Structural Determinants of Metal-induced Conformational Changes in HIV-1 Integrase*

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Human immunodeficiency virus type 1 (HIV-1) integrase (IN) undergoes a reversible metal-induced conformational change that activates the enzyme (Asante-Appiah, E., and Skalka, A. M. (1997) J. Biol. Chem. 272, 16196–16205). In this report, key structural features that mediate this conformational change have been identified by site-directed mutagenesis, limited proteolysis, and mass spectrometry studies. The results reveal two separable metal-induced effects. One depends on residues in the N-terminal domain (amino acids 1–50) and a C-terminal tail (amino acids 274–288) and is detected by increased resistance of the full-length protein to proteolytic digestion. This effect appears to depend on metal binding at an undefined location distinct from the known sites in the N-terminal and catalytic core domains. The second conformational change depends on metal binding at the active site in the catalytic core domain. Substitution of acidic residues Asp64 or Glu152 in the catalytic core D,D(35)E motif or truncation of the Src homology 3 (SH3)-like domain in the C-terminal region of the enzyme abolishes this metal-induced change. Comparison of tryptic digests of an HIV-1 IN derivative competent for metal-induced conformational change and a conformation-defective D64N derivative identified specific regions in HIV-1 IN that are affected by this second change. A region in the N terminus that spans Lys14, an extended loop and the adjacent region in the core domain (including lysines 136, 156, and 160 and Arg272), and residues at the C terminus beyond the SH3-like domain all become less accessible to proteolysis in the conformation-competent protein. In contrast, a region that encompasses Lys258 in the putative DNA binding groove of the SH3-like domain becomes more sensitive to proteolysis in the presence of Mn2+. The results are consistent with a model in which the binding of the metal ion by residues of the D,D(35)E motif elicits specific changes in all three domains of HIV-1 IN, inducing the restructurization of the enzyme for catalytic competence.

EXPERIMENTAL PROCEDURES

The retroviral enzyme integrase (IN)* catalyzes the integration of viral DNA into the host genome (for recent reviews, see Refs. 1 and 2). The protein comprises three independently folding domains that include several conserved residues (Fig. 1). At the N terminus is the invariant HHCC motif (3) that binds zinc ions (4–6). Biochemical studies show that the binding of zinc ions stabilizes the N-terminal domain, induces multimerization, and enhances the activity of human immunodeficiency virus type 1 (HIV-1) IN (6, 7). Solution of the three-dimensional structure of an isolated N-terminal fragment (residues 1–55) of HIV IN by NMR has confirmed the role of zinc ions and revealed a helix-turn-helix structural motif (8, 9). Amino acid substitutions or truncations that disrupt this motif impair catalytic activity of HIV IN in vitro (10) and block viral infection (11).

The catalytic core domain of retroviral integrases and related proteins is characterized by the presence of a triad of invariant acidic amino acids referred to as the D,D(35)E motif (12–14). These acidic residues are essential for catalysis and are evolutionarily conserved; even conservative substitutions severely impair enzymatic activity (14–16). X-ray crystallographic analyses of the core domain of avian sarcoma virus (ASV) IN and HIV-1 IN have revealed that these residues coordinate the divalent metal ion(s) required for catalysis with the same topology (17–21, 41).

The three-dimensional structure of a C-terminal fragment of HIV-1 IN (residues 220–270) has also been determined (22, 23). It resembles the Src homology 3 (SH3) domain, and analysis of a solution dimer by NMR revealed a saddle-shaped feature that may play a role in nonspecific DNA binding. The structure and contribution of the C-terminal tail (residues 271–288), however, remains unknown (Fig. 1). Although the structures of all of the independently folding domains of HIV-1 integrase have been determined and models of how they may fit together have also been presented (8, 24), very little is known about the structural basis of catalysis. Previously, we reported that the metal cofactor induces a change in the conformation of HIV-1 IN that involves a reorganization of the core and C-terminal domains and leads to an activated competent enzyme (25). To obtain further insight into this metal-dependent structural change, we have introduced specific deletions and amino acid substitutions into HIV-1 IN and assessed their influence on this property.

