion, causing the K_d value to be artificially high. The Tb^{3+} titration for wild-type KF (Fig. 11) is similar to that of the single mutant, fitting to a single-site model with a K_d of 6.9 μM, once again higher than that measured for Eu^{3+} (1.3 μM). We conclude from these experiments that wild-type KF and single-mutant enzymes bind one Tb^{3+} ion tightly and perhaps a second more weakly, while the double mutant binds a single Tb^{3+} ion with a lower affinity. These conclusions are consistent with the Eu^{3+} luminescence data.

Polymerase and exonuclease activity studies

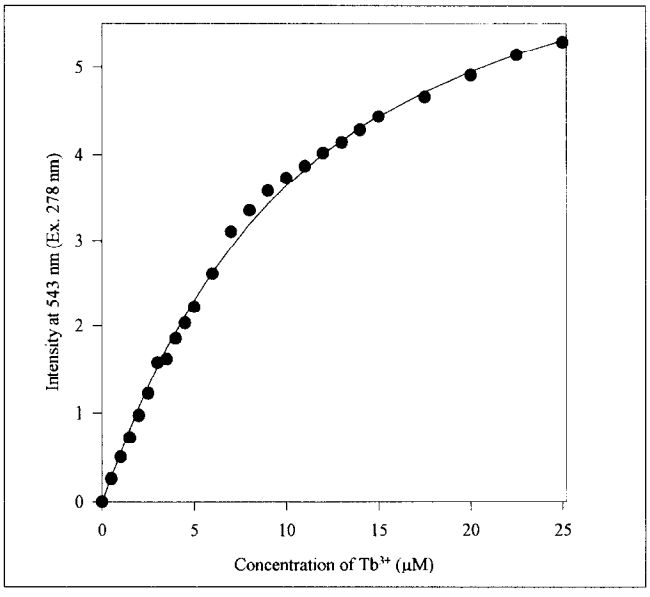
The polymerase and exonuclease activities of both the T4 polymerase and KF were evaluated on a 13/20-mer DNA substrate with Mg^{2+}, Eu^{3+}, or both metal ions as the metal source. Similar results were obtained for the exonuclease activities of T4 pol and KF. Control experiments with Mg^{2+} gave exonucleolytic activity as expected for both enzymes, but when Eu^{3+} was employed as the exogenous metal there was complete inhibition of activity. The results of the Mg^{2+} control and Eu^{3+} spike experiments for T4 polymerase exonuclease activity are shown in Figure 12. The spike reactions were initiated in the presence of 1 mM Mg^{2+} with 10 μM, 1 mM or 20 mM Eu^{3+} added after 40 s. In every case the addition of Eu^{3+} completely terminated activity. Identical results were obtained with KF.

In the presence of Mg^{2+}, both enzymes exhibited typical steady-state reaction profiles for polymerase activity with rate constants similar to that previously reported (0.08 s^{-1} for KF and 3–6 s^{-1} for wild-type T4 DNA polymerase) [29,30]. In the presence of Eu^{3+}, however, no polymerase activity was detected. To further examine this inhibition of polymerase activity by Eu^{3+}, spike experiments were employed in which the reaction was begun with Mg^{2+} and spiked with Eu^{3+} after a set amount of time (40 s for T4 pol or 120 s for KF). The timecourse for both enzymes mirrored the corresponding Mg^{2+} controls until Eu^{3+} addition, after which all polymerase activity was completely abolished, suggesting that Eu^{3+} is indeed a competitive inhibitor of Mg^{2+} binding.

Discussion

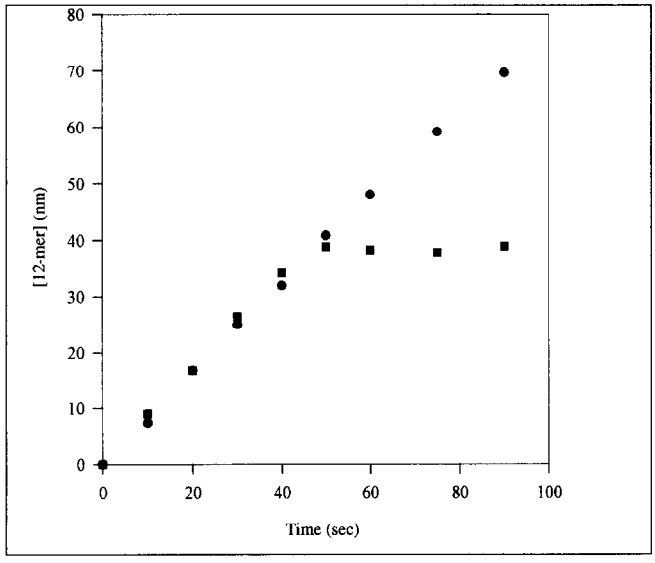
Exonucleolytic cleavage of phosphodiester bonds is catalyzed by polymerase enzymes from a wide variety of sources, but only in the presence of divalent metal ions. A requirement for these cofactors has long been appreciated,

