Zn^{2+} Promotes the Self-Association of Human Immunodeficiency Virus Type-1 Integrase *in Vitro*

S. Paul Lee,[‡] Jianming Xiao,[§] Jay R. Knutson,[§] Marc S. Lewis,[||] and Myun K. Han*,[‡]

Department of Biochemistry and Molecular Biology, Georgetown University Medical Center, Washington, DC 20007, and Laboratory of Cell Biology, National Heart, Lung, and Blood Institute, and Biomedical Engineering and Instrumentation Program, National Institutes of Health, Bethesda, Maryland 20892

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ABSTRACT: It has been recently demonstrated that the Mg^{2+}-dependent 3′-processing activity of purified human immunodeficiency virus type-1 (HIV-1) integrase is stimulated by the addition of exogenous Zn^{2+} [Lee, S. P., & Han, M. K. (1996) *Biochemistry 35*, 3837−3844]. This activation was hypothesized to result from integrase self-association. In this report, we examine the Zn^{2+} content of purified HIV-1 integrase by atomic absorption spectroscopy and by application of a thiol modification reagent, *p*-(hydroxymercuri)benzenesulfonate, with a metallochromic indicator, 4-(2-pyridylazo)resorcinol. We find that the Zn^{2+} content of HIV-1 integrase varies from 0.1 to 0.92 equiv of Zn^{2+} per monomer depending on the conditions of protein purification. *In vitro* activity assays, time-resolved fluorescence emission anisotropy, and gel filtration chromatographic analyses all indicate that EDTA yields an apoprotein which is predominantly monomeric and less active with Mg^{2+}. Further, sedimentation equilibrium studies reveal that reconstitution of the apoprotein with Zn^{2+} results in a monomer−tetramer−octamer transition. These results suggest that Zn^{2+} promotes a conformation with enhanced oligomerization and thereby stimulates Mg^{2+}-dependent 3′-processing. This may also imply that multimers larger than dimers (tetramers and possibly octamers) are required for *in vitro* activity of integrase in the presence of Zn^{2+} and Mg^{2+}. It should be noted, however, that the content of Zn^{2+} did not significantly affect the 3′-processing and strand transfer reactions with Mn^{2+} *in vitro*.

Retroviral integration is an essential step in the retrovirus life cycle. For the human immunodeficiency virus type-1 (HIV-1),[1] integration consists of distinct biochemical reactions that are catalyzed by integrase (Kulkosky & Skalka, 1994). In the presence of divalent cation (Mg^{2+} or Mn^{2+}), HIV-1 integrase cleaves two nucleotides from the 3′-ends of the viral DNA (product of reverse transcription), a step referred to as "3′-processing". The recessed 3′-ends are then covalently joined into the host target DNA via a concerted cleavage−ligation reaction, the "strand transfer" reaction. This results in a gapped recombination intermediate which is presumably repaired by the host DNA repair mechanism. In addition, two other activities, disintegration (Chow et al., 1992) and alternative disintegration (Lee et al., 1995), have been observed *in vitro* upon incubation of integrase with the recombination intermediate.

Three distinct domains in HIV-1 integrase have been identified by sequence homology with retroviral integrases, retrotransposons, and bacterial insertion sequence elements: the N-terminal domain, the central core domain, and the C-terminal domain (Kulkosky & Skalka, 1994). The N-terminus contains a HHCC motif, a pair of histidine and cysteine residues, which has been postulated to form a zinc-finger domain (Johnson et al., 1986). Direct spectroscopic studies (Burke et al., 1992; Haugan et al., 1995) and zinc blotting studies (Bushman et al., 1993) have demonstrated that the HHCC region coordinated Zn^{2+}, while point mutations of His or Cys residues in the HHCC region resulted in a loss of Zn^{2+} binding. The role of Zn^{2+} coordination in integrase function is presently unclear.

In an effort to elucidate the role of the HHCC region, point and deletion mutants have been previously examined. Deletion of the HHCC motif and the point mutants (H9N and H13N) of RSV integrase were active *in vitro* in the presence of Mn^{2+} (Khan et al., 1991; Bushman & Wang, 1994; Katz et al., 1996). Similarly, mutants of HIV-1 integrase (H16C and H16V) retained nearly wild-type activities in the presence of Mn^{2+} (Leavitt et al., 1993). These observations indicated that alterations in the HHCC region did not significantly affect the integrase-related activities with Mn^{2+}. Zn^{2+} binding, therefore, did not appear to be essential for these particular activities of integrase *in vitro*. In contrast to these Mn^{2+}-dependent activities, however, the HHCC mutants of RSV integrase were virtually inactive with Mg^{2+} (Khan et al., 1991). In addition, T-cell line infectivity assays for a panel of HIV-1 integrase mutants demonstrated that mutations in the HHCC motif do not produce infectious particles (Cannon et al., 1994; Wiskerchen & Muesing, 1995; Leavitt et al., 1996). These results suggested that the activities with Mn^{2+} *in vitro* may not be reliable predictors of viral infectivity. Stimulation of 3′-cleavage reactions by the addition of Zn^{2+} with Mg^{2+} (Lee & Han, 1996), but not with Mn^{2+}, further supports this assertion. Increasing evidence suggests that, although the *in vitro* activities with Mg^{2+} and

* To whom correspondence should be addressed.
[‡] Georgetown University Medical Center.
[§] National Heart, Lung, and Blood Institute.
[||] National Institutes of Health.
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[1] Abbreviations: HIV-1 IN, human immunodeficiency virus type-1 integrase; RSV, Rous sarcoma virus; OP, 1,10-phenanthroline; PAR, 4-(2-pyridylazo)resorcinol; PMPS, *p*-(hydroxymercuri)benzenesulfonate.