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1 The abbreviations used are: IN, integrase; HIV-1, human immunodeficiency virus type 1; ASV, avian sarcoma virus; SH3, Src homology 3; TPK, 1-tosylamido-2-phenylethyl chloromethyl ketone; HMR, heart muscle kinase; mAb, monoclonal antibody; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MS, mass spectrometry.
the active site D,D(35)E motif substitutions (D64A, D64N, E152A, and D64A,E152A) were introduced into HIV-1 IN coding sequences by oligonucleotide-directed mutagenesis following established methods (26). DNA from positive clones was subsequently introduced into E. coli BL21(DE3) cells for protein expression. All positive clones were verified by DNA sequencing.

Construction of HIV-1 IN Deletion Mutants—Two primers complementatory to the initiating and terminating sequences and in opposite orientations were used in a polymerase chain reaction to amplify the sequences that encode residues 1–285, 1–274, 1–253, and 22–288 of HIV-1 IN by established protocols (26). pET29bNY5IN F185K,C280S was used as the template DNA in order to include the solubility-promoting F185K and C280S substitutions into these constructs. Construction of the isolated catalytic core domain IN-(50–212) and the core plus the C terminus, IN-(50–288), has been reported (25).

Construction of pET29bHMK-HIV-1 IN—The sequence that encodes the kinase site, RRA$\phi$SV (the underlined serine residue is phosphorylated), recognized by the catalytic subunit of cyclic AMP-dependent protein kinase from heart muscle (HMK) was introduced by polymerase chain reaction-based mutagenesis (26). The cloning strategy ensured that the kinase-specific sequences preceded those that specify the N-terminal residues of HIV-1 IN. The restriction site for KpnI was also introduced between the sequences that encode the kinase site and HIV-1 IN in order to facilitate future subcloning of other IN derivatives into the vector backbone. The introduction of the KpnI restriction site leads to the expression of a Gly-Thr dipeptidyl linker between the kinase site and the N-terminal residue of integrase, Phe-1. Thus, a total of 8 amino acids (including the initiating methionine) precede the authentic 288 amino acids of HIV-1 IN when the protein is expressed. The correct construction of the clone was confirmed by DNA sequencing.

Protein Purification—HIV-1 IN proteins were expressed in, and purified from, *Escherichia coli* BL21(DE3) as reported recently (25, 27).

Limited Proteolysis—The protocol for assessing the effect of the metal cofactor on limited proteolysis of HIV-1 IN has been described previously (25). These assays were performed in a 75-µl reaction containing 20 mM HEPES-KOH at a pH of 7.5, 100 mM KCl, 10 mM glycerol and 310 pmol of IN. The reactions were incubated at 37 °C in the absence or presence of 10 mM MnCl$_2$. Protease K or TPCK-treated trypsin at a final concentration of 4 ng/µl was used in all experiments. At the times indicated in the figures, a 15-µl aliquot was removed and quenched by boiling at 95 °C for 5 min with SDS-polyacrylamide gel electrophoresis loading buffer (15 µl). The protein samples were subjected to electrophoresis in a 15% polyacrylamide gel and then silver-stained (Bio-Rad).

The extent of limited proteolysis of $^{32}$P-end-labeled HIV-1 IN was quantitated following the separation of the proteolysis products (by SDS-polyacrylamide gel electrophoresis), by exposing the gel to a Fuji imaging plate that was read on a Fuji MacBas 2000 imaging system. Protein samples earmarked for mass spectrometric analysis were quenched by adding trifluoroacetic acid to a final pH of 2. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS was performed on a PerSeptive Biosystems Voyager DE mass spectrometer in linear mode at an accelerating voltage of 20,000 V. The tryptic peptides were analyzed on a matrix of a-cyano-4-hydroxycinnamic acid. Melittin and a-cyano-4-hydroxycinnamic acid matrix dimer were used as internal mass standards. Several assigned integrase tryptic peptides in each mass spectrum were used subsequently for mass calibrations in the final reported results. Data from 128 scans were averaged.

Immunoblotting—Following the separation of proteolysis products by electrophoresis in a 15% denaturing polyacrylamide gel, the protein bands were transferred to a nitrocellulose membrane. The SuperSignal chemiluminescent immunoblot kit (Pierce) was used for protein detection following the manufacturer’s suggested protocol. Anti-HIV-1 IN monoclonal antibodies, mAbs 17 and 33, were used at a final concentration of 0.04 µg/ml, while mA4 was used at a final concentration 2 µg/ml.

