Quantitation of Metal Ion and DNA Junction Binding to the Holliday Junction Endonuclease Cce1†

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ABSTRACT: Cce1 is a magnesium-dependent Holliday junction endonuclease involved in the resolution of recombining mitochondrial DNA in *Saccharomyces cerevisiae*. Cce1 binds four-way DNA junctions as a dimer, opening the junction into an extended, 4-fold symmetric structure, and resolves junctions by the introduction of paired nicks in opposing strands at the point of strand exchange. In the present study, we have examined the interactions of wild-type Cce1 with a noncleavable four-way DNA junction and metal ions (Mg^{2+} and Mn^{2+}) using isothermal titration calorimetry, EPR, and gel electrophoresis techniques. Mg^{2+} or Mn^{2+} ions bind to Cce1 in the absence of DNA junctions with a stoichiometry of two metal ions per Cce1 monomer. Cce1 binds to four-way junctions with a stoichiometry of two Cce1 dimers per junction molecule in the presence of EDTA, and one dimer of Cce1 per junction in 15 mM magnesium. The presence of 15 mM Mg^{2+} dramatically reduces the affinity of Cce1 for four-way DNA junctions, by about 900-fold. This allows an estimation of $\Delta G°$ for stacking of four-way DNA junction 7 of −4.1 kcal/mol, consistent with the estimate of −3.3 to −4.5 kcal/mol calculated from branch migration and NMR experiments [Overmars and Altona (1997) *J. Mol. Biol. 273*, 519−524; Panyutin et al. (1995) *EMBO J. 14*, 1819−1826]. The striking effect of magnesium ions on the affinity of Cce1 binding to the four-way junction is predicted to be a general one for proteins that unfold the stacked X-structure of the Holliday junction on binding.

Holliday junction resolving enzymes are a class of structure-specific DNA endonucleases whose function is the resolution of four-way DNA junctions arising during homologous recombination. Junction resolution is accomplished by the introduction of paired cleavages in opposing strands, usually within two nucleotides of the junction center, giving rise to recombinant nicked duplex DNA products. The Holliday junction specific endonucleases are all dimeric enzymes, require divalent metal ions (typically magnesium) for catalysis, and exhibit structure-specific DNA binding (reviewed in ref *1*). They differ from one another in many other respects, in particular in their protein fold, sequence dependence for junction cleavage, and manipulation of the junction structure on binding.

Many questions remain to be answered concerning the mechanism of junction recognition, manipulation, and cleavage by junction resolving enzymes. The mitochondrial junction resolving enzyme Cce1 of *S. cerevisiae* has become a model for much of the detailed biochemical studies in the past few years. Cce1 resolves recombining mtDNA genomes, and *Cce1* mutants display highly branched mtDNA, linked by unresolved Holliday junctions (*2*), and have an increased frequency of petite mutants due to impaired mitochondrial function (*3*). A homologue of Cce1, Ydc2, has been identified in the distantly related fission yeast *Schizosaccharomyces pombe* (*4*−*6*), suggesting that this enzyme has a conserved role in mtDNA recombination in the fungi, and possibly in higher eukaryotes.

Junction cleavage by Cce1 is sequence dependent, with the consensus cleavage sequence 5′-ACT/A, and any alterations in the central CT sequence result in at least a 600-fold drop in the cleavage rate (*7*). Junctions are cleaved preferentially at the point of strand exchange, but can cleave at one nucleotide 5′ or 3′ of the junction center, depending on the position of the cleavage consensus sequence, suggesting significant manipulation of the center of the junction by Cce1 during catalysis (*7*). Four-way DNA junctions are bound by Cce1 in the extended, 4-fold symmetric structure seen for free junction in the absence of metal ions (*8*). Sequence variations do not significantly affect the equilibrium binding affinity of Cce1 to four-way junctions, with dissociation constants for a variety of junction sequences all approximately 1 nM in EDTA (*7*).

One significant question that has not been addressed for the junction resolving enzymes concerns the role of divalent metal ions in the binding and resolution of Holliday junctions by these enzymes. The presumed catalytic role of the metal ion(s), in stabilization of the transition state and activation of a catalytic hydroxyl ion, is a requirement of all nucleases, though there are many variations in the identity, number, and specific roles of the cations required by different

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enzymes. For the Holliday junction resolving enzymes, there is the added complication that divalent metal ions such as magnesium exert a large influence over the conformation of the four-way junction structure. Four-way DNA junctions adopt an extended, 4-fold symmetric conformation in the absence of metal ions, but fold on addition of divalent metals by pairwise stacking of helices to adopt an antiparallel, stacked X-structure with 2-fold symmetry [(*9*); for recent reviews, see refs *10* and *11*)]. This structure is likely to be the predominant form of the free Holliday junction in vivo, where magnesium ion levels are typically in the millimolar range.

