Retroviral integrases (INs) contain two known metal binding domains. The N-terminal domain includes a zinc finger motif and has been shown to bind Zn\(^{2+}\), whereas the central catalytic core domain includes a triad of acidic amino acids that bind Mn\(^{2+}\) or Mg\(^{2+}\), the metal cofactors required for enzymatic activity. The integration reaction occurs in two distinct steps; the first is a specific endonucleolytic cleavage step called “processing,” and the second is a polynucleotide transfer or “joining” step. Our previous results showed that the metal preference for in vitro activity of avian sarcoma virus IN is Mn\(^{2+}\) > Mg\(^{2+}\) and that a single cation of either metal is coordinated by two of the three critical active site residues (Asp-64 and Asp-121) in crystals of the isolated catalytic domain. Here, we report that Cu\(^{2+}\), Zn\(^{2+}\), and Cd\(^{2+}\) can also bind in the active site of the catalytic domain. Furthermore, two zinc and cadmium cations are highly conserved, and both include metal-binding sites. The central, catalytic domain (amino acids 51–50) contains a zinc finger-like motif, HHCC. Binding of Zn\(^{2+}\) at this site stabilizes the structure of HIV-1 IN and enhances multimerization and activity (5–7). The central, catalytic domain (amino acids 50–200) is characterized by a triad of invariant acidic amino acids (Asp-64, Asp-121, and Glu-157 in ASV IN), the last two separated by 35 amino acids comprising the D,D(35)E motif. These three acidic residues are essential for both processing and joining activity and have been proposed to bind the divalent metal cofactors during catalysis (8).

Retroviral integrases contain approximately 300 amino acids and are composed of three domains (4). The first two domains are highly conserved, and both include metal-binding sites. The N-terminal domain (amino acids 1–50) contains a zinc finger-like motif, HHCC. Binding of Zn\(^{2+}\) at this site stabilizes the structure of HIV-1 IN and enhances multimerization and activity (5–7). The central, catalytic domain (amino acids 50–200) is characterized by a triad of invariant acidic amino acids (Asp-64, Asp-121, and Glu-157 in ASV IN), the last two separated by 35 amino acids comprising the D,D(35)E motif. These three acidic residues are essential for both processing and joining activity and have been proposed to bind the divalent metal cofactors during catalysis (8).

Solution of the crystal structures of the isolated catalytic core domains of HIV-1 (9, 10) and ASV IN (11, 12) have revealed that these retroviral enzymes belong to a superfamily of nucleases and polynucleotidyltransferases, all of which contain a cluster of conserved acidic amino acids at their presumed active sites. HIV-1 reverse transcriptase ribonuclease H (RNase H) domain, another member of this superfamily, was shown to bind two divalent cations in this site (13), prompting the suggestion (14) that all members of this family may use a two-metal catalytic mechanism like that deduced for the 3′-5′ exonuclease of Escherichia coli DNA polymerase I (15, 16). We have shown that side chains of two of the acidic triad residues in the D,D(35)E motif in ASV IN also form a metal binding pocket. A single ion, Mn\(^{2+}\) or Mg\(^{2+}\), is complexed to Asp-64 and Asp-121 when crystals of the isolated catalytic domain of ASV IN are soaked in metal-containing solutions (12). We hypothesized that a second metal might be bound between the Asp-64 and Glu-157 in the full-length protein or in the presence of substrate.
Here we show that additional divalent cations can also bind in the active site of crystals of the isolated catalytic core domain of ASV IN. Moreover, in the case of Zn$^{2+}$ and Cd$^{2+}$, two ions are complexed by side chains from all three of the acidic amino acids of the D,D(35)E motif. To investigate the significance of these observations, we measured enzymatic properties in the presence of these metals with the isolated catalytic core, full-length ASV IN protein and an N-terminal deletion derivative previously (11), and the crystals used here were grown by the same protocol confidence in the structural results described here. The coordinates of the complexes with Ca$^{2+}$, Zn$^{2+}$, and Cd$^{2+}$ were deposited previously (11), and the complexes were deposited previously (19). Assays were carried out exactly as described (11). Briefly, for nicking and processing, the substrate was an 18-base pair duplex corresponding to the U3 end of ASV DNA. The 5’-end of the plus strand was labeled with $^{32}$P. The reaction mix contained 50 mM Tris, pH 8.4, 2 mM $\beta$-mercaptoethanol, 200 $\mu$g/ml bovine serum albumin, 50 mM NaCl, and 4% glycerol. Reactions were carried out in substrate excess. For processing and joining, the concentration of IN was 2 $\mu$M and the DNA substrate was 15 $\mu$M. For disintegration reactions, the concentration of IN was 1 $\mu$M, and the DNA substrate was 8 $\mu$M. The disintegration substrate was prepared by annealing four separate DNA strands of 19, 16, 10, and 9 nucleotides with the 10-nucleotide strand end-labeled with $^{32}$P. Reactions were carried out at the indicated times and metal concentrations. Reaction products were fractionated on 20% acrylamide/urea sequencing gels, and bands were quantified using a Fuji phosphorimager. Results are expressed as $\mu$mol of product/10$^{-6}$ of IN monomer or IN fragment.