### Results

**Active Site Residues of the D,D(35)E Motif Are Required for a Metal-dependent Change in HIV-1 IN Conformation**—Previously, we showed that metal cofactor-induced conformational changes can occur in HIV-1 integrase (25). These changes were detected by antibody sensitivity and by a difference in the pattern of digestion products after limited proteolysis with protease K. As illustrated by immunoblotting with domain-specific monoclonal antibodies, two clear differences are observed in the presence of Mn$^{2+}$ (Fig. 2). Increased stability is observed among products that migrate at and just below the position of full-length IN, referred to as band I. These products are recognized by antibodies that are specific for all three domains. However, the more rapid loss of binding of the N-terminal-specific antibody indicates that this domain is most susceptible to proteolysis. In addition, a new band of products is observed in the presence of Mn$^{2+}$ that migrates just above the position of the catalytic core. These products, referred to as band II, are recognized by the catalytic core domain-specific antibody but not by the antibodies that are specific for the N-terminal or C-terminal domains.

Metal-dependent production of band II was observed in a truncated HIV-1 IN derivative that lacked the N-terminal, zinc-binding domain (25). Thus, metal binding to this domain cannot be responsible for these conformational changes. As the D,D(35)E motif in the catalytic core domain is the only other known metal-coordinating motif in the protein, we examined its role in the metal-induced conformational changes, using site-directed mutagenesis. All proteins tested included the solubility-promoting substitutions, F185K and C280S (28). These changes have been shown to affect neither the activity of HIV-1 IN (28) nor its ability to undergo metal-induced conformational changes (25). Substitution of residues Asp$^{64}$ (to Ala or Asn) and Glu$^{152}$ (to Ala) were introduced singly and in combination. As expected, the resulting, active site substituted proteins were soluble. As reported previously (14–16), we found that substitutions of acidic residues in the D,D(35)E motif abolished catalytic activity of the protein (data not shown). Antibody recognition (a panel of five monoclonals including two that are conformation-sensitive were tested) and chromatographic profiles of the D,D(35)E substituted derivatives were identical to that of the parental protein, IN F185K,C280S, indicating that the proteins do not fold aberrantly (data not shown).

A comparison of silver-stained products of limited proteolysis of the D64N- and D64A-substituted proteins with the parental protein is presented in Fig. 3, A, C, and D. The results show that the Mn$^{2+}$-dependent product species of band II were absent when the Asp$^{64}$-substituted derivatives were digested with protease K. However, the band I products of both the Asp$^{64}$-substituted and the parental protein were more resistant to proteolysis in the presence of metal ions. Limited proteolysis of HIV-1 IN derivative E152A gave a cleavage pattern that was similar to that of the Asp$^{64}$-substituted proteins (not shown). Because the crystal structures of metal complexes of the core domain of the homologous ASV IN protein show that two metal ions can be coordinated by the acidic residues of the D,D(35)E

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**Figure 1. A linear model of HIV-1 IN protein showing the three major domains of the protein whose structures are known and the unresolved tail region.** The conserved and catalytically important residues are indicated, and the corresponding residue numbers are shown above the model. The residue numbers that delimit the domains and the features that describe the domains are indicated below:
motif (18), the effect of introducing two amino acid changes simultaneously into HIV-1 IN, D64A and E152A, was also investigated. As Asp$^{64}$ donates ligands to bridge both metal ions, substituting this residue and another member of the motif should eliminate all metal binding by this motif. As with the single Asp$^{64}$ and Glu$^{152}$ substitutions, this derivative also failed to yield the metal-dependent band II products but exhibited metal-dependent resistance of band I products against proteolysis (data not shown; see tabulation in Fig. 4). We conclude, therefore, that the appearance of band II products depends on metal ion binding by active site residues of the D,D(35)E motif, whereas the relative resistance of band I does not.