In this paper, we combine the techniques of EPR[1] and ITC, together with established gel electrophoretic retardation analysis, to address the role of metal ions in junction binding and cleavage by the resolving enzyme Cce1. Our results suggest a pivotal role for divalent metal ions in the binding of Cce1 to the DNA junction, possibly exerted through stabilization of the stacked X-structure.

MATERIALS AND METHODS

Four-Way DNA Junction. The four-way DNA junction used in this study was junction 7, described previously (*12*). Junction 7 was chosen as it has no cleavage sites for Cce1, allowing binding experiments with wild-type Cce1 to be carried out in the presence of magnesium. Junction 7 was assembled from the following four oligonucleotides:

b-strand: 5′ CCCGTCCTAGCAAGCCGCTGCTACC-GGAGG 3′

h-strand: 5′ CCTCCGGTAGCAGCGCGAGCGGTGG-TTGGG 3′

r-strand: 5′ CCCAACCACCGCTCGGCTCTTCTGCA-GTGG 3′

x-strand: 5′ CCACTGCAGAAGAGCGCTTGCTAGG-ACGGG 3′

Recombinant Cce1 Protein. Wild-type Cce1 protein was purified as described previously (*8*).

Gel Electrophoretic Retardation Analysis. A range of concentrations of purified Cce1 protein was incubated with a known concentration of four-way DNA junction 7 radioactively 5′-^{32}P-labeled on each of the four strands in binding buffer (20 mM Tris, pH 8.0, 200 mM NaCl, 0.2 mM DTT, and 0.1 mg/mL bovine serum albumin) containing either 1 mM EDTA or 15 mM $MgCl_2$, in 10 μL total volume. After 15 min at 20 °C, one-sixth volume of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 35% Ficoll type 400) was added, and samples were loaded onto 5% polyacrylamide gels (29:1 acrylamide to bisacrylamide ratio) and electrophoresed in 1×TBE buffer (for binding in the presence of EDTA) or in 1×TB + 15 mM $MgCl_2$ buffer with recirculation (for binding in the presence of magnesium). After electrophoresis, gels were dried and exposed to X-ray film for documentation or to phosphor screens for quantification as described previously (*7*). The values for the dissociation constants reported in the text represent the means of duplicate experiments, with the standard error indicated.

Isothermal Titration Calorimetry. ITC experiments were carried out at 25 °C using a VP-ITC titration calorimeter (MicroCal, Northampton, MA). All solutions were degassed before the titrations. Cce1 and DNA junction samples were extensively dialyzed against 20 mM Tris-HCl buffer, pH 8.0, containing 200 mM NaCl, and $MgCl_2$ solutions were prepared in the same buffer. Titration was carried out using a 370 μL syringe with stirring at 400 rpm. Each titration consisted of a preliminary 1 μL injection followed by 20−30 subsequent 10 μL injections into a cell containing approximately 1.4 mL enzyme or DNA solutions. Calorimetric data were analyzed using MicroCal ORIGIN software. All measurements of binding parameters presented are the means of duplicate experiments.

Electron Paramagnetic Resonance. EPR spectra were recorded on a Bruker 200D spectrometer (Bruker UK Ltd., Coventry, U.K.) fitted with an Oxford Instruments ER900 liquid helium cooled flow cryostat (Oxford Instrument Company Ltd., Osney Mead, Oxford, U.K.). Instrument settings are given in the figure legend. All data points are the means of duplicate or triplicate measurements, with standard errors less than 5%.

RESULTS

EPR Measurements of Mn^{2+}−Cce1 Binding. EPR was used to examine the interaction of Cce1 with divalent metal ions. As Mg^{2+} ions are difficult to detect spectroscopically, the chemically similar Mn^{2+} is commonly used as a substitute. Mn^{2+} contains five unpaired electrons, giving a total electron spin $S = 5/2$ and a readily detected EPR spectrum. The rates of Cce1-dependent catalysis of four-way DNA junction resolution with Mg^{2+} and Mn^{2+} are broadly similar (White, M. F., and Lilley, D. M. J., unpublished observation). For the EPR measurements, purified Cce1 was dialyzed against 20 mM MOPS buffer, pH 7.0, containing 200 mM NaCl. The dialyzed enzyme had no EPR-detectable signal, indicating that it does not contain any integral manganese. A 100 μM $MnCl_2$ solution, prepared from a 20 mM stock in the same buffer, gave a typical Mn^{2+} spectrum with a sharp six-line pattern (arising from nuclear hyperfine interactions) with broad shoulders either side centered about $g \sim 2.0$ (Figure 1A).