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Mn^{2+} may be similar, the Mg^{2+}-dependent reactions *in vitro* better reflect the functional events which occur *in vivo* (Lee et al., 1995a,b; Blain & Goff, 1996).

In this report, both atomic absorption spectroscopy and *p*-(hydroxymercuri)benzenesulfonate (PMPS)-promoted Zn^{2+} release in the presence of the metalloindicator, 4-(2-pyridylazo)resorcinol (PAR), were employed to determine the Zn^{2+} content of purified HIV-1 integrase. Reconstitution experiments with different divalent cations were also performed. The effects of Zn^{2+} and other divalent cations were examined by activity assays, size-exclusion chromatography, fluorescence emission anisotropy, and sedimentation equilibrium techniques. These studies correlate Zn^{2+} content, reversible self-association, and the Mg^{2+}-dependent activity of HIV-1 integrase. In particular, the sedimentation equilibrium studies quantitatively differentiate the metal-dependent modes of reversible self-association.

EXPERIMENTAL PROCEDURES

Purification of HIV-1 Integrase. Plasmid pINSD, which contains the gene encoding HIV-1 integrase, and a plasmid encoding deletion mutant IN(50−212/F185K) were both generously provided by Dr. Robert Craigie. These plasmids were overexpressed in *Escherichia coli* strain BL21(DE3). Wild-type integrase protein was purified according to a procedure previously described (Sherman & Fyfe, 1990) with the following modifications: EDTA was omitted, and 10 μM $ZnSO_4$ was added to all buffers. The purified protein was dialyzed against buffer containing 25 mM HEPES, pH 7.5, 2 mM DTT, 1 M NaCl, and 20% glycerol (v/v) prior to storage at −80 °C. The deletion mutant integrase (50−212/F185K) was purified according to the previously described procedure (Jenkins et al., 1995). Protein concentrations were determined by the method of Bradford with BSA as the standard (Bradford, 1976) and by absorbance using an extinction coefficient of 1.31 mL mg^{-1} cm^{-1} at 280 nm for wild-type integrase, calculated from the amino acid sequence of integrase for a temperature of 25 °C according to the values of Perkins (1986). Dialyses of the protein samples were performed using acid-washed plastic containers in order to avoid metal ion contamination from glass.

3′-Processing Assays. Preparations of oligonucleotides and 3′-processing reactions were performed as previously described (Lee & Han, 1996). The terminal U5 LTR sequences of the substrates are:

35 bp:5′-GACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAGT

3′-CTGGGAAAATCAGTCACACCTTTTAGAGATCGTCA

Reaction of HIV-1 Integrase with PMPS in the Presence of PAR. HIV-1 integrase was dialyzed against Chelex 100 resin (Bio-Rad) treated buffer containing 25 mM HEPES, pH 7.0, 1 M NaCl, and 10% (v/v) glycerol. PMPS, PAR, and Chelex-treated buffer solutions were prepared and tested by the procedures previously described (Hunt et al., 1985). The concentration of Zn^{2+} in solution was determined by using a molar extinction coefficient at pH 7.0 for $(PAR)_2$·Zn complex formation of 66 000 M^{-1} cm^{-1} at 500 nm (Hunt et al., 1984, 1985). Absorbance measurements were performed using a Milton Roy Spectronic 3000 diode array spectrophotometer. Zn^{2+} analysis by atomic absorption spectroscopy was performed by Galbraith Laboratories, Inc., using a Perkin-Elmer 4100 ZL atomic absorption spectrophotometer equipped with a HGA-graphite furnace (detection limit was 3.0 ppb).

FPLC Gel Filtration Chromatography. Upon dialysis in the indicated buffers (see Results and Discussion), HIV-1 integrase proteins were chromatographed on a Pharmacia FPLC Superose 12 HR 10/30 column. The column was equilibrated with 25 mM HEPES, pH 7.0, 1 M NaCl (buffer LC), and calibrated with blue dextran 2000, bovine serum albumin, hen ovalbumin, and chymotrypsinogen A (Pharmacia). Proteins were eluted with a flow rate of 0.4 mL/min, and the elutions were recorded by continuously monitoring the A_{280}. Prior to chromatographic analysis, the samples were centrifuged for 30 min at 14 000 rpm in a microcentrifuge to remove any large protein aggregates.

Fluorescence Studies. Steady-state and time-resolved fluorescence studies were performed using an SLM-8000 spectrophotofluorometer and a time-correlated single-photon counting instrument with a frequency-doubled dye laser light source as previously described (Han et al., 1990; Lee et al., 1994).

Analytical Ultracentrifugation. Sedimentation equilibrium measurements of wild-type integrase were performed using a Beckman XL-A centrifuge at 14 000 rpm and at 4 °C with six-channel centerpieces (wild-type integrase) or at 20 000 rpm and at 4 °C with two-channel centerpieces (50−212, F185K). Sample volumes were 0.12 mL, giving column heights of approximately 3 mm. All scans were performed at 280 nm after allowing 48 h to reach equilibrium. The data were edited by using the XL-A software and analyzed by mathematical models using MLAB (Civilized Software, Bethesda, MD) to perform nonlinear least-squares curve fitting.