The N and C Termini of HIV-1 IN Are Required for the Resistance to Proteolysis of Band I—Additional derivatives of HIV-1 IN were analyzed in attempts to uncover other structural features that mediate the conformational changes. Deletion of three amino acids from the C terminus or 22 amino acids from the N terminus produced no change relative to full-length HIV-1 IN. A similar resistance of band I and the appearance of band II were observed upon proteolysis in the presence of metal ions (not shown). However, metal-dependent resistance of band I was lost upon truncation of 14 amino acids from the C terminus (Fig. 3B) or truncation of 50 amino acids from the N terminus (not shown). On the other hand, band II was produced upon digestion of both of these derivatives (e.g. Fig. 3B). These results provide additional evidence that the resistance of band I fragments to proteolysis and the appearance of the core-derived band II product can be uncoupled and are likely to reflect distinct metal-dependent structural features of HIV-1 IN. Neither increased resistance of band I nor appearance of band II fragments in the presence of Mn$^{2+}$ was observed upon digestion of HIV-1 IN derivatives that either lacked 35 amino acids from the C terminus, IN-(1–253), which disrupts the SH3-like domain, or lacked both the N- and C-terminal domains, IN-(50–212) (25). These data are summarized in Fig. 4.

The Presence of Metal Ions Alters Accessibility of Proteolytic Target Sites—In earlier (25) and in the preceding studies, limited proteolysis with proteinase K was used to monitor structural changes in HIV-1 IN. This enzyme is very useful to detect and highlight global conformational changes in proteins because of its broad specificity. However, this same broad specificity limits the ability to identify susceptible cleavage sites. Analysis of specific tryptic digestion products allows detection of structural changes that may mask or expose cleavage sites. Fig. 5A shows the silver-stained pattern obtained after digestion of HIV-1 IN F185K,C280S with trypsin in the absence and presence of Mn$^{2+}$. As expected, differences were somewhat less conspicuous than observed with the broadly specific proteinase K (cf. Figs. 3A and 5A). Nevertheless, trypsin digestion also revealed a different pattern in the presence of metal ions (Fig. 5A). As with proteinase K, the full-length protein was more resistant to digestion in the presence of Mn$^{2+}$. In addition, the arrows to the left in Fig. 5A point to the positions of cleavage products whose origin (solid) or stability (open) are also metal-

**Fig. 2.** Characterization of proteinase K proteolytic products of HIV-1 IN, generated in the absence and presence of Mn$^{2+}$, by monoclonal antibodies. The products of limited proteolysis by proteinase K were immunblotted with N-terminal specific mAb 17 (A) catalytic core domain-specific mAb 4 (B), and C-terminal domain-specific mAb 33 (C). The positions of full-length IN and the catalytic core domain are indicated on the left. The products of proteolysis recognized by all three antibodies are referred to as band I. Band II products are recognized only by mAb 4. Note the recognition of some proteolytic products with mAb 33 but not mAb 17 in the absence of Mn$^{2+}$, indicating that the N-terminal domain is most susceptible to proteolysis. Due to the high sensitivity of the SuperSignal immunoblot kit, half the amount of protein removed at each time point was loaded. The same blot was used for all three experiments.

**Fig. 3.** Limited proteinase K proteolysis of HIV-1 IN derivatives in the absence or presence of Mn$^{2+}$. At the indicated times, aliquots of the reaction were removed, quenched, separated on 15% SDS-polyacrylamide gels, and silver-stained as described under “Experimental Procedures.” A, full-length IN-(1–288) F185K,C280S; B, IN-(1–274) F185K; C, D64N,F185K,C280S; D, D64A,F185K, C280S.
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Fig. 4. A summary of the deletions and substitutions introduced into HIV-1 IN and their effects on resistance of band I to proteolysis and appearance of band II. The inclusion of the core domain in all band II products was verified by immunoblotting with the core-specific monoclonal antibody mAb 4 (see Fig. 2). A linear model of HIV-1 IN showing the organization of domains is presented at the top to relate the location of the introduced deletions and substitutions to the full-length protein.

Dependent. To establish that the concentration of metal ions used in our assay does not affect the activity of trypsin, we digested the homologous enzyme ASV IN under the same conditions. We showed previously that ASV IN does not exhibit a metal-induced differential resistance to proteolysis with protease K (24). As expected, the tryptic cleavage pattern of ASV IN was similar in the absence and presence of Mn2+ (Fig. 5B). We conclude from this result that trypsin is not affected by the divalent metal ion under the conditions of our assay. We also analyzed the active site-substituted protein, HIV-1 IN D64N,F185K,C280S, with trypsin. As expected from results of Fig. 3C, no new species were observed with trypsin, but the slowly migrating species, analogous to protease K band I, did show increased resistance in the presence of metal ions. The tryptic digest of HIV-1 IN and ASV IN showed a prominent protein band of approximately 27 kDa. Immunoblot analysis (data not shown) revealed that these products include the catalytic core and C-terminal domains. Hence, as in proteolysis with protease K (cf. Fig. 1), the N-terminal domains of these proteins appear to be most susceptible to digestion.