The spectrum from a 100 μM $MnCl_2$ solution containing 120 μM Cce1 was significantly different. The amplitude of the six-line hyperfine pattern increased, and the underlining broad features became less broad (Figure 1A). The simplest interpretation of these changes is that Mn^{2+} has bound to the Cce1. By titrating Cce1 into a solution of 100 μM Mn^{2+}, we observed a progressive conversion between two forms, with increasing amplitude of the sharp hyperfine signal (Figure 1B). Stoichiometric binding was observed, allowing calculation of an end-point of the titration. This was achieved at 53 μM Cce1, indicating that approximately 1.9 mol of Mn^{2+} bound/mol of Cce1 monomer.

ITC Measurements of Mg−Cce1 Binding. The intrinsic limits in the sensitivity of the EPR technique precluded the estimation of affinity constants for metal ion binding to Cce1. ITC is a versatile method for detection and quantitation of ligand binding in solution. From heats of mixing of small

[1] Abbreviations: ITC, isothermal titration calorimetry; EPR, electron paramagnetic resonance.

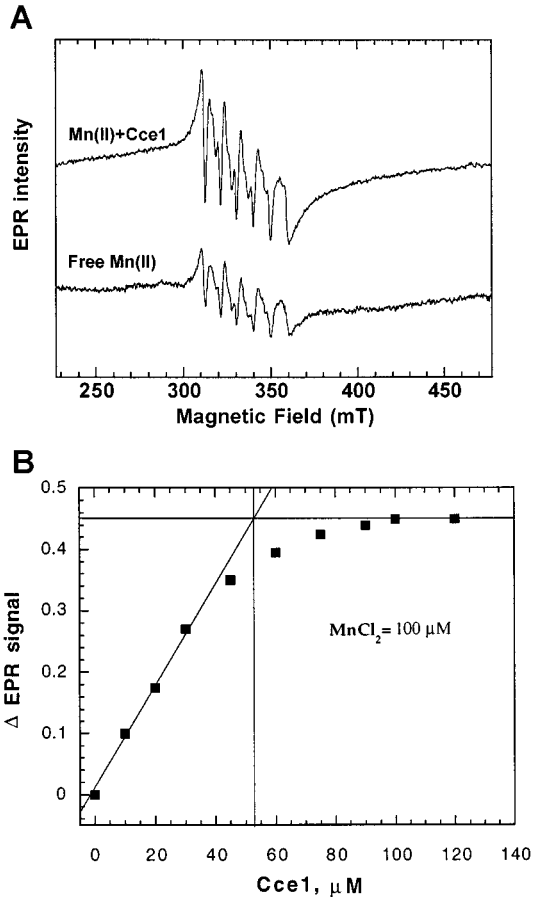

FIGURE 1: Interaction of Mn^{2+} ions with Cce1 monitored by EPR spectroscopy. EPR spectra were recorded as an average of 5 scans at a temperature of 30 K; with 2 mW microwave power, 9.417 GHz microwave frequency, 0.52 mT modulation amplitude, and 1.0e6 receiver gain. (A) X-band EPR spectrum of 100 μM $MnCl_2$ in 20 mM MOPS, 200 mM NaCl, pH 7.0, and that after addition of 120 μM Cce1. (B) Cce1 was titrated into a 100 μM solution of $MnCl_2$, and the resultant increase in the sharp hyperfine EPR signal amplitude was monitored, and analyzed by least-squares fitting. Stoichiometric binding was observed. The intercept of the initial slope and an estimated infinite limit is indicated. This shows that approximately 53 μM Cce1 is required to bind all of the 100 μM Mn^{2+}, with a Mn^{2+}:Cce1 stoichiometry of 2:1.

aliquots of Mg^{2+} ions with Cce1, we obtained thermal titration curves which after correction for appropriate dilution controls were analyzed by standard methods to give the apparent dissociation constant (K_D), stoichiomery (n), and enthalpy ($\Delta H°$) for the binding process. By using both protein and Mg ions in the micromolar concentration range, we were able to measure equilibrium dissociation constants in the micromolar range. The titration of Cce1 with Mg^{2+} ions was strongly exothermic (Figure 2), $\Delta H° = -4.5 \pm 0.4$ kcal·mol^{-1}, and the data fit is consistent with simple stoichiometric binding with $n = 2.1$ Mg^{2+} ions per monomer of Cce1, in strong agreement with the estimate of 1.9 Mn^{2+} ions per Cce1 monomer obtained from EPR. The apparent dissociation constant (at 298 K) was estimated as 0.9 μM.