**RESULTS**

Mn$^{2+}$, Mg$^{2+}$, and Ca$^{2+}$ Bind to a Single Site in ASV Catalytic Domain—In our earlier studies, we soaked crystals of the isolated ASV IN catalytic domain with the two known divalent cation cofactors, Mn$^{2+}$ and Mg$^{2+}$ (12). We observed coordination of both cations between Asp-64 and Asp-121 of the catalytic triad but no participation of its third member, Glu-157. To further investigate the possible participation of Glu-157 in metal binding, we carried out a systematic analysis encompassing a variety of divalent cations and a wider range of concentrations.

Crystals of the catalytic domain of ASV IN (52–207) were soaked in 2–500 mM solutions of five divalent cations: Mg$^{2+}$, Mn$^{2+}$, Zn$^{2+}$, Ca$^{2+}$, and Cd$^{2+}$. Structures of the metal-soaked crystals were solved and refined at moderately high resolution (Table I). As reported earlier, the electron density map corresponding to the structure of the Mg$^{2+}$ complex obtained at the highest concentration of its salt is exceptionally clear and shows a cation bound between the carboxylates of Asp-64 and Asp-121 of the D,D(35)E motif (denoted site I). However, no indication of binding of a second cation could be found in this map. Less than 30% occupancy was observed at a lower concentration of Mg$^{2+}$ (20 mM), indicating that binding was much weaker. A putative metal/water cluster did not refine well. However, a similarly positioned, single metal ion was detected at full occupancy after soaking the crystals in an even lower concentration of Mg$^{2+}$ (9 mM). Table I shows a cation bound between the carboxylates of Asp-64 and Asp-121 of the catalytic triad but no participation of its third member, Glu-157. To further investigate the possible participation of Glu-157 in metal binding, we carried out a systematic analysis encompassing a variety of divalent cations and a wider range of concentrations.

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(10 mM) concentration of MnCl$_2$. No additional metal-binding sites were observed, even in 500 mM MnCl$_2$ (Fig. 1A). A higher apparent affinity for Mn$^{2+}$ is consistent with observations that ASV IN is approximately 30-fold more active in Mn$^{2+}$ than Mg$^{2+}$.

Crystals soaked in 100 mM CaCl$_2$ were also found to bind a divalent cation at site I, with interactions between the calcium ion and the active site residues similar to those observed with Mg$^{2+}$ and Mn$^{2+}$. With Ca$^{2+}$, coordination is an incomplete octahedron in appearance, a square pyramid with the metal in the center of the square base and three water molecules in the coordination sphere.