Quantitation of the Relative Resistance of HIV-1 IN to Digestion by Trypsin in the Presence of Mn2+—Several of our attempts to quantitate the products of proteolysis performed in the absence and/or presence of metal ions by reverse-phase high pressure liquid chromatography and other chromatographic methods failed, due to the tendency of the protein fragments to self-associate (data not shown). As an alternative approach to measuring the degree of resistance of the metal-bound conformation of HIV-1 IN, a derivative of the protein was constructed, HMK-HIV-1 IN, that includes the recognition sequences of the catalytic subunit of cyclic AMP-dependent protein kinase. The kinase site, RRASV, was fused to the N terminus of HIV-1 IN, because previous studies have shown that N-terminal fusion derivatives of HIV-1 IN retained catalytic activity similar to the wild-type protein (30). As expected, this fusion of an additional 8 amino acids to the N terminus of integrase did not affect catalytic activity (data not shown). The

HMK-HIV-1 IN was labeled with radioactive phosphate, 32P, at the kinase site, and then loss of radioactivity at the position of the full-length protein was followed upon the addition of trypsin. Fig. 6A shows an autoradiogram of products from a limited tryptic digest of the labeled protein in the absence and presence of Mn2+ with increasing time. As expected (25), no 32P-labeled full-length protein was observed after a 2-h digestion in the absence of Mn2+, whereas a significant amount remained in the presence of Mn2+. Quantitation of the results with samples from additional time points is shown in Fig. 6B. Data from three separate experiments indicated that under our assay conditions, the half-life of HIV-1 IN in the absence of a divalent cation was 19 ± 4 min compared with 59 ± 7 min and 178 ± 15 min when digestion was carried out in the presence of Mg2+ or Mn2+, respectively.

Induction of Metal-dependent Resistance of HIV-1 IN to Proteolysis Can Be Blocked by Conformation-specific mAb 33—Our previous studies have shown that if HIV-1 IN is preincubated with Mn2+, binding of C-terminal specific monoclonal antibody mAb 33 to the metal-bound protein is severely impaired (25). In the experiment described in Fig. 7, we asked whether the
binding of this antibody would alter the pattern of trypsin digestion in the absence or presence of Mn$^{2+}$. As a control, the effects of the same antibody on ASV IN were assessed. As expected, the results showed no effect of metal or antibody on the pattern of digestion of ASV IN (Fig. 7, lanes 11–14). On the other hand, a comparison of results with HIV-1 IN shows a metal-induced stabilization of the full-length protein against proteolysis (Fig. 7, compare lanes 2 and 3 with lanes 6 and 7; dark arrow). This effect was blocked by the addition of mAb 33 (compare lanes 6 and 7 with lanes 8 and 9). The addition of mAb 33 to HIV-1 IN thus produced a result that was similar to that seen if no metal ion had been added to the protein (compare lanes 4 and 5 with lanes 8 and 9). We conclude that the metal induces a unique conformation of HIV-1 IN that results in increased resistance against proteolysis and that this effect can be specifically blocked by binding of the C terminus-specific antibody.

A comparison of lanes 2 and 3 with lanes 4 and 5 in Fig. 7 shows that the addition of mAb 33 resulted in the appearance of a new proteolytic band (open arrow at bottom left). The fact that this band is not observed with ASV IN enzyme suggested that it was derived from HIV-1 IN and not from mAb 33. Furthermore, the intensity of this band was diminished when metal ions were added to the incubation (compare lanes 4 and 5 with lanes 8 and 9). This observation is consistent with previous studies (25), which revealed that addition of the metal cofactor to HIV-1 IN impairs the binding of conformation-sensitive mAb 33. To identify this product, the protein band was isolated and sequenced. Data obtained for the first 20 N-terminal amino acids (IQFRVYRDSNPLWKGPA) mapped the protein fragment to the C-terminal domain of HIV-1 IN beginning at residue 220. The relative molecular mass of the fragment (approximately 5.5 kDa) suggested that it spans the stable C-terminal domain (residues 220–270) whose NMR structure has been solved (22, 23). Thus, binding of mAb 33 protects the C-terminal domain against trypsin digestion. This protein footprint is consistent with our previous deletion mutagenesis studies that mapped the binding site of mAb 33 to the C terminus of HIV-1 IN (29).