RESULTS AND DISCUSSION

Determination of the Zinc Content. It has been previously established that recombinant integrase effectively uses Mg^{2+} as a metal cofactor, and that this activity is enhanced either by longer DNA substrates (Lee et al., 1994, 1995a,b) or by higher protein concentrations (Engelman & Craigie, 1995; Lee & Han, 1996). In addition, we have recently demonstrated that addition of exogenous Zn^{2+} yielded more significant effects on Mg^{2+}-dependent 3′-processing at low integrase concentrations than at higher protein concentrations (Lee & Han, 1996). The Zn^{2+} chelator, 1,10-phenanthroline (OP), was found to inhibit Mg^{2+} activity without affecting the Mn^{2+}-dependent 3′-processing activity (Lee & Han, 1996). As previously mentioned, the N-terminal domain is known to bind Zn^{2+} (Burke et al., 1992; Haugan et al., 1995; Bushman et al., 1993), and this domain has been implicated in the multimerization of HIV-1 integrase (Ellison et al., 1995). It was suggested, therefore, that the stimulation by Zn^{2+} was due to the likely presence of apoproteins produced during purification and dialysis in EDTA, and that Zn^{2+} was in fact important for the self-associations of retroviral integrases (Lee & Han, 1996).

We determined the Zn^{2+} content of two different HIV-1 integrase preparations by two methods: (1) atomic absorption and (2) a spectroscopic method using PMPS and PAR. The two protein samples differed only in their method of purification. The first sample of integrase was purified according to the previously described procedure (Lee & Han,

1996) in the presence of 0.1 mM EDTA with a final dialysis in 1 mM EDTA. The second sample of integrase was purified in the presence of 10 μM Zn^{2+}, and EDTA was omitted in all purification steps to optimally preserve bound Zn^{2+}. Both samples were dialyzed against Chelex-treated buffer A containing 25 mM HEPES, pH 7.5, 1 M NaCl, and 10% glycerol prior to Zn^{2+} analyses.

Atomic absorption analyses determined that integrase purified in the presence of EDTA (sample 1) contained 0.12 equiv of Zn^{2+} per integrase monomer. The presence of some bound Zn^{2+} suggests that the affinity of integrase for Zn^{2+} is such that 1 mM EDTA could not effect total removal. In contrast, the Zn^{2+} content of integrase purified in the presence of Zn^{2+} and without EDTA (sample 2) was 0.67 equiv of of Zn^{2+} per integrase monomer. This result suggests that an integrase monomer is capable of binding 1 equiv of Zn^{2+}; however, some loss of bound Zn^{2+} during purification must be expected. These results make plausible the previous view that the stimulatory effect of exogenous Zn^{2+} was due to partial restoration of Zn^{2+} lost during purification in the presence of EDTA.

The concentration of "tightly bound" Zn^{2+} (e.g., in a zinc finger) can also be spectroscopically determined with a thiol-specific reagent such as PMPS and the metallochromic indicator PAR. PMPS, a common modification agent for proteins with metal−cysteinate coordination, reacts with cysteine to form a mercury−thiolate complex. This triggers the release of bound Zn^{2+} by significantly weakening the protein−metal ion interaction. The released Zn^{2+} will then form a $Zn^{2+}/(PAR)_2$ complex accompanied by an enhanced absorbance at 500 nm (Hunt et al., 1984, 1985). This method was used to determine the Zn^{2+} contents of aspartate transcarbamoylase (Hunt et al., 1984, 1985), TFIIIA (Han et al., 1995), and gene 32 protein from T4 (Giedroc et al., 1986).

Utilizing this method, the Zn^{2+} contents of the aforementioned preparations were determined to be 0.2 equiv of Zn^{2+} per integrase monomer for sample 1 and 0.6 ± 0.15 equiv of Zn^{2+} per integrase monomer for sample 2. These results are in good agreement with the atomic absorption analyses, further supporting our previous assertion that there is a considerable amount of Zn^{2+} lost due to purification in the presence of EDTA. Overall, the Zn^{2+} appears to be tightly bound (e.g., dialysis-resistant) to HIV-1 integrase.

The two integrase samples were then examined for their 3′-processing activity (Mg^{2+} or Mn^{2+}) using the U5 35 bp oligonucleotide LTR substrate. Zn^{2+}-bound integrase (sample 2) removed the two terminal nucleotides in the presence of either Mg^{2+} or Mn^{2+} (data not shown). In contrast, integrase purified in EDTA with reduced Zn^{2+} content (sample 1) was fully active in 3′-processing with Mn^{2+}, while significantly reduced activities were observed with Mg^{2+} (data not shown). Therefore, consistent with our previous results, Zn^{2+} is important for the Mg^{2+}-dependent activity but is not as significant for the Mn^{2+} activity (Lee & Han, 1996). The Zn^{2+} requirement may explain early work which found that HIV-1 integrase did not catalyze 3′-processing reactions efficiently in Mg^{2+}.

To confirm that the Zn^{2+} bound was not due to coordination to the core domain, the PMPS/PAR reactions were performed on deletion mutant (50−212, F185K) which lacks both the N- and C-terminal domains (Jenkins et al., 1995).