Gel Electrophoretic Retardation Analysis of Cce1−Junction Interactions. Gel electrophoretic retardation analysis has been used extensively to follow the interaction of resolving enzymes with DNA junctions. For Cce1, a wide range of four-way junctions of differing sequence were found to bind with dissociation constants in the range 0.6−6 nM in EDTA, without any obvious correlation of binding affinity with the

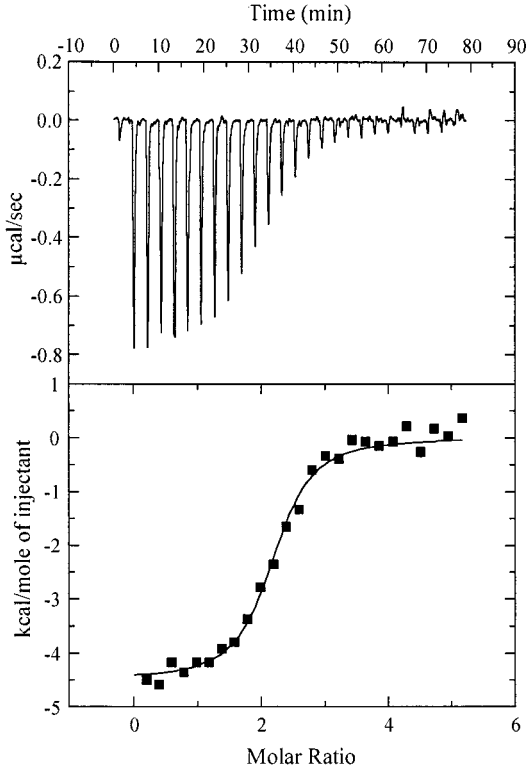

FIGURE 2: Isothermal titration calorimetry of Mg binding to Cce1. Mg(II) was titrated into a 8 μM solution of Cce1, and the exothermic reaction was monitored by ITC. Upper panel: Raw data for sequential 10 μL injections of 500 μM $MgCl_2$ into a solution containing 8 μM Cce1 in binding buffer (50 mM Tris, pH 8.0, 200 mM NaCl) at 25 °C. Lower panel: Integrated heat data with theoretical fit to a stoichiometric binding model. A stoichiometry of 2.1 ± 0.2 Mg ions per monomer of Cce1 was observed, in agreement with the data from EPR. The apparent dissociation constant (at 298 K) was calculated as 0.85 ± 0.2 μM, and the enthalpy change $\Delta H° = -4.5 \pm 0.4$ kcal·mol^{-1}.

presence or absence of strong cleavage sites for the enzyme (*7*). We chose to study four-way DNA junction 7 (J7) (*12*), which has no sequences suitable for cleavage by Cce1 on any of the four arms. In EDTA, Cce1 formed two distinct complexes with the junction (Figure 3A), as observed previously, with complex I corresponding to one dimer of Cce1 binding per junction and complex II corresponding to two dimers of Cce1 binding per junction (*8*). Quantification yielded a value for the apparent dissociation constant (at 293 K) of 1 ± 0.1 nM for complex I (Figure 3C), in good agreement with the range of affinities reported previously (*7, 8*).

The experiment was next repeated in the presence of 15 mM magnesium, both in the binding buffer and recirculated in the gel during electrophoresis. In this case, Cce1−junction complexes appeared less stable, with a broad smear below the retarded complex due to in-gel dissociation (Figure 3B). Only a single complex was observed, suggesting that complex II may not be stable in the presence of high concentrations of magnesium ions. The apparent dissociation constant was estimated by quantifying the amount of free junction, yielding a K_D value (at 293 K) of 520 ± 40 nM for the complex in 15 mM Mg(II) (Figure 3C). Magnesium ions therefore appear to have the effect of severely reducing the affinity of Cce1 for the four-way junction.

ITC Analysis of Cce1−J7 Interaction. Binding of Cce1 to four-way junction J7 in solution was investigated by