The Metal Cofactor Induces Specific Structural Changes in HIV-1 IN—In order to probe structural changes in HIV-1 IN that are dependent on metal binding to the active site residues in the catalytic core (see Fig. 5), we analyzed the products of limited trypsin proteolysis by MS. From results in Figs. 3–6, it is clear that substitutions introduced into residues of the D,D(35)E motif abolish the appearance of the Mn$^{2+}$-dependent core-related band II products but do not affect the resistance of band I to proteolysis. Thus, to specifically reveal changes that give rise to the band II products, we compared the peptides produced by trypsin digestion of IN D64N,F185K,C280S with those of the parental IN F185K,C280S. Both proteins were digested for 15 min in the presence of Mn$^{2+}$, and the mass spectra of released peptides were analyzed by MALDI-TOF MS. The differences observed are highlighted by solid arrows in the D64N spectrum shown in Fig. 8. A similar MS analysis performed with wild type HIV-1 IN gave results that were indistinguishable from those with the parental IN F185K,C280S when the spectra from three independent experiments were compared (data not shown). Peptides in the spectra were assigned (Table I) by comparing their masses to those expected from predicted tryptic sites in the amino acid sequence of these proteins.

The differences shown in Fig. 8 included both the appearance and the loss of specific peptides. Several peptides were observed only in the spectrum of the D64N protein. Their absence among products from the parental protein identifies sites that must become masked as a result of the metal-induced conformational change. These peptides mapped to all three domains of HIV-1 IN. Peptides f and k are produced by trypsin cleavages in the N terminus of the protein and generate (M)Phe$^{1}$–Lys$^{14}$ and (M)Phe$^{1}$–Arg$^{20}$, respectively (Table I; the letter M in parentheses signifies the presence of the initiating methionine residue in the bacterially expressed protein), suggesting that both Lys$^{14}$ and Arg$^{20}$ become less accessible as a consequence of the conformational change. However, because peptides b and e, which span residues Ala$^{21}$–Lys$^{34}$ and Ala$^{8}$–Arg$^{20}$, respectively, are present in the spectra of products from both D64N and
parental proteins, Arg20 must be accessible in both the apo and metal-induced conformations of HIV-1 IN. An intense ion assigned to Tyr15–Arg20 (m/z 863.2, Table I) is observed for the trypsin digest of the D64N protein and is not observable for the "wild-type" protein. Hence, only the trypsic site at Lys14 becomes masked as a result of the conformational changes. Peptides j and k map to adjacent regions in the core domain of HIV-1 IN. Peptide j, whose band is quite small in the spectrum shown in Fig. 8, was nonetheless a reproducible product of D64N digestion. It comprises a trypsin fragment that spans Gln137–Lys166 and encompasses the extended (20) or disordered (19) loop (residues 141–153) in the crystal structure of the core domain of HIV-1 IN, near the metal-binding site (see Figs. 9 and 10). Peptide c (Ile161–Lys173) includes a region within a-helix 4 and the loop between a-helix 4 and a-helix 5 in the crystal structure of the core domain of HIV-1 IN (see Fig. 10). This result indicates that the disordered loop and regions that flank it are restructured upon metal binding at the active site. Another peptide, d,d′, found abundantly in the spectrum of the D64N protein but barely detectable in the parental spectrum is derived from the extreme C terminus of HIV-1 IN, beginning with Gln274 and extending to the very last residue, Asp288 (see Table I). This observation indicates that the structurally unresolved C-terminal "tail" is accessible to proteolysis when no metal is bound at the active site but becomes protected when metal is bound at that site.