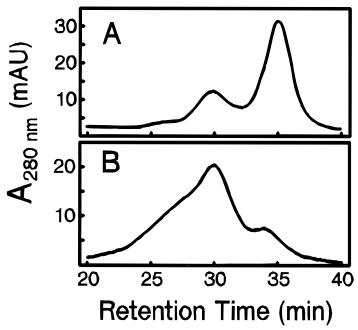

FIGURE 1: Gel filtration chromatography of HIV-1 integrase. HIV-1 integrase proteins (500 μL; 10 μM) were chromatographed on a FPLC Superose 12 HR 10/30 column equilibrated with buffer LC. Elution profiles of HIV-1 integrase sample 1 (panel A) and sample 2 (panel B) were monitored at 280 nm. See text for sample preparation.

If Zn^{2+} binds to the putative zinc finger, this mutant should not contain Zn^{2+}. In fact, PMPS-promoted Zn^{2+} release was not detectable with this mutant (data not shown). These results further suggest that the N-terminal domain is responsible for the observed phenomenon.

Purified Recombinant HIV-1 Integrase Exhibits Zn^{2+}-Dependent Multimerization. Since the integrase samples with different Zn^{2+} contents displayed different activities *in vitro*, we sought a relationship between Zn^{2+} content and possible alterations in conformation as reflected by multimerization of HIV-1 integrase. The apparent molecular sizes of the two aforementioned HIV-1 integrase samples were examined on a FPLC Superose 12 column (Pharmacia) equilibrated with buffer LC at 4 °C. Sample 1 (EDTA) exhibited a small peak eluting at 30 min and a predominant peak eluting at 35 min (Figure 1A). These protein peaks correspond roughly to dimeric (30 min) and monomeric (35 min) integrase species as determined from a calibration curve of molecular weight standards. Sample 2, in contrast, eluted predominantly at or before 30 min with a small peak at 35 min (Figure 1B). The broad contribution near 27 min may represent tetrameric and larger complexes of integrase. This elution profile indicates that the recombinant HIV-1 integrase purified in the presence of Zn^{2+} (absence of EDTA) is predominantly multimeric, whereas that which is purified in the presence of EDTA is primarily monomeric.

It has been previously reported that purified integrase is a dimer as determined by gel filtration and glycerol gradient studies (Sherman & Fyfe, 1990; Vincent et al., 1993; Grandgenett et al., 1978). This is in contrast to sedimentation studies which indicate that, in the presence of 1 mM EDTA, both full-length (1−288) and truncated (50−212) HIV-1 integrases undergo a monomer−dimer transition with dissociation constants of 25 and 80 μM, respectively (Hickman et al., 1994). The Zn^{2+} analyses presented above, however, suggest that in the presence of 1 mM EDTA a significant fraction of the integrase protein is in the form of apoprotein. Further, the gel filtration studies above showed that Zn^{2+} loss promotes dissociation of multimeric integrase. Therefore, the relatively weak interactions (25 μM for dimer) determined by prior sedimentation equilibrium studies might be accounted for by the presence of EDTA. It should be noted, however, that roles for other divalent cations have yet to be ruled out.

As demonstrated above, the loss of Zn^{2+} produced predominantly monomeric species with reduced activity in

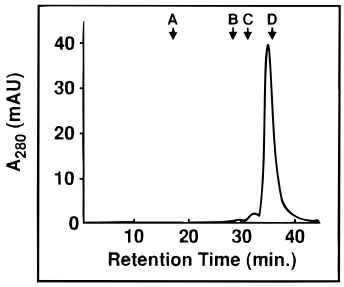

FIGURE 2: FPLC elution profile of the apoprotein of HIV-1 integrase. HIV-1 integrase apoprotein was chromatographed on the Superose 12 HR 10/30 column equilibrated with buffer LC supplemented with 1 mM DTT (see text for sample preparation). Arrows A, B, C, and D show the elution times of blue dextran-2000, bovine serum albumin (67 kDa), hen ovalbumin (45 kDa), and chymotrypsinogen A (25 kDa), respectively.

Mg^{2+}. In contrast, the predominantly multimeric sample 2 exhibited significant Mg^{2+}-dependent activity. Previous studies have indicated that multimeric integrase is active (Ellison et al., 1995; Jones et al., 1992; van Gent et al., 1993; Engelman et al., 1993). If so, the activity with sample 1 in the presence of Mn^{2+} might be explained by the propensity of integrase to form multimers or aggregates in the presence of this divalent cation (van Gent et al., 1991; Ellison et al., 1995; Pemberton et al., 1996; Wolfe et al., 1996).

Reconstitution of Apo-Integrase with Metal Ions. To investigate the possible role of other divalent cations on the self-associative interactions of HIV-1 integrase, general metal ion reconstitution experiments were performed. These experiments require the initial removal of any intrinsic metal ions with chelating agents. The chelating agent must then be removed without appreciable reactivation of the apoenzyme by "adventitious" metal ions. Accordingly, sample 3 was prepared by dialyzing integrase (~10 μM) against several changes of buffer A supplemented with 1 mM DTT and 10 mM EDTA, followed by dialysis against the same buffer without EDTA. Figure 2 shows the elution profile of the resulting sample 3, which is primarily monomeric.