**Zn$^{2+}$ and Cd$^{2+}$ Bind at Multiple Sites in the ASV IN Catalytic Domain**—Unexpectedly, we found that Zn$^{2+}$ binds in four separate sites on the surface of the isolated catalytic domain of ASV IN (Table I), with partial occupancy observed at a concentration as low as 2 mM and full occupancy of the sites at 100 mM. At the higher concentration of Zn$^{2+}$, two of the ions bind in the catalytic center, one at site I between Asp-64 and Asp-121 and a second, denoted site II, coordinated by Asp-64 and Glu-157 (Fig. 1B). The distance between the zinc cations in the active site is 3.62 Å. Site III (not shown) is in a loop defined by amino acids 92–107, with the ion directly coordinated to His-103, coordinated to His-93 via a water molecule, and liganded with two other water molecules. The fourth zinc ion is bound in the vicinity of the C terminus of the catalytic domain and is coordinated by residues His-198 and Tyr-194 (site IV).

Zinc ions bound at sites III and IV show clear tetrahedral coordination, whereas the cations in sites I and II are coordinated to two oxygens of the carbonylates and one water molecule each. One additional water molecule may complete a tetrahedral coordination of cations in the catalytic center by bridging both zinc ions. This putative water is not seen clearly in the electron density maps; it does, however, appear as a peak in $2F_o - F_c$ maps. During the refinement process, the water molecule located in this position shifts from one edge...
of one Zn\(^{2+}\) electron density to the other, so it was placed in the
final model with null occupancy just to indicate its putative
placement. As this water molecule faces bulk solvent, it is not
surprising that its position is not well defined. The other water
molecules coordinating the zinc ions are found in very clear
electron density. With the crystal soaked in 2 mM Zn\(^{2+}\), we
observed a pattern of metal binding similar to that at 100 mM.
In this case, both catalytic binding sites showed less than
complete occupancies, with site II lower than site I. Tempera-
ture factors are correspondingly higher for site II than for site
I. However, the structure with 100 mM Zn\(^{2+}\) had complete
occupancy and relatively low temperature factors for both cations
located in the active site.

Two Cd\(^{2+}\) ions were visible in the catalytic center after
soaking in 100 mM CdCl\(_2\) and were again coordinated to all
three carboxylate residues of the essential triad (Fig. 1C). How-
ever, there was lower occupancy of site II and higher tempera-
ture factor for this ion than for the ion in site I. This is similar
to binding observed at the lowest concentration (2 mM) of Zn\(^{2+}\)
and again suggests that the cations in site II may be bound less
strongly than those occupying site I. The distance between the
cadmium ions in the catalytic center is 4.06 Å (see Fig. 2). The
Cd\(^{2+}\) in site I has an almost perfect octahedral coordination
sphere, very similar to the coordination of Mn\(^{2+}\) and Mg\(^{2+}\). The
Cd\(^{2+}\) in site II has deformed octahedral coordination, sharing a
water ligand with the first cation. Singularly in this case, both
Glu-157 carboxyly oxygen coordinates with this one cation. The
most solvent-accessible water molecules liganded to each
metal, bound opposite to the Asp-64 ligand, have slightly longer
hydrogen bonding distances and higher temperature factors.

The atomic coordinates of the three crucial carboxylic acids
are only slightly affected when one or more cations are bound.
There is practically no change in the position of the side chain
of Asp-64 (mean shift of the atomic positions from their average
values, calculated for all structures listed in Table I, is 0.125
Å), a very slight movement of the side chain of Asp-121 (0.187
Å), and a more pronounced difference in location of the side
chain of Glu-157 (0.857-Å shift) (Fig. 2). The rotation of the side
chain of Glu-157 results in approximately the same difference
in carboxylate positions as seen in two different, uncomplexed
forms of ASV IN crystallized from different precipitants, poly-
ethylene glycol and ammonium sulfate (11). The minimal de-
viation in carboxylate positioning upon metal binding is con-
sistent with the observation that even single conservative
substitutions in these residues drastically reduced activity of
both ASV and HIV-1 IN (8).

**Competition for Cation Binding at the Active Site**—The Zn\(^{2+}\)
structures show the only active sites that are fully occupied
with two metal cations at 100 mM concentration (Table I). To
determine which cations are preferred by the enzyme, we
soaked ASV IN catalytic domain crystals in various concentra-
tions of Zn\(^{2+}\) plus either Mg\(^{2+}\) or Mn\(^{2+}\) salts. In the highest
concentration (100 mM for each salt), both structures were
essentially identical to the structure with 100 mM Zn\(^{2+}\) alone.