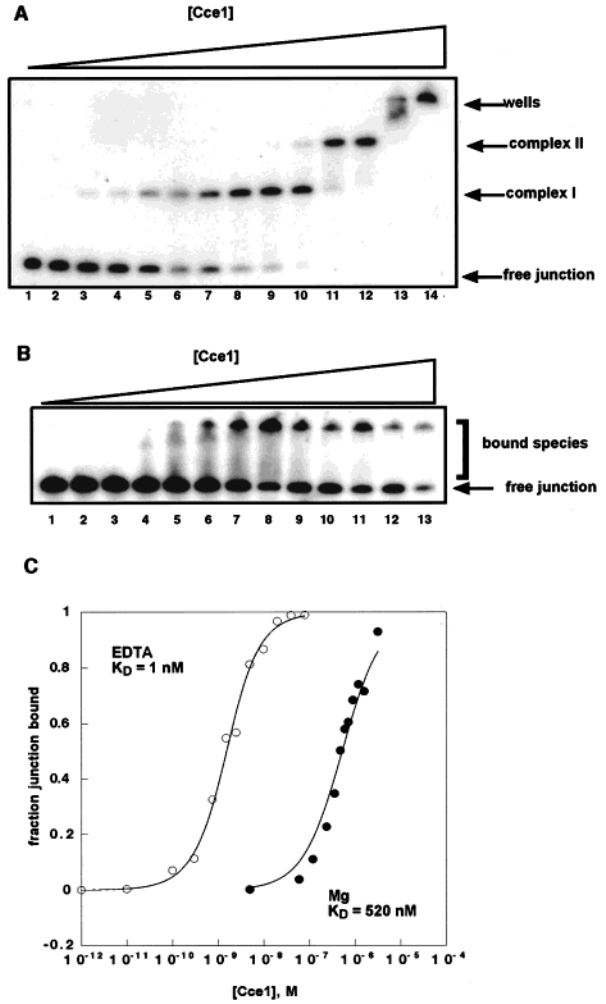

FIGURE 3: Gel electrophoretic analysis of Cce1−junction binding affinity. (A and B) Electrophoretic analysis of binding. Radioactively 5′-^{32}P-labeled junction 7 (1 nM) was incubated with increasing concentrations of Cce1 at 20 °C in binding buffer in the presence of (A) 1 mM EDTA or (B) 15 mM $MgCl_2$. Free junction and DNA−junction complexes were separated by electrophoresis in 5% acrylamide gels [with 15 mM $MgCl_2$ recirculated in the case of (B)] and visualized by autoradiography. Two complexes were observed in EDTA, as noted previously, but only one complex was apparent in the presence of magnesium. Cce1 concentrations used in (A) were as follows: lane 1, 0.001 nM; lane 2, 0.01 nM; lane 3, 0.1 nM; lane 4, 0.3 nM; lane 5, 0.75 nM; lane 6, 1.5 nM; lane 7, 2.5 nM; lane 8, 5 nM; lane 9, 10 nM; lane 10, 20 nM; lane 11, 40 nM; lane 12, 80 nM; lane 13, 200 nM; lane 14, 500 nM. Cce1 concentrations used in (B) were as follows: lane 1, 5 nM; lane 2, 20 nM; lane 3, 60 nM; lane 4, 120 nM; lane 5, 240 nM; lane 6, 360 nM; lane 7, 480 nM; lane 8, 600 nM; lane 9, 720 nM; lane 10, 900 nM; lane 11, 1.2 μM; lane 12, 1.6 μM; lane 13, 3.2 μM. (C) Binding isotherms for the interaction of Cce1 with junction in the presence and absence of magnesium. The fraction of DNA junction bound to protein was calculated for each concentration of Cce1 by phosphorimaging, and plotted against the logarithm of the protein molarity (with Cce1 assumed to be dimeric). The data were fitted to a model for the binding process (see Materials and Methods), from which the binding affinities were calculated. Symbols: (○) binding in EDTA; (●) binding in the presence of 15 mM $MgCl_2$.

titrating the enzyme into a sample of the junction in binding buffer, and monitoring heat changes by ITC. The binding was characterized by a strong endothermic effect (heat uptake). In EDTA, an initial very tight binding interaction was observed with a stoichiometry of one Cce1 dimer per junction (Figure 4A), consistent with the formation of complex I observed by gel electrophoresis. This was followed by a second binding event of lower affinity, yielding a final stoichiometry of two dimers per junction, consistent with the formation of complex II.

When the experiment was repeated in the presence of 15 mM magnesium, a single binding curve was observed, with a stoichiometry of one Cce1 dimer per junction molecule, and an apparent dissociation constant of 920 ± 90 nM (at 298 K) (Figure 4B). The dramatic effect of magnesium ions on Cce1 binding to the DNA junction observed by gel electrophoresis was thus confirmed by ITC. As an in-solution method, ITC has clear advantages over gel-based methods for the calculation of dissociation constants in the micromolar range.

DISCUSSION

Metal Ion Binding to Cce1. Much effort has gone into elucidating the role of metal ions in phosphodiester bond cleavage by nucleases. While it is clear that divalent metals play a key role in DNA hydrolysis, the detailed function and number of metal ions involved in catalysis for many nucleases remain obscure. There are examples of one, two, and three metal ion mechanisms in the literature, but the most common class are the two metal ion nucleases, typified by the exonuclease domain of *E. coli* DNA polymerase 1 (*13*). In these enzymes, one cation is thought to provide a strong electrostatic stabilization to the OH^- nucleophile, and the second cation is involved in stabilizing the negative charge that comes to reside on the phosphate center. In nucleases with a single catalytic ion at the active site, such as *Serratia* nuclease, the divalent cation is thought to play a dual role with the ion being able to both reduce the pK_a of bound water and also to move the OH^- nucleophile toward the phosphate center (*14*).