Initially, we wanted to determine whether reconstituted protein would bind appreciable levels of Zn^{2+}. We measured the Zn^{2+} content of reconstituted integrase by the previously described PMPS/PAR method. Sample 3 was dialyzed against buffer A containing 10 μM Zn^{2+}, followed by dialysis against Chelex-treated buffer A to remove any free Zn^{2+}. Figure 3 shows the PMPS titration of this reconstituted protein. The addition of PMPS released 0.92 equiv of Zn^{2+} per integrase monomer. Thiol reagents such as β-mercaptoethanol can reverse PMPS cysteine modification. This reversal might permit Zn^{2+} rebinding to the protein, depending on the apoprotein stability and the relative affinities of Zn^{2+} to PAR and to integrase. As demonstrated by the return of the absorbance at 500 nm to the base line, β-ME reversal of the PMPS reaction resulted in the rebinding of Zn^{2+} to HIV-1 integrase (Figure 4). Hence, PMPS-modified integrase retains enough structure to be easily returned to a state capable of tighter Zn^{2+} binding than the metalloindicator. Rebinding of the Zn^{2+} to the protein also suggests that functional reconstitution is attainable.

We performed reconstitution studies with other divalent metal cations (Mg^{2+} and Mn^{2+}). Aliquots (1 mL each) of sample 3 (~10 μM) were dialyzed against buffer A supplemented with 1 mM DTT and either 7.5 mM Mg^{2+}

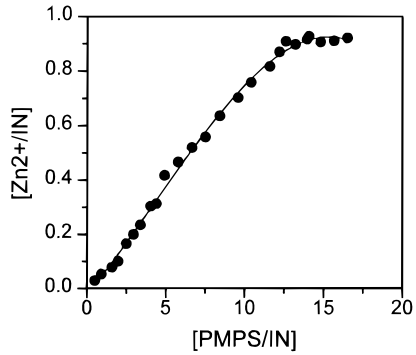

FIGURE 3: Determination of Zn^{2+} content of reconstituted HIV-1 integrase by PMPS/PAR analysis. PMPS/PAR experiments were performed using Chelex-treated buffer containing 25 mM HEPES, pH 7.0, 1 M NaCl, 10% glycerol (v/v), and 0.2 mM PAR. The integrase apoprotein (5 μM) dialyzed in the presence of 10 μM Zn^{2+} was titrated with PMPS in a total volume of 400 μL (see text for sample preparation). The absorbance was recorded upon each addition of PMPS, and the concentrations of Zn^{2+} in solution were determined as described under Experimental Procedures.

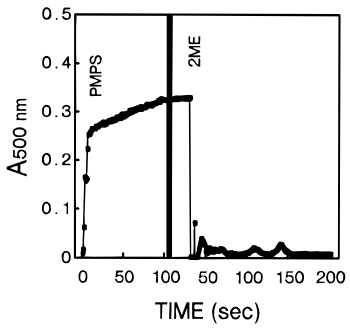

FIGURE 4: Reversal of PMPS modification. IN (5 μM) was incubated with 0.2 mM PAR in Chelex-treated 25 mM HEPES, pH 7.0, 1 M NaCl, and 10% glycerol (v/v) buffer and used as the reference. An excess of PMPS was then added, and the absorbance changes at 500 nm were monitored. Once a plateau was reached, an excess of β-ME was added. Rebinding of the Zn^{2+} to the protein caused the absorbance at 500 nm to return to the base line.

(sample 4), 7.5 mM Mn^{2+} (sample 5), 10 μM Zn^{2+} (sample 6), or 10 μM Zn^{2+} with 7.5 mM Mg^{2+} (sample 7). These samples were then centrifuged for 30 min at 14 000 rpm to remove any large aggregates. The protein reconstituted with Mn^{2+} exhibited significant aggregation even in the presence of 1 M NaCl. Mn^{2+}-dependent aggregation of the integrase protein has been previously reported (Ellison et al., 1995; van Gent et al., 1991; Pemberton et al., 1996). In contrast, dialysis against Mg^{2+} and/or Zn^{2+} did not result in significant aggregation as indicated by the absence of a visible pellet after centrifugation.

The elution profiles of these protein samples are shown in Figure 5. Sample 4 (8.8 μM after dialysis in the presence of 7.5 mM Mg^{2+}) contained both monomeric and larger species (Figure 5A). Sample 5 (dialyzed with 7.5 mM Mn^{2+}), although tested at a very low protein concentration (due to depletion by centrifugal removal of aggregate), also showed both monomeric and larger species (Figure 5B). Sample 6 (dialyzed with 10 μM Zn^{2+}) exhibited mostly multimeric species (Figure 5C). Similarly, sample 7 (dialyzed with both 10 μM Zn^{2+} plus 7.5 mM Mg^{2+}) eluted mostly as multimers (Figure 5D). From these results, it is apparent that Zn^{2+} is not the only cation with influence on protein−protein interactions of HIV-1 integrase.