However, a Zn\(^{2+}\), Mn\(^{2+}\) structure at 10 mM concentrations
of each salt produced a mixed image. The water coordination
around the metal occupying active site I appears as a superpo-
sition of both Zn\(^{2+}\) and Mn\(^{2+}\) structures. We interpreted these
maps as a mixture of Zn\(^{2+}\) and Mn\(^{2+}\) in site I and only Zn\(^{2+}\)
in site II. As summarized in Table I, the occupancy of zinc cations
in sites I and II are refined to be equal. This result may indicate
the existence of some cooperativity between the sites, such that
if site II is occupied by Zn\(^{2+}\), then site I can no longer be
occupied by Mn\(^{2+}\) or Mg\(^{2+}\).

**Activity of the ASV IN Catalytic Domain with Various Divalent
Cations**—Having observed Ca\(^{2+}\), Zn\(^{2+}\), and Cd\(^{2+}\) occupa-
cy of site I (or I and II) in the active site of the catalytic
domain, we asked whether any of these cations could function as
cofactors for enzymatic activity of IN. The isolated catalytic
core domain of ASV IN displays two metal-dependent activi-
ties, a DNA endonuclease “nicking” activity, and a DNA cleav-
age- ligation “disintegration” activity (11, 20). DNA nicking by
the ASV IN catalytic domain is quite efficient and similar in
specific activity to that of the full-length protein. Therefore, we
first assayed for this nicking activity, which is characterized by
preferred cleavage between the C and A of the conserved CA
dinucleotide near the viral DNA termini (Fig. 3A, –3). This
endonucleolytic activity is distinct from “−2” processing, and
its biological relevance is not yet understood. The catalytic core
domain was incubated with a short DNA duplex substrate that
represents a viral DNA end in the presence of varying concen-
trations of ZnCl\(_2\), ZnSO\(_4\), CaCl\(_2\), and CdSO\(_4\) as well as the
known metal cofactors, MgCl\(_2\) and MnCl\(_2\). Of the new metals
tested, only Zn\(^{2+}\) supported significant activity (comparison
not shown). As shown in Fig. 3A, the Zn\(^{2+}\)-dependent activity
exhibits a sharp peak at approximately 2 mM ZnSO\(_4\); similar
results were obtained with ZnCl\(_2\) (data not shown). At this
peak, activity is approximately half of that observed with the
same concentration of MnCl\(_2\). However, significantly less ac-
tivity is observed at higher ZnSO\(_4\) concentrations. The opti-
 mum concentration of MnCl\(_2\) is approximately 10 mM, with
little change in activity up to 25 mM. The decreased activity at
ZnSO\(_4\) concentrations >5 mM is not due to the anion, as similar
results were observed with the chloride and sulfate salts. We
conclude that the ASV IN catalytic domain displays significant
nicking activity with Zn\(^{2+}\), as a cofactor at a concentration in
which site I is likely to be fully occupied and site II is likely to
be at least partially occupied. Higher concentrations of Zn\(^{2+}\)
are inhibitory. Although Mg\(^{2+}\) and Ca\(^{2+}\) could bind to sites I and
II in the crystal, no nicking activity could be detected in the presence of a broad range of
concentrations of these metals (data not shown).

We compared the time-dependent nicking activity of the
catalytic domain in the presence of Zn\(^{2+}\), Mg\(^{2+}\), and Mn\(^{2+}\).
The metal concentrations used were 2 mM ZnSO\(_4\), 10 mM MnCl\(_2\)
(see the optimal concentrations determined above), and 5 mM
MgCl\(_2\). As illustrated in Fig. 3B, nicking activity is readily
detected in the presence of ZnSO\(_4\), although the rate and extent
of the reaction are approximately one-tenth that observed with Mn$^{2+}$. However, no activity was detected with MgCl$_2$. Thus, at least for the isolated catalytic core domain, we find no activity with the cation presumed to be important in vivo but significant activity with Zn$^{2+}$ as a cofactor.