We have demonstrated by both EPR and ITC experiments that Cce1 binds two magnesium or manganese ions in the absence of DNA. In addition to determining the stoichiometry of metal binding by Cce1, the two techniques each provide further unique information. EPR has the potential to distinguish between integrally bound and solvent-exposed metal ions, as the former can give rise to a distinct broadening of the sharp component of the hyperfine EPR signal (*15*). No broadening was detected in this case, which is consistent with both manganese ions being exposed to the solvent. The higher sensitivity of ITC has allowed an estimation of the affinity of magnesium ion binding to Cce1, indicating that both magnesium ions are bound with very similar affinities (K_D approximately 1 μM at 298 K). Taken together, both divalent metals bound to Cce1 seem to be exposed to the solvent, consistent with a role in catalysis. Indeed in many nucleases, metals are located at the surface of the protein, coordinated by only two protein ligands (*16*−*18*). While we have clear evidence for the binding of two divalent metal ions by Cce1, we cannot rule out the possibility that one or more further ions are present in the complex of Cce1 bound to the Holliday junction. In principle, this could be measured by ITC, but in practice, this is complicated by the destabilizing effect of the divalent metal ions upon the complex. Many nucleases that use three metal ions for phosphodiester bond cleavage comprise the trinuclear Zn^{2+} cluster rather than magnesium or manganese ions (*19*).

Cce1 is the first Holliday junction resolving enzyme for which divalent metal binding has been investigated by EPR