Figure 11



A Tb^{3+} binding curve for wild-type KF. Emission intensity at 543 nm (excitation at 278 nm) of a solution containing 5 μM wild-type KF is plotted as a function of Tb^{3+} ion concentration. The resulting curve is fit to a single-binding-site model with a K_d of 6.9 μM.

Figure 12



The effect of Eu^{3+} ion spike on T4 DNA polymerase exonuclease activity. The T4 pol exonuclease control reaction (●) yields a standard reaction profile. When the reaction is spiked with 20 mM Eu^{3+} (■; 1 mM or 10 μM, data not shown) all activity ceases. Similar results were obtained with wild-type KF.

although the details of their interaction with the protein scaffolding were not available until the determination of the crystal structure of *E. coli* pol I KF. The structural studies [7,8,10,12] resulted in the identification of a two-metal-ion binding pocket located in the exonuclease active site. In the presence of dNMP, the two metal ions, separated by ~4 Å, are bridged by a non-esterified oxygen of the 5' phosphate of dNMP. Metal ion A is further coordinated by the carboxylate moieties of Asp355, Glu357 and Asp501. Metal ion B, observed only when dNMP is present, shares the Asp355 residue with metal ion A, and finishes its octahedral coordination sphere with three water molecules, the carboxylate group of Asp424, and two 5' phosphate oxygens. Alteration of Asp424 (D424A single mutant) or of Asp355 and Glu357 (D355A, E357A double mutant) results in mutant proteins with greatly compromised exonuclease activities. In the D424A mutant, metal binding at site B is abolished, while the D355A and E357A mutations disrupt both sites A and B.

Amino acid sequence alignment demonstrates that these KF residues are conserved in several DNA-dependent DNA polymerases, and their general importance for exonuclease activity is further supported by site-directed mutagenesis studies in ϕ29, T7 and *Saccharomyces cerevisiae* δ polymerases [18]. For bacteriophage T4 DNA polymerase, the analogous residues were identified as Asp112, Glu114 and Asp219. Subsequent mutagenesis of these residues [19,20] resulted in mutant T4 polymerase enzymes that are indeed devoid of exonuclease activity.

Both exonuclease and polymerase activities require the presence of divalent metal ions, and Eu^{3+} is shown to competitively inhibit both of these. Our Eu^{3+} and Tb^{3+} spectroscopic studies on the wild-type KF and on wild-type T4 polymerase reveal, in each case, the presence of a single, tight Ln^{3+} ion binding site and perhaps a weaker site which becomes apparent only at higher concentrations of added metal ion. The fact that our spectroscopy shows that the tight Eu^{3+} or Tb^{3+} site is markedly altered by mutations in the region known to be critical to exonuclease activity proves that it corresponds to the exonuclease active site. One can speculate that the weaker interaction may involve Ln^{3+} ion binding at the site of polymerase activity; this site is known to bind divalent metal ions strongly only in the presence of DNA.

To determine the utility of Eu^{3+} luminescence spectroscopy in the characterization of exonuclease active sites in polymerases and mutants thereof, we first examined the well-characterized KF and its single (D424A) and double (D355A, E357A) mutants. Both Eu^{3+} excitation spectroscopy and tyrosine-sensitized Tb^{3+} emission spectroscopy of wild-type KF polymerase demonstrate the existence of a single tight Ln^{3+}-binding site with the possibility of a second weaker site. The observation of a single tight-binding site for Eu^{3+} is consistent with other metal-binding studies which reveal a single tight-binding site for divalent metal ions in the absence of dTMP [8,9,31]. Because of the complexity introduced to the Eu^{3+} excitation spectrum by dTMP, we were unable to obtain spectral data providing evidence for a second Ln^{3+} site as was seen in the crystal structure and Mn^{2+} solution studies [8,9,31]. The Eu^{3+} excitation spectroscopy reveals two isomeric forms of the tight-binding site. The isomeric forms of this single binding site are likely due to a competition between two imperfectly positioned carboxylates, or a carboxylate and water molecule. The observation of isomeric forms of the Eu^{3+} environment suggests that the tight binding site does not accommodate Eu^{3+} perfectly. It is likely that the tripositive charge and large ionic radius of Eu^{3+} causes the binding pocket to reorganize somewhat from the configuration appropriate for the native metal ions. This would then explain the inhibition of exonucleolytic activity of KF by Eu^{3+} (*vide infra*).