The ASV catalytic domain was also assayed for disintegration activity (Fig. 3C), which proceeds at 0.2% of the rate observed with the full-length protein. In the presence of 10 mM MnCl$_2$, disintegration is clearly detectable with the catalytic domain, as described previously (20). However, no disintegration activity was observed with 2 mM ZnSO$_4$, even after prolonged exposure of the autoradiogram. We conclude that Zn$^{2+}$ is unable to support significant disintegration activity of the isolated catalytic domain under these conditions.

Effects of Metal Combinations on the Nicking Activity of the Catalytic Core—As noted above, using equimolar (10 mM) amounts of Zn$^{2+}$ and Mn$^{2+}$, we observed predominant occupancy of site I by Mn$^{2+}$ and exclusive occupancy of site II by Zn$^{2+}$. For comparative activity studies, incubations were carried out using a fixed but suboptimal concentration of Mn$^{2+}$ (3 mM) in the presence of increasing concentrations of the divalent metals to be tested, and production of the −3 product was followed as in Fig. 3A and B. We observed a slight increase in activity at low or equal concentrations of Mg$^{2+}$ relative to Mn$^{2+}$ (Fig. 4). As Mg$^{2+}$ does not support nicking activity on its own, the significance of this increase is not yet clear. Ca$^{2+}$ had no significant effect at the same concentrations, and there was only a slight decrease in activity at higher concentrations of Mg$^{2+}$ or Ca$^{2+}$.

In contrast to results with Mg$^{2+}$ and Ca$^{2+}$, both ZnCl$_2$ and ZnSO$_4$ showed potent inhibition of Mn$^{2+}$-dependent nicking activity (Fig. 4). At equal concentrations of Zn$^{2+}$ and Mn$^{2+}$, the reaction was inhibited approximately 75%. The residual activity observed at this concentration may reflect the Zn$^{2+}$-dependent nicking. Although other interpretations are possible, these results are consistent with inhibition of the Mn$^{2+}$-dependent activity in favor of the Zn$^{2+}$-dependent activity. This potent inhibition by Zn$^{2+}$ is also consistent with the apparent high affinity of the isolated catalytic domain for Zn$^{2+}$ ions, as exemplified by high occupancy in the crystals even at relatively low

FIG. 3. Activities of the ASV IN catalytic domain in the presence of Mn$^{2+}$, Mg$^{2+}$, and Zn$^{2+}$. A, the $^{32}$P 18-mer duplex viral DNA substrate (S) was incubated with the ASV IN catalytic domain at the indicated metal concentrations for 150 min. A diagram of the substrate is shown below; the asterisk indicates the 5′-end label. Products were fractionated on 20% sequencing gels. The major product results from cleavage three nucleotides from the 3′-end of the labeled strand (indicated as −3). Quantitation using a Fuji phosphoimager is shown below (averaged from three separate experiments). B, time course reaction at fixed divalent cation concentrations: ZnCl$_2$ (2 mM), MgCl$_2$ (5 mM), and MnCl$_2$ (10 mM). Reactions were carried out for the indicated times, and products were fractionated as in panel A. Gels were analyzed using a Fuji phosphoimager, and results are displayed graphically. Mn$^{2+}$ and Zn$^{2+}$ data are the average from three separate experiments. Note the difference in scale for reactions in Mn$^{2+}$ versus Zn$^{2+}$ and Mg$^{2+}$. C, disintegration activity of the catalytic domain. Substrate is shown below. Thick lines indicate viral sequences; the asterisk indicates the 5′-end label. Concerted cleavage-ligation activity results in release of viral sequences and generation of a labeled 19-mer product. The time points were 10, 30, 60, 90, 120, and 150 min. nt, nucleotides.