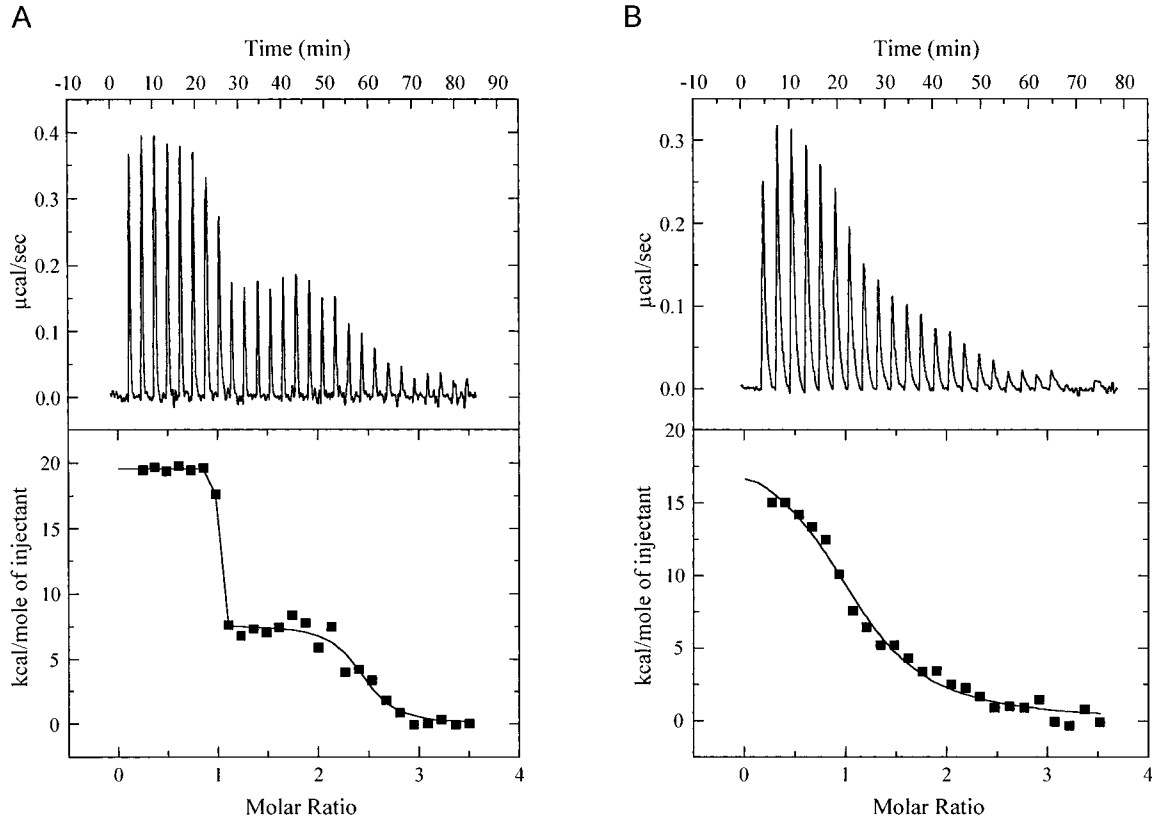

FIGURE 4: Isothermal calorimetry of Cce1 binding to junction in EDTA and 15 mM Mg. **(**A) Titration of Cce1 into a solution containing 3.5 μM junction in the presence of EDTA. Upper panel: Raw data for sequential 10 μL injections of Cce1 into a solution containing junction 7 in binding buffer. Lower panel: Integrated heat data with theoretical fit to a stoichiometric binding model. Two binding steps were observed, consistent with the formation of high-affinity complex I (at one Cce1 dimer per junction) and lower affinity complex II (two dimers per junction), in agreement with the gel electrophoresis data. For complex I, $n = 0.96 \pm 0.1$, $\Delta H^\circ = 19.6 \pm 1.2$ kcal·mol^{-1}; complex II, $n = 2.2 \pm 0.2$, $\Delta H^\circ = 7.6 \pm 0.3$ kcal·mol^{-1}. Values for dissociation constants could not be determined accurately, due to the high affinity of binding of both complexes. (B) Titration of Cce1 into a solution containing 5.5 μM junction in the presence of 15 mM Mg(II). Upper panel: Raw data for sequential 10 μL injections of Cce1 into a solution containing junction 7 in binding buffer containing 15 mM $MgCl_2$. Lower panel: Integrated heat data with theoretical fit to a stoichiometric binding model. A single binding event was observed, with $\Delta H^\circ = 19 \pm 1.1$ kcal·mol^{-1}, stoichiometry $n = 1.1 \pm 0.15$ Cce1 dimer per junction molecule, and an apparent dissociation constant K_D (298 K) = 920 ± 90 nM.

and ITC. The only other evidence for metal ion binding by Holliday junction resolving enzymes comes from crystallographic studies of RuvC and T4 endonuclease VII. RuvC crystals soaked in 5 mM $MnCl_2$ solution suggested the presence of only one manganese ion ligated to the carboxylate moieties of Asp7 and Asp141 (*17*), and biochemical studies suggested that these two residues, together with Glu66 and Asp138 in the same region of the protein, are catalytically important (*20*). The first three of these acidic residues are conserved in *E. coli* RnaseH1, which has the same fold as RuvC (*21*). In this case also, crystal-soaking with magnesium ions led to the identification of a single metal binding site, in the vicinity of the conserved acidic residues (*21*). However, another member of the RnaseH1 superfamily, the retroviral integrase, has two metal binding sites in the same vicinity (*22*, *23*).

In the case of bacteriophage T4 endonuclease VII, the crystal structure revealed a single calcium ion (*18*), directly liganded to Asp40 and Asn62 and close to Glu65, all of which are essential for activity (*18*, *24*, *25*). This is likely to indicate the active site of the enzyme, where the metal binding pocket would normally be occupied by a magnesium ion, but no direct evidence for magnesium occupancy at this or any other position has been obtained to date. The question of whether T4 endonuclease VII and RuvC (and indeed other members of the RnaseH1 superfamily) have a one- or two-metal catalytic mechanism is therefore an open one at present. Application of the ITC and EPR techniques described here to metal ion binding by RuvC and T4 endonuclease VII could provide valuable new information in this regard. Likewise, for Cce1, analysis of metal ion binding by mutants with specific alterations in conserved acidic residues could help define the residues that form the metal binding pocket.

Role of Mg^{2+} in the Cce1−Junction Interaction. The vast majority of binding studies of junction resolving enzymes with branched DNA ligands have been carried out by gel electrophoretic retardation analysis in the presence of EDTA (i.e., the absence of divalent metal ions). The most notable difference in vivo is that free magnesium concentrations are in the millimolar range, and this has two consequences. First, we have shown by ITC that Cce1 binds two magnesium ions per monomer, with an apparent dissociation constant of approximately 1 μM. Thus, under in vivo conditions, Cce1 is likely to exist as a metalloenzyme, with consequent changes to the surface charge, particularly around the active site, compared to the apoenzyme. Second, as discussed earlier, four-way DNA junctions themselves undergo a significant structural change in the presence of divalent metal ions, folding into the 2-fold symmetric, antiparallel stacked