Perhaps the two divalent metal ion sites which were identified in the crystal structure and Mn^{2+} solution studies [31] (in the presence of dNMP) are only able to accommodate a single Eu^{3+} ion. This type of behavior has been observed previously in a study of thermolysin in which a single Ln^{3+} ion was found to bind to a dinuclear divalent metal-ion-binding site [32]. The D424A single mutant of KF is also observed to contain one tight Ln^{3+} ion binding site and possibly one weaker site. The affinity of the single mutant tight site is nearly the same as that of the wild-type, but results from only one isomeric form of this site. This

mutation appears to have forced the Eu^{3+} ion to adopt a single configuration. Eu^{3+} and Tb^{3+} luminescence spectroscopies reveal the loss of tight metal ion binding in the KF double mutant (D355A, E357A), which exhibits a weak binding site with two isomeric forms. Whether this weak site is a remnant of the original two divalent metal ion binding sites or exists somewhere else in the protein is unclear. However, several weak binding sites for Mn^{2+} were also discovered in a previous study of this mutant [31]. The conclusion is, therefore, that the divalent metal ion sites of the double mutant have, at least, been severely compromised.

Examination of the wild-type T4 DNA polymerase by Eu^{3+} excitation spectroscopy reveals one tight Eu^{3+} ion binding site with two isomeric forms, analogous to what was seen for wild-type KF. The weak site noted in the KF study is, however, absent from the wild-type T4 DNA polymerase Eu^{3+} excitation spectrum. As the D424A mutation in KF results in the disruption of the isomeric nature of the tight Ln^{3+} binding site, it was expected that the analogous mutation in T4 polymerase would yield similar results. However, a tight site with two isomeric forms remains in the T4 mutant enzyme with a slightly greater affinity for Ln^{3+} ions. This result suggests that the mutated carboxylate residue (D219A) does not enhance the coordination of Ln^{3+} ions in the wild-type enzyme, but, in fact, detracts from it. We speculate that geometric constraints do not allow the carboxylate (Asp219) to coordinate to Ln^{3+} ions in the binding site, and its presence actually congests the metal-ion-binding pocket.

Evidence supporting this hypothesis exists in the spectroscopic data. For instance, the Eu^{3+} excited state lifetimes are identical for both the wild-type and mutant T4 polymerases, suggesting that the number of liganding atoms supplied by the enzyme does not differ. Moreover, the position of the Eu^{3+} $^7F_0 \rightarrow {}^5D_0$ excitation bands, which is diagnostic of the types of atoms coordinated to the metal ions [33], remains unchanged as a result of the mutation. The only observed difference between the wild-type polymerase and mutant excitation spectra is the relative intensity of the bands corresponding to the two isomeric forms. The band intensities are reversed in the mutant as compared to the wild-type T4 polymerase. As intensity is a function of symmetry about the Eu^{3+} ion, this reversal suggests a difference in orientation of the coordinating atoms around the metal ion between the two isomers. We believe that the mutation has opened up the binding pocket to better accommodate the Ln^{3+} ions resulting in tighter binding.

To provide evidence for exonuclease active-site binding of Ln^{3+} ions in the Eu^{3+} and Tb^{3+} luminescence studies, both the polymerase and exonuclease activities of T4 DNA polymerase and KF were examined in the presence of Mg^{2+}, Eu^{3+} or both metal ions. The exonuclease experiments for both the T4 polymerase and KF discussed herein demonstrate that Eu^{3+} is a competitive inhibitor of Mg^{2+}. As the addition of Eu^{3+} (10 μM) completely abolishes activity in the presence of Mg^{2+} (1 mM), the K_i of Eu^{3+} must be <10 μM, in agreement with our spectroscopically measured K_d (KF K_d = 1.3 μM; wild-type T4 pol K_d = 0.8 μM, D219A T4 pol K_d = 0.17 μM). Our results are consistent with a Mg^{2+} K_d value of 38 μM, and an apparent K_m for this ion of 1.7 mM [31].