In contrast to the above, peptide m, a prominent peptide ion in the spectrum of products from the parental protein, is absent from the spectrum of the D64N derivative. This peptide represents residues Val259–Arg284 in the C terminus of HIV-1 IN. Its absence among the D64N protein products suggests that K258, located on b-strand 4 in the SH3-like domain (see Fig. 9) becomes accessible as a consequence of metal binding. The fragment comprising Gln274–Arg284 in the unresolved portion of the C terminus (see Fig. 1) is observed only among products of the D64N protein derivative (not shown). This indicates that both Lys273 and Arg284 are inaccessible when metal is bound at the active site. Thus, the apparent accessibility of Arg284 in the parental protein seems to be a result of a secondary cleavage following the initial cutting at Lys258. This conclusion is further supported by the fact that peptide d (residues Gln274–Asp288) is also observed only among products of the D64N protein and is consistent with the conclusion that the C-terminal tail becomes resistant to cleavage upon the addition of metal ions to the parental protein. Cleavages in the basic stretch from residues...
262–269 invariably give rise to single amino acids or tripeptides that cannot be accurately assigned. This portion of the spectra which was not informative has not been presented.

### DISCUSSION

The catalytic activity of HIV-1 IN is dependent upon the presence of a metal cofactor usually Mn\(^{2+}\) or Mg\(^{2+}\). It is now evident that in addition to acting as electrophiles, metal ions also play a structural role for the enzyme (25, 31–33) that explains the presence of the initiator methionine. The metal-bound conformation that gives rise to new proteolytic products with both proteinase K and trypsin, we used the conservative D64N deletion (Fig. 4), and nearby Asp 279 can be substituted with alanine without affecting this metal-induced change. Thus, binding of metal ions to a still undefined locus is postulated to stabilize the changes that ensue as a consequence of metal binding coincides with their ability to support catalysis. A 10-fold increase in half-life of HIV-1 IN contributed by Mn\(^{2+}\) binding (Fig. 6) translates to a change in free energy of \(-1.6\) kcal/mol available for catalysis.

The second effect that we have observed is the metal-induced appearance of new proteolytic digestion products (denoted band II) that include the catalytic core domain. Binding of metal ions to a still undefined locus is postulated to explain this resistance to proteolysis. We are currently introducing single and multi-amino acid substitutions into HIV-1 IN in an attempt to uncover this putative new metal-binding site. The metal-bound conformation that gives rise to new proteolytic products with both proteinase K and trypsin, we used the conservative D64N deletion (Fig. 4), and nearby Asp 279 can be substituted with alanine without affecting this metal-induced change. Thus, binding of metal ions to a still undefined locus is postulated to stabilize the changes that ensue as a consequence of metal binding coincides with their ability to support catalysis. A 10-fold increase in half-life of HIV-1 IN contributed by Mn\(^{2+}\) binding (Fig. 6) translates to a change in free energy of \(-1.6\) kcal/mol available for catalysis.

The second effect that we have observed is the metal-induced appearance of new proteolytic digestion products (denoted band II) that include the catalytic core domain. Binding of metal ions to the D,D(35)E motif in HIV-1 IN abolishes the conformational change that gives rise to new proteolytic products with both proteinase K and trypsin, we used the conservative D64N deletion (Fig. 4), and nearby Asp 279 can be substituted with alanine without affecting this metal-induced change. Thus, binding of metal ions to a still undefined locus is postulated to explain this resistance to proteolysis. We are currently introducing single and multi-amino acid substitutions into HIV-1 IN in an attempt to uncover this putative new metal-binding site. The metal-bound conformation that gives rise to new proteolytic products with both proteinase K and trypsin, we used the conservative D64N deletion (Fig. 4), and nearby Asp 279 can be substituted with alanine without affecting this metal-induced change. Thus, binding of metal ions to a still undefined locus is postulated to explain this resistance to proteolysis. We are currently introducing single and multi-amino acid substitutions into HIV-1 IN in an attempt to uncover this putative new metal-binding site. The metal-bound conformation that gives rise to new proteolytic products with both proteinase K and trypsin, we used the conservative D64N deletion (Fig. 4), and nearby Asp 279 can be substituted with alanine without affecting this metal-induced change. Thus, binding of metal ions to a still undefined locus is postulated to explain this resistance to proteolysis. We are currently introducing single and multi-amino acid substitutions into HIV-1 IN in an attempt to uncover this putative new metal-binding site. The metal-bound conformation that gives rise to new proteolytic products with both proteinase K and trypsin, we used the conservative D64N deletion (Fig. 4), and nearby Asp 279 can be substituted with alanine without affecting this metal-induced change. Thus, binding of metal ions to a still undefined locus is postulated to explain this resistance to proteolysis. We are currently introducing single and multi-amino acid substitutions into HIV-1 IN in an attempt to uncover this putative new metal-binding site.
rivative and trypsin digestion to identify the regions of the protein that are affected by this change. This approach had the advantage that it allowed the addition of metal ions to both the D64N and the conformation-competent parental proteins and thus excluded nonspecific cation effects and the changes that result in increased resistance of full-length and slightly shorter fragments described above. By comparing products of the D64N and parental proteins using mass spectrometry, we identified the following specific metal-induced changes in the conformation of HIV-1 IN.