FIG. 4. Effect of metal combinations on the nicking activity of the ASV IN catalytic domain. Increasing concentrations of several metal salts (as indicated) were added to reactions containing a suboptimal 3 mM MnCl$_2$ concentration. Assays were carried out as in Fig. 3A, and the results from phosphoimager analyses are shown graphically. Results are expressed as the percentage activity relative to the MnCl$_2$ reaction with no added metal salt. The vertical dashed line indicates the point at which the MnCl$_2$ concentration is equal to that of the second metal salt.

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metal concentrations. Cd$^{2+}$, which can also bind to sites I and II but is not a cofactor for nicking by the isolated catalytic core domain, is even a more potent inhibitor than Zn$^{2+}$; no significant activity was detected with this divalent cation at the higher concentrations (Fig. 4). If we assume that the inhibition reflects the abilities of these metals to bind to the catalytic center, these results suggest a relative affinity corresponding to Cd$^{2+}$, Zn$^{2+}$ $>$ Mn$^{2+}$ $>$ Ca$^{2+}$, Mg$^{2+}$ and is consistent with the results shown in Table I.

**Zn$^{2+}$ Can Serve as a Cofactor for the Processing Activity of Full-length ASV IN**—We next asked if Zn$^{2+}$ or the other previously untested divalent metals could function as a cofactor for the processing and joining activities of full-length ASV IN. Our initial survey revealed no significant activity with full-length IN in a range of concentrations of Ca$^{2+}$ or Cd$^{2+}$ (data not shown). Zn$^{2+}$ did support activity with an optimal concentration of 2 mM (not shown). We then compared the activities in the presence of 2 mM ZnSO$_4$ or 10 mM MgCl$_2$ (the optimal concentration, data not shown). As illustrated in Fig. 5A, the full-length IN showed significant processing activity (~2 nicking) in the presence of Zn$^{2+}$; this activity is slightly reduced compared with that observed with Mg$^{2+}$, but both are approximately 10-fold lower than that observed with Mn$^{2+}$ as the cofactor (not shown). Joining activity can be detected as insertion events into the viral DNA substrate, which produces a ladder of products that is longer than the substrate (Fig. 5C). As expected, joining activity is readily detected in the presence of Mg$^{2+}$; however, no significant joining activity was observed with Zn$^{2+}$.

The N-terminal Domain Is Not Required for Zinc-dependent Processing Activity—We previously showed that IN-(39–286), a non-fused ASV IN derivative lacking the N-terminal zinc binding domain, displays near wild type levels of processing and non-fused ASV IN derivative lacking the N-terminal zinc binding domain, is even a more potent inhibitor than Zn$^{2+}$; no significant activity was detected with this divalent cation at the higher concentrations (Fig. 4). If we assume that the inhibition reflects the abilities of these metals to bind to the catalytic center, these results suggest a relative affinity corresponding to Cd$^{2+}$, Zn$^{2+}$ $>$ Mn$^{2+}$ $>$ Ca$^{2+}$, Mg$^{2+}$ and is consistent with the results shown in Table I.

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**FIG. 5.** Processing, joining, and disintegration activities of full-length IN (WT IN) and a zinc finger domain deletion mutant (IN-(39–286)) in the presence of Mg$^{2+}$ and Zn$^{2+}$. Assays were carried out as described in Fig. 3A. Processing activity is characterized by 3’ cleavage of the conserved CA two nucleotides from the 3’-end of the labeled strand (~2 site). Joining activity is characterized by the appearance of joined products (JP) longer than substrate length. Reactions were carried out in 2 mM ZnSO$_4$ and 10 mM MgCl$_2$ from 1 to 75 min. Panels C and D show a prolonged exposure of the region above the substrate bands in panels A and B in order to display joined products. Panel E shows disintegration activity as described in Fig. 3C. The time points were 10, 30, 60, 90, 120, and 150 min. S, $^{32}$P 18-mer duplex viral DNA substrate; WT, wild type; nt, nucleotides.

II ligands are Asp-64 and Glu-157, suggesting a role for this third member of the invariant triad in the D,D(35)E motif. Two more Zn$^{2+}$ cations are located away from the active site of the enzyme. As it is not yet known if these sites can be occupied in the full-length protein, the significance of this binding cannot be evaluated. It is possible that metal ions bound to one or both of these sites could play a role in structural stabilization and/or activity control, as reported for other enzymes (21).