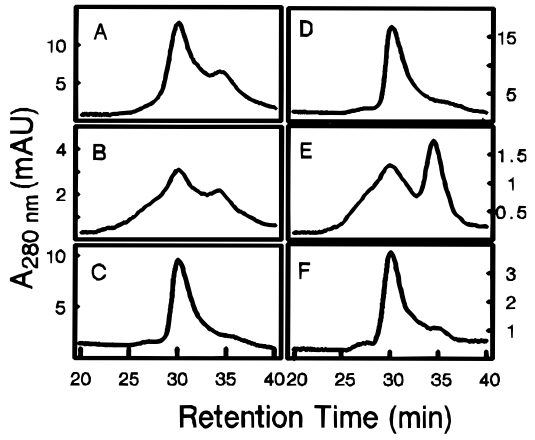

FIGURE 5: FPLC elution profiles of HIV-1 integrase reconstituted with various metal ions. Elution profiles of the different reconstituted samples (150 μL; 8.8 μM) with (A) 7.5 mM Mg^{2+}, (B) 7.5 mM Mn^{2+}, (C) 10 μM Zn^{2+}, and (D) 7.5 mM Mg^{2+} plus 10 μM Zn^{2+}. Elution profiles (E) and (F) represent 1:4 diluted reconstituted samples 4 and 6. Samples were prepared as described in the text and chromatographed with buffer LC supplemented with 1 mM DTT. The elution times of the protein standards were 29.3 min for bovine serum albumin (67 kDa), 32.2 min for hen ovalbumin (45 kDa), and 36.2 min for chymotrypsinogen A (25 kDa), respectively.

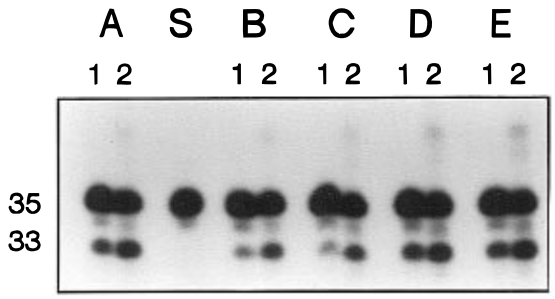

FIGURE 6: 3′-Processing activity of reconstituted HIV-1 integrase. 3′-Processing activity was assayed with the 35 bp DNA substrate as described under Experimental Procedures. Reactions were performed with 0.25 μM HIV-1 integrase for all instances. Lanes 1 and 2 represent reactions with 7.5 mM $MgCl_2$ and 7.5 mM $MnCl_2$, respectively. S represents the substrate in the absence of HIV-1 integrase. Panel A represents a control reaction performed with integrase purified in the presence of Zn^{2+} (sample 2). Panels B−E represent reactions with sample 3 (B) and reconstituted integrase with Mg^{2+} (C), Zn^{2+} (D), and Mg^{2+} plus Zn^{2+} (E).

The role of divalent cations was further probed by diluting samples 4 and 6 4-fold. A redistribution of the monomer and multimer species was observed when sample 4 (Mg^{2+}) was diluted (Figure 5E), with dilution increasing the proportion of apparent monomer. In contrast, when sample 6 (Zn^{2+}) was diluted 4-fold, the protein still eluted in the multimeric peak (Figure 5F). Zn^{2+}, therefore, is more potent than Mg^{2+} in stabilizing the multimers of HIV-1 integrase.

In vitro activity assays of these metal-reconstituted samples indicated that all samples assayed were active with Mn^{2+}, while only samples 6 (with Zn^{2+}) and 7 (with Zn^{2+}/Mg^{2+}) were active in the presence of Mg^{2+} (Figure 6). Sample 5 could not be assayed due to the low protein concentration after aggregation. These results suggest that the apoenzyme was reactivated more efficiently upon reconstitution with either Zn^{2+} (Figure 6D) or Zn^{2+}/Mg^{2+} (Figure 6E). The small amount of Mg^{2+}-dependent activity observed with apoprotein (Figure 6B) may be attributed to the extreme difficulty in removing all the adventitious Zn^{2+} in the assay conditions. Nevertheless, these results strongly implicate

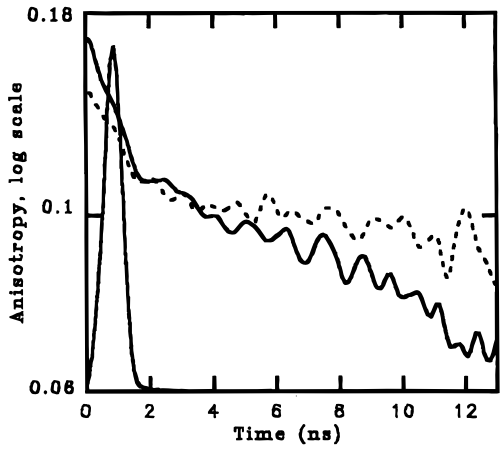

FIGURE 7: Fluorescence emission anisotropy decay of HIV-1 integrase. Time-resolved emission anisotropy of the "monomeric apoprotein" (solid line) and "mixed multimeric holo-enzyme" (dashed line) was performed as described in the text. The raw anisotropy data were smoothed 3 times with 5-point convolution smoothing to remove high-frequency noise. log anisotropy curves with a linear "lamp" (instrument response) curve are displayed to show that the anisotropy curves at longer times are unaltered by convolution.

Zn^{2+} in promoting the appropriate conformer for Mg^{2+}-dependent 3′-processing activity.

Fluorescence Studies of HIV-1 Integrase. We examined the apparent molecular sizes of HIV-1 integrase in various states using time-resolved fluorescence anisotropy. In such experiments, Brownian motion yields a rotational correlation time indicative of molecular size. For short-lived intrinsic fluorophores like tryptophan, such data can only distinguish monomers from multimers since fluorescence from the probes decays before significant differences can be seen between dimer, tetramer, and higher oligomer rotation curves. Longer lived (extrinsic covalent) probes such as IAEDANS (∼18 ns) or pyrene maleimide (∼100 ns) will be needed to make more detailed size analyses.