The addition of metal ions rendered a trypsin cleavage site at Lys14 in the N terminus of HIV-1 IN less accessible to digestion. In the solution structure of an isolated N-terminal fragment of HIV-1 IN (residues 1–55) determined by NMR, two conformations, D and E, of the first helix of the N terminus have been illustrated by depicting the susceptible last six residues of the helix with faint, dashed, crisscrossing rectangles.
have a role in catalysis. The location of Lys\textsuperscript{258} on the presumed DNA binding surface of the SH3-like domain (22, 23) allows us to speculate that changes in this region may lead to reorganization of the DNA binding domain and that this may also be important for catalysis.

What do these observed metal-induced conformational changes reveal about the mechanism of catalysis? The results reported here indicate that the conserved residues of the D,D(35)E motif mediate both catalytic activity and conformational change. Amino acids Lys\textsuperscript{258} and Lys\textsuperscript{259} in the core domain of HIV-1 IN have recently been implicated in specific binding of substrate DNA (36). The region that includes Lys\textsuperscript{258} has also been implicated in sequence-independent DNA interactions that may involve the target host DNA (37). We have shown previously that a metal-induced conformational change activates HIV-1 IN and that blocking the structural change abolishes catalytic activity (25). Results reported here indicate that the structures of regions that include both the metal-binding and substrate-binding residues are altered upon binding to the required metal cofactor. It seems likely that the restructuring of these regions produces a conformation that can interact more productively with the substrate DNA. We suggest that the stabilization of the N-terminal α-helix 1, the organization of the substrate-binding basic residues in the C-terminal SH3-like domain, and ordering of the metal-coordinating residues of the D,D(35)E motif and the extended loop (Fig. 10) may all contribute to the enhanced specific activity associated with the preincubation of HIV-1 IN with metal ions (25, 31, 32). As the active form of IN is a multimer, it is possible that some of the protein reorganization induced by metal ions affects inter- as well as intramolecular interactions. Additional experiments will be required to address this question.

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Fig. 10. Ribbon models of the three-dimensional structures of the three domains of HIV-1 IN showing regions affected by metal binding at the core D,D(35)E motif. Regions corresponding to peptides Phe\textsuperscript{1}–Lys\textsuperscript{20} and Tyr\textsuperscript{15}–Arg\textsuperscript{20} in the N-terminal domain that become protected against trypsin proteolysis as a consequence of metal ion coordination by residues of the D,D(35)E motif (in red) are indicated in pink and red, respectively. The green Zn\textsuperscript{2+} ion is exaggerated to make it more visible. Regions corresponding to peptides Phe\textsuperscript{1}–Lys\textsuperscript{20} and Tyr\textsuperscript{15}–Arg\textsuperscript{20} in the N-terminal domain that become protected against trypsin in the metal-bound conformation of HIV-1 IN are labeled and shown in a ball and stick representation. Evolutionarily conserved residues in the N-terminal and catalytic core domain are similarly represented. The region corresponding to the peptide highlighted with green in the C-terminal domain, Val\textsuperscript{295}–Arg\textsuperscript{298} becomes more accessible to trypsin proteolysis in the presence of the metal ion. The accession number for the crystal structure of the apocore domain of HIV-1 IN from the Protein Data Bank (Brookhaven National Laboratory, Upton, NY) is 2ttg; numbers for the NMR structures of the N-terminal (E form) and the C-terminal domain are 1WJC and 1HWH, respectively. The figure was created with the program MOLSCRIPT (39) and rendered with RASTER3D (35, 40). aa, amino acids.

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