It is not certain why the complexes with different divalent cations show differential occupancy of site II. Each of the two sites contains two direct links to the protein through carboxyl oxygens, but in all structures of the isolated catalytic domain,
the temperature factors for Glu-157 are considerably higher than for the two aspartates in the active site, indicating higher mobility of that side chain. This may cause more difficulty for binding of the octahedrally coordinated Cd\(^{2+}\) than the tetrahedrally coordinated Zn\(^{2+}\), which has less stringent requirements for the geometry of its binding site and a lower preferred coordination number (22). This does not explain why the octahedrally coordinated Cd\(^{2+}\) would bind in site II, but we note that this ion is able to utilize both oxygens of Glu-157 for its binding, and this might enhance its stability.

The structural features of zinc ions in the active site agree well with the description of other co-catalytic sites in multi-Zn\(^{2+}\) enzymes discussed by Vallee and Auld (23). As is commonly the case, both ions are close to each other (the distance is 3.62 Å in the high occupancy structure), and they are bridged by the carboxylate group of an aspartic acid (e.g. Asp-64). Similar arrangements have been reported in the past for other enzymes, such as phospholipase C (24) and nuclease P1 (25). In common with these structures, there is also indication of a shared water molecule bridging the two cations, although this putative water is not well ordered in ASV IN.

Active Sites of Polynucleotidyltransferases Bear Considerable Similarity—In the absence of a bound DNA substrate, the current structural data do not allow us to propose any specific model concerning metal binding to site II and the mechanism by which hydrolysis or nucleotidyl transfer occurs with ASV IN. Yang and Steitz (14) have noted the similarity of the cluster of acidic residues forming the active sites of enzymes in the structural superfamily to which integrase belongs, and more detailed comparisons of metal complexes of some of the members of this family were presented by Bujacz et al. (12). The binding of two metal ions in the ASV IN active site reported here invites comparison with the 3-5' exonuclease domain of the E. coli DNA polymerase I (Klenow fragment) in which two metals are proposed to cooperate to activate an attacking hydroxide and stabilize a pentacoordinate DNA phosphate transition state in the active site (14, 15). In Fig. 6, we compare metal complexes of the active sites of ASV IN, HIV-1 reverse transcriptase RNase H, and the exonuclease domain of the Klenow fragment of DNA polymerase I (15). Despite significant differences in topologies of the three proteins, the similarity in positions of both divergent cations and the coordinating carboxylates is apparent. In this alignment, site I of ASV IN is superimposed on site A of the Klenow fragment, both of which have been shown to have similar metal binding properties in terms of occupancy and metal ligand geometry. Alignment of site I of ASV IN with site B of the exonuclease domain does not yield as good a superposition of the coordinating residues.

Some differences between these enzymes are also clear. When a mixture of divalent cations is present, site A in the Klenow fragment is occupied by a Zn\(^{2+}\) ion, whereas site B is occupied by Mg\(^{2+}\), with clear differences in the octahedral versus tetrahedral coordination. This is not the case for integrase, because Zn\(^{2+}\) clearly is preferred in both sites under higher metal concentrations, both with tetrahedral coordination. As there are only two carboxylate oxygens binding each ion, there are no geometric constraints that favor or necessitate one type of coordination. These differences may be due to the absence of coordinating ligands contributed by other portions of ASV IN or the DNA substrate. For both integrase and exonuclease, the concentration of Zn\(^{2+}\) necessary to observe binding is more than an order of magnitude lower than that for Mg\(^{2+}\). Active sites that contain more than one type of divalent cation are observed in other Zn\(^{2+}\)-containing enzymes that act upon phosphate esters. The second metal ion is often Mg\(^{2+}\) as, for example, in phospholipase C or alkaline phosphatase (24, 25).