First, we proceeded on the hypothesis that the elution profiles above (Figure 1) were dominated by true heterogeneity rather than by kinetic trapping in the gel; e.g., that each sample was a mix of apo- and holoprotein eluting at representative times. Some kinetic trapping of monomer−multimer equilibria in the gel must be expected, leading to tailing of peaks and bias toward longer retention species. However, if heterogeneity is dominant, different fractions will still retain different average sizes.

Using tryptophan anisotropy, we found greater than 2-fold difference in apparent molecular size between protein peaks eluted at 30 vs 35 min (Figure 1). The former peak is largely (70−90%) comprised of holoprotein, and the latter is ∼90% apoprotein. We recovered average rotational correlation times of 27 and 64 ns, respectively, for the "monomeric" apoprotein and for the "mixed multimeric holo-integrase" (Figure 7). Steady-state emission anisotropies for "monomeric" and "mixed" integrase were 0.102 ± 0.004 and 0.123 ± 0.008, respectively. Both steady-state and time-resolved emission anisotropy were measured with an excitation wavelength of 295 nm and an emission wavelength of 350 nm at 25 °C.

Following these fluorescence benchmarks, Zn^{2+} promotion of associations was monitored via steady-state anisotropy. The "monomeric apoprotein" previously eluted at 35 min

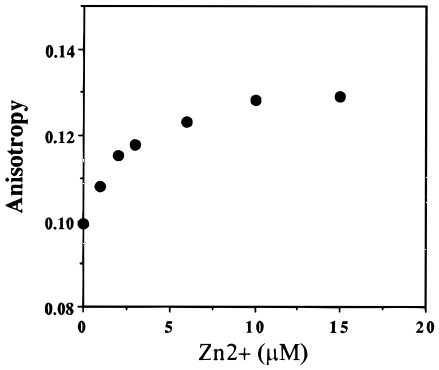

FIGURE 8: Reconstitution of monomeric HIV-1 integrase with Zn^{2+} monitored by steady-state anisotropy. Steady-state anisotropy of the protein fraction that eluted at 35 min (Figure 1A) was measured with increasing concentrations of Zn^{2+} in 25 mM HEPES, pH 7.0, 1 mM DTT, and 1 M NaCl at 25 °C. The protein concentration was 1.5 μM. Anisotropy was monitored with excitation and emission wavelengths of 295 and 350 nm, respectively.

was titrated with Zn^{2+} stock solution prepared in 25 mM HEPES, pH 7.0, and 1 M NaCl. As shown in Figure 8, the steady-state anisotropy increased from approximately 0.10 to 0.128 with increasing Zn^{2+} concentrations. These data indicate that restoration of Zn^{2+} favors multimerization of integrase.

We have also examined the effects of Zn^{2+} removal using fluorescence; IAEDANS-labeled holo-integrase (labeled in the presence of Zn^{2+}) exhibited an average rotational correlation time, ϕ, of 74 ns at room temperature (90% confidence $\phi > 50$ or MW > 100 000). When treated with EDTA, this sample provided a reduced rotational correlation time near 27 ns (with 90% confidence between 23 and 38 ns, data not shown). We are working to reconcile the ϕ of 27 ns with monomeric species; we have yet to determine whether shape, heterogeneity, or hydration is responsible for this value (intermediate between spherical monomer and dimer). Kinetic anisotropy studies of both abstraction and reconstitution processes are in progress; meanwhile, both the intrinsic and extrinsic fluorophores have shown us that multimer equilibria are dominated by metal binding; i.e., competent, complexed holoprotein vs nearly monomeric apoproteins.

Analytical Ultracentrifugation of Wild-Type HIV-1 Integrase. The gel filtration profiles (Figure 5) suggest that metal ions have profound effects on multimerization of integrase. Gel filtration chromatography, however, can distort the equilibrium process between self-associating species of integrase and may not present the true molecular size of the protein since the sample may interact with the column resin. Thus, gel filtration studies are not suitable for quantitative analysis of equilibrium between the self-associating species of integrase. To obtain quantitative information, we performed sedimentation equilibrium analyses to examine the effects of Zn^{2+} on multimerization.

Sedimentation equilibrium measurements were simultaneously performed with two concentrations (0.11 and 0.2 mg/mL) of both sample 3 and sample 3 reconstituted with Zn^{2+} at 4 °C. As described earlier, sample 3 was prepared by dialysis against several changes of buffer A supplemented with 1 mM DTT and 10 mM EDTA, followed by dialysis against the same buffer without EDTA. Zn^{2+} reconstitution was performed by dialysis of sample 3 against buffer A supplemented with 0.5 mM DTT and 10 μM Zn^{2+}.

The self-association models of these integrase samples were analyzed by fitting the sedimentation data using the general equation:

$$A_r = A_{b,1}\exp[AM_1(r^2 - r_b^2)] + A_{b,1}^2\exp[\ln K_{12} - \ln(E_{280}/2) + 2AM_1(r^2 - r_b^2)] + A_{b,1}^4\exp[\ln K_{14} - \ln(E_{280}^3/4) + 4AM_1(r^2 - r_b^2)] + A_{b,1}^8\exp[\ln K_{18} - \ln(E_{280}^7/8) + 8AM_1(r^2 - r_b^2)] + \epsilon \quad (1)$$

where A_r is the total absorbance at a given radius (r), $A_{b,1}$ is the absorbance of monomer at the radial position from the cell bottom (r_b), M_1 is the compositional molecular mass of the monomer (M_1 = 32 123.6 Da), E_{280} is the molar extinction coefficient of the integrase monomer at 280 nm (E_{280} = 42 050 M^{-1} cm^{-1}), and ϵ is a small base line error correction term. A is defined as $(1 - \bar{\nu}\rho)\omega^2/2RT$, where $\bar{\nu}$ is the compositional partial specific volume of integrase ($\bar{\nu}$ = 0.729 mL/g at 4 °C), ρ is the solvent density (1.0643 g/mL at 4 °C), ω is the angular velocity of the rotor, R is the gas constant, and T is the absolute temperature.