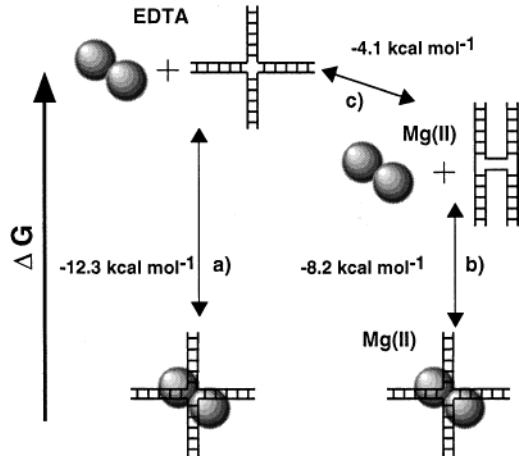

FIGURE 5: Schematic representation of the free energy diagram of Cce1−junction interaction. $\Delta G°$ values for equilibria (a) and (b) have been determined experimentally by ITC and gel electrophoretic retardation analysis in this study. The value of $\Delta G°$ for equilibrium (c) is inferred if the free energy difference arising from metal ion binding in the Cce1−junction complex is assumed to be small. With this assumption, our estimate for $\Delta G°$ of equilibrium (c) is −4.1 kcal·mol^{-1}, which agrees well with independent observations by Overmars and Altona (*30*).

X-structure. In complex with Cce1, however, the DNA junction adopts an open, 4-fold symmetric global structure similar to that of the free junction in EDTA, regardless of the presence or absence of divalent metal ions (*8*). This is a characteristic shared by RuvA, which may help to catalyze branch migration by holding the junction in the open, EDTA form (*26*).

For this study, we used a magnesium ion concentration of 15 mM, with a sodium ion concentration of 200 mM. These conditions were chosen principally because Cce1 has a sharp optimum activity at 15 mM Mg^{2+} under single-turnover conditions [(*27*); Schofield and Lilley, unpublished]. The stability of the stacked X-structure of the four-way junction is also dependent on the concentration of divalent ions in solution, and has been assessed by a variety of methods. Original experiments using gel electrophoretic analysis and protection from osmium tetroxide judged stacking to be essentially complete at 80 μM $MgCl_2$ (*28*). Subsequently, the stability of the stacked X-structure has been assessed by measuring rates of branch migration of Holliday junctions under different ionic conditions, and these studies confirmed that the major effect of magnesium ions occurred at submillimolar levels, though further effects were seen above 10 mM Mg^{2+} (*29*). Most recently, an NMR study has revealed changes in the stability of a stacked X junction up to 12 mM $MgCl_2$ (*30*). Our experiments were therefore carried out under ionic conditions that most favored the stacked X over the open structure of the DNA junction.

We have examined the effect of magnesium ions on the interaction of Cce1 with the four-way DNA junction by two independent techniques: gel electrophoresis and isothermal titration calorimetry. The two methods are in agreement that high concentrations of magnesium ions abolish the formation of complex II, and dramatically reduce the affinity of Cce1 (complex I) for the four-way DNA junctions (Figures 3 and 4). This is a striking effect with potential implications for all proteins that bind four-way DNA junctions: Can we rationalize it? Figure 5 shows a thermodynamic diagram comprising three equilibria: (a) Cce1 binding to the four-way junction in the presence of EDTA; (b) binding in the presence of 15 mM Mg^{2+}, and (c) the equilibrium between the stacked and open forms of the four-way junction in 15 mM Mg^{2+} (*8*). We have measured equilibrium (b) by both gel electrophoresis and ITC, and the latter yields a value for $\Delta G°$ of −8.2 kcal·mol^{-1} (using the relationship $\Delta G° = -RT \ln K_A$). Similarly, from the gel electrophoretic retardation analysis in EDTA, we obtain a value of $\Delta G°$ for equilibrium (a) of −12.3 kcal·mol^{-1}. Though we have not measured the thermodynamics of metal ion binding to the Cce1−junction complex, we do know that the global structure of the bound junction is similar both in the presence and in the absence of magnesium (*8*), and thus predict that the free energy change associated with this equilibrium is relatively small. This assumption allows us to make an estimate of $\Delta G°$ for junction stacking in 15 mM magnesium of approximately −4 kcal·mol^{-1}. This value has been estimated independently using data from step times of branch migration and NMR experiments as approximately −3.3 to −4.5 kcal·mol^{-1} in 15 mM Mg^{2+} (*29*, *30*), in good agreement with the data presented here. As virtually all proteins that bind to the Holliday junction manipulate the stacked X-structure to some extent (reviewed in ref *1*), we predict that the strong effect of magnesium concentration on the affinity of junction binding observed for Cce1 will be a general phenomenon.

There are two possibilities for the recognition of Holliday junctions by Cce1 in the presence of high magnesium ion concentrations. One is that the enzyme binds the stacked X-form of the junction that predominates under these conditions, unfolding it to the open structure with a concomitant energetic penalty of about 4 kcal·mol^{-1}. The other is that the enzyme binds the small amount of the junction that is present in the open form (a few molecules in every thousand under these conditions), gradually displacing the equilibrium between the stacked and open forms until all the junction is bound. Measurements of thermodynamics cannot distinguish between the two models, but future experiments will address this question by utilizing stopped-flow kinetics to examine rates of Cce1−junction binding.

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