For the polymerase activity studies of both enzymes, the Eu^{3+} ion was again shown to be a competitive inhibitor of Mg^{2+}. It therefore appears that Eu^{3+} binds to the polymerase active site of T4 DNA polymerase and KF in the presence of DNA. In the absence of DNA we did not observe Eu^{3+} binding to the polymerase active site of either enzyme. The presence of DNA must therefore serve to stabilize this interaction. This behavior is consistent with the crystallographic studies in which metal-ion binding was observed only in the presence of dNTP or DNA [34,35].

Significance

While it is evident that Ln^{3+} ions do not bind to KF in the same fashion as the native metal ions, Mg^{2+} or Zn^{2+}, this study suggests that Eu^{3+} and Tb^{3+} luminescence can be used to probe changes in the binding sites that result from various mutations. Furthermore, the fact that the Eu^{3+} luminescence excitation spectroscopy for T4 DNA polymerase is so similar to that of KF implies that the latter is a good model for the former, and that metal binding in polymerases as a whole might be quite uniform. Finally, while the single mutation of the T4 DNA polymerase does not disrupt Ln^{3+} binding, the sensitivity of the Eu^{3+} excitation spectroscopy reveals a change in the metal binding site. This probably reflects the disruption of divalent-metal-ion binding, which ultimately abolishes the exonuclease activity of this enzyme. This work establishes Ln^{3+} luminescence as a sensitive spectroscopic screening method for determining similarities and differences in the metal-ion-binding sites of different DNA polymerases. Furthermore, this technique can be used to determine when particular mutations significantly affect the metal ion binding pocket. Future experiments involving competition of dipositive ions, such as Mg^{2+}, Mn^{2+} and Zn^{2+}, with Eu^{3+} or Tb^{3+} should allow the determination of the binding affinities of physiologically relevant metal ions and provide a further characterization of this important class of enzymes.

Materials and methods

Materials

All chemicals were of the highest grade commercially available. The water used was distilled and deionized. D_2O, $EuCl_3{\cdot}(H_2O)_6$ and $TbCl_3{\cdot}(H_2O)_6$ were purchased from the Aldrich Chemical Company. Ultrex NaCl was supplied by the Baker Chemical Company. DNA

oligonucleotides (13-mer and 20-mer) were purchased from Operon Technologies, Incorporated; single-stranded and duplex DNA were purified as previously described [19]. Ultrapure, unlabeled dNTPs were obtained from Pharmacia. [γ-^{32}P]ATP was purchased from New England Nuclear, and duplex DNA (13/20-mer) was 5'-^{32}P end labeled as described previously [19].

Enzyme solutions were buffered with 50 mM HEPES, pH or pD 7.4, and 150 or 250 mM NaCl. All stock buffer and salt solutions were passed through a Chelex-100 (BIO-RAD) column equilibrated with 50 mM HEPES, pH 7.4, to remove exogenous metal ions. Metal stock solutions were standardized by an arsenazo titration [36].

Wild-type and mutant T4 DNA polymerases were purified as described previously [37] except that EDTA was omitted from all steps following DEAE-Sephacel chromatography, and any residual EDTA was removed by desalting in the Amicon concentration apparatus with a YM30 membrane.

The KF plasmids were the kind gift of Dr Cathy Joyce at Yale University. The KF proteins (wild-type, D424A single mutant, and D355A, E357A double mutant) were purified according to the methods of Derbyshire [9] except that EDTA was added to the lysis buffer, and final dialysis was into 50 mM HEPES pH 7.4 (4 x 2 l) that had been chelex treated.

Enzyme concentrations were determined by absorbance measurements at 280 nm and verified by active-site titrations. Enzyme samples were enriched in D_2O by exchanging the H-buffer for D-buffer in Centricon-30 concentrators using standard methods.