This model is written in terms of an equilibrium constant on a molar concentration scale, where $K_{12} = C_2/C_1^2 = (A_2/2E)/(A_1/E)^2$; $K_{14} = C_4/C_1^4 = (A_4/4E)/(A_1/E)^4$; and $K_{18} = C_8/C_1^8 = (A_8/8E)/(A_1/E)^8$. This model also assumes that the apparent partial specific volumes of the monomer and the oligomers are identical. The use of ln K precludes obtaining negative values for K_{12}, K_{14}, and K_{18}; the curve fitting returns a large negative number for an absent species.

Solvent density was calculated according to Laue et al. (1992). The partial specific volume of integrase was calculated at 25 °C from the amino acid sequence using the values of Perkins (1984). A value of $\Delta\bar{\nu}/\Delta T$ = 0.000425 cm^3 g^{-1} deg^{-1} was used to calculate the value of the partial specific volume at 4 °C.

Simultaneous analysis of the data obtained with two different concentrations of sample 3 demonstrated that the optimum self-association model for this protein was a monomer−dimer−tetramer, based on the criteria of minimum sum of squares of the residuals, the most random distribution of the residuals, and minimum estimated standard errors of the parameter values. This model was consistent with previous results (Jones et al., 1992). From the data analysis, the recovered ln K_{1-2} and ln K_{1-4} values were 10.9 ± 0.6 and 33.9 ± 0.4, respectively. These values lead to apparent dissociation constants, K_d, of 1.85 × 10^{-5} M for dimer dissociation and a K_d of 1.27 × 10^{-5} M^3 for tetramer dissociation.

In contrast, the data obtained with Zn^{2+}-reconstituted integrase were best fit by a monomer−tetramer−octamer model as illustrated in Figure 9. When attempts were made to include dimer in the model, the presence of dimer was always rejected in the fitting procedure with a returned large negative value of ln K_{12}. This indicates that the dimeric species was not detectable under the experimental conditions. The recovered ln K_{1-4} and ln K_{1-8} values were 38.3 ± 0.2 and 89.1 ± 0.3, respectively. These values correspond to apparent dissociation constants, K_d, of 2.85 × 10^{-6} M^3 for tetramer dissociation and K_d of 2.97 × 10^{-6} M^7 for octamer dissociation.

These results indicate that Zn^{2+} alters both the mode and affinities of self-association of integrase. This may explain

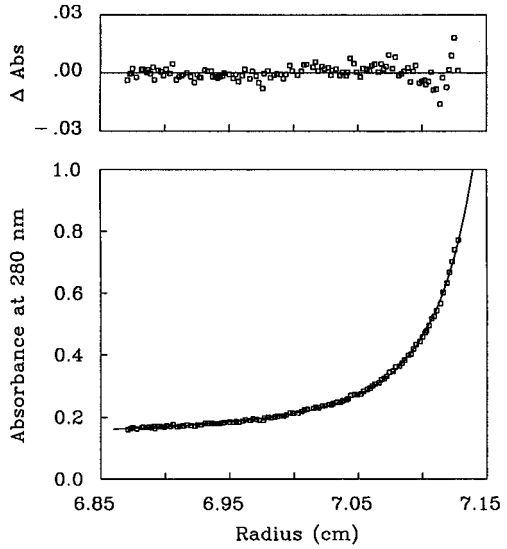

FIGURE 9: Distributions of concentration and residuals of HIV-1 integrase at centrifugal equilibrium at 14 000 rpm. Sedimentation equilibrium studies of apo-integrase reconstituted (8.8 μM) with Zn^{2+} were performed in 25 mM HEPES, pH 7.5, 1 M NaCl, 0.5 mM DTT, 10 μM $ZnSO_4$, and 10% glycerol at 4 °C. The bottom panel presents the concentration distribution fit for a monomer−tetramer−octamer equilibrium model, and the top panel displays the distribution of the residuals for this fit.

the ϕ of 74 ns obtained with the active IAEDANS-labeled integrase (larger than expected for dimer; corresponding better to tetramer or higher oligomeric size). The data obtained from sedimentation studies together with *in vitro* activity assays suggest that the enzymatically active form of integrase is oligomeric in the presence of Zn^{2+}, although it is not clear whether the tetramer form, octamer form, or both forms are active.

Analytical Ultracentrifugation of Mutant HIV-1 Integrase (50−212,F185K). To ensure that the observed tetramerization and octamerization were the result of Zn^{2+} coordination, sedimentation equilibrium studies were also performed with mutant HIV-1 integrase (50−212,F185K) which contains the conserved "D,D,E" motif, but lacks both the N- and C-terminal domains. Sedimentation equilibrium studies were performed with two different protein concentrations (0.1 and 0.2 mg/mL) of the mutant protein with two-channel centerpieces at