It is important to note that active site configurations different from those shown in Fig. 6 are observed for other enzymes with DNA-processing or polymerizing activity. Two divalent cations are reported in one of the structures of rat polymerase β (26), separated by about 3.7 Å and also coordinated by three acidic residues, yet we could obtain no convincing superposition on the active site of integrase. Even more different is the active site of phage T4 RNase H (27) where the two Mg\(^{2+}\) ions are 6.3 Å apart, with one of them coordinated by only a single carboxylate oxygen and five waters molecules, whereas the other is surrounded by six water molecules and does not make direct contacts with any protein atom. These examples show the limits of the comparisons and are a caveat against drawing conclusions that might be too far-reaching.

Biochemical Activity of Divalent Cations—Our biochemical analyses show that although Zn\(^{2+}\) is less effective than Mn\(^{2+}\), it can serve as a cofactor for nicking activity of the isolated catalytic core domain of ASV IN, whereas there is no detectable
activity with Mg\(^{2+}\), Ca\(^{2+}\), or Cd\(^{2+}\). Comparisons of nicking activities in mixtures of Mn\(^{2+}\) and the other cations suggest that both Zn\(^{2+}\) and Cd\(^{2+}\) bind with higher affinity than Mn\(^{2+}\) and that Mg\(^{2+}\) and Ca\(^{2+}\) bind with lower affinity. This is consistent with the occupancies of these metals observed in our structural analyses of the catalytic domain. We also observed a sharp peak for the optimal concentration of Zn\(^{2+}\) at 2 mM; higher concentrations were inhibitory. The reason for this decreased activity at higher Zn\(^{2+}\) concentrations is not yet apparent. However, the most striking result from these studies was the observation that Zn\(^{2+}\) supported the endonuclease activity of the catalytic domain, whereas Mg\(^{2+}\), the presumed physiologically relevant cation, did not. This observation prompted us to ask whether Zn\(^{2+}\) or any of the other catalytic domain binding cations could serve as cofactors for processing and joining by the full-length enzyme.

As ASV IN can perform both processing and joining in vitro, in the absence of the N-terminal domain it is possible to separate the presumed structural role of Zn\(^{2+}\) bound to the N-terminal domain from a catalytic role. We found that both full-length ASV IN and IN-(39–286), which lacks the N-terminal Zn\(^{2+}\) binding motif, display both Mg\(^{2+}\)- and Zn\(^{2+}\)-dependent endonucleolytic processing activity. The Mg\(^{2+}\)-dependent activity is somewhat higher with the full-length protein than with IN-(39–286), indicating that the N-terminal domain is required for optimal activity. Most importantly, both full-length and IN-(39–286) showed Zn\(^{2+}\)-dependent processing activity almost equal to that observed with Mg\(^{2+}\). Other investigators have reported that the addition of Zn\(^{2+}\) can enhance the Mg\(^{2+}\)-dependent activity of HIV-1 IN (6, 7). However, in their studies, stimulation was shown to be mediated by the N-terminal zinc binding domain. Thus, ours is the first report that Zn\(^{2+}\) can also act as a cofactor for catalysis by a retroviral integrase.

In contrast to results with processing, we could detect no joining activity by either full-length ASV IN or IN-(39–286) in the presence of Zn\(^{2+}\). We also observed that Zn\(^{2+}\) fails to support significant disintegration activity by either the catalytic core domain or IN-(39–286). Thus, the two reactions in which the nuclease is derived from a DNA moiety and contact with “target” DNA sequences is required (i.e., joining and disintegration) are greatly impaired with Zn\(^{2+}\) as a cofactor. As the same triad of acidic amino acids in the catalytic center is essential for both processing and joining in the presence of Mn\(^{2+}\) and Mg\(^{2+}\) and these steps employ similar chemistry, there is no obvious explanation for this difference. It is possible that the lower preferred coordinating number of Zn\(^{2+}\) compared with Mn\(^{2+}\) or Mg\(^{2+}\) disfavors interactions with target DNAs or links to the protein that affect target DNA binding. Further structural analyses should help us to understand the basis for these distinct metal preferences. Lastly, the fact that Zn\(^{2+}\) binds tightly to the active site but can only support one step in integration may be relevant to the design of active site inhibitors of retroviral integrases.

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