Methods

Eu^{3+} luminescence spectroscopy

Eu^{3+} excitation spectra and excited-state lifetimes were recorded with a Continuum YG-581C pulsed (10 Hz) Nd:YAG laser-pumped tunable TDL-50 dye laser as an excitation source. A mixture of Rhodamine 590 and 610 dyes was used to access the $^7F_0 \rightarrow {}^5D_0$ transition of Eu^{3+} (577–581 nm) while monitoring the $^5D_0 \rightarrow {}^7F_2$ emission at 614 nm. The wavelength of the dye laser was calibrated by recording an excitation spectrum of solid $EuCl_3(H_2O)_6$ which has a $^7F_0 \rightarrow {}^5D_0$ transition peak maximum at 579.273 nm. Details of the data acquisition system have been discussed previously [38]. In a typical experiment, 5–10 μM polymerase enzyme was incubated in a cuvette with the titration buffer described above and varying amounts of Eu^{3+} ion. Spectral peak fitting and lifetime analysis was achieved using the program Peak Fit (Jandel Scientific), which employs a non-linear regression analysis based on the Marquardt algorithm. Excitation spectra were fit to individual peaks describable by a Lorentzian-Gaussian product function discussed elsewhere [39]. Binding curves for the wild-type and mutant T4 DNA polymerase enzymes were generated by plotting the dilution-corrected intensities of the resolved peaks in the excitation spectra versus the concentration of Eu^{3+} ion. Binding curves were produced for the KF proteins by exciting samples containing varying amounts of Eu^{3+} at 579.5 nm. At this excitation wavelength, a contribution from free Eu^{3+} ion is avoided. Dilution-corrected intensities were plotted against total Eu^{3+} concentrations. The binding curves were fit using the program EQUIL, which utilizes a nonlinear regression analysis based on the Marquardt algorithm to fit multiple, independent binding events. (EQUIL ver. 1.02 was developed at the Computer Center of the University of Illinois at Chicago by Robert F. Goldstein in 1990.)

Tyrosine-sensitized Tb^{3+} luminescence

Fluorescence emission spectra were recorded on an SLM Aminco Fluorescence spectrometer. Intrinsic enzyme tyrosine residues were excited at 278 nm while the emission spectrum was recorded from 400 to 600 nm. In a typical experiment, 5–10 μM of polymerase enzyme was incubated in a cuvette with the titration buffer described above and varying amounts of Tb^{3+} ion. Binding curves for the KF wild-type and mutant enzymes were created by exciting the samples at 278 nm while monitoring the most intense Tb^{3+} emission band at 543 nm. Dilution-corrected intensities were plotted as a function of Tb^{3+} concentration. Binding curves were fit using the program EQUIL mentioned above which assumes that the metal-binding sites are independent.

Effect of Eu^{3+} on polymerase and exonuclease activities

All KF reactions were carried out in assay buffer containing 50 mM Tris HCl pH 7.4 and 100 μg ml^{-1} acetylated BSA, while T4 polymerase assays were done in a buffer system consisting of 50 mM Tris OAc pH 7.5, 150 mM KOAc, 10 mM β-mercaptoethanol and 100 μg ml^{-1} acetylated BSA. Products were separated on 20 % denaturing polyacrylamide gels, and visualized and quantitated using a Molecular Dynamics Phosphorimager and ImageQuant 3.3 software.

Polymerase assays

Polymerase reactions contained 5 mM $MgCl_2$ or $EuCl_3$, dATP (10 μM for KF assays or 100 μM for T4 polymerase assays) and 1 μM 13/20-mer. Reactions were initiated by the addition of enzyme (15 nM KF or 5 nM T4 polymerase). Time points were obtained by removing 5-μl aliquots at various times and quenching into 5 μl EDTA (0.5 M, pH 8.0). Load buffer (10 μl of 90 % deionized formamide, 1x TBE, 0.25 % bromophenol blue, 0.25 % xylene cyanol) was added to each time-point aliquot and products separated as described above. Further polymerase studies were performed by initiating the reaction (as above) in the presence of $MgCl_2$ (1 mM) and spiking the reaction with $EuCl_3$ (20 mM) at 40 s (T4 pol) or 120 s (KF). The timecourse was followed as described above.

Exonuclease assays

Exonuclease reactions contained 13/20-mer (500 nM for KF assays and 1 μM for T4 pol assays) and 5 mM $MgCl_2$ or $EuCl_3$ in assay buffer. Exonuclease reactions were initiated by the addition of enzyme (1 nM T4 pol or 100 nM KF). The reactions were terminated at variable times by removing 5-μl aliquots and quenching into 5 μl EDTA (0.5 M, pH 8.0). All products were analyzed as described above. Additional exonuclease studies were performed by initiating the exonuclease reaction in the presence of $MgCl_2$ (1 mM) and spiking the reaction at either 40 s (T4 pol) or 120 s (KF) with $EuCl_3$ (10 μM, 1 mM, or 20 mM). Products were analyzed as described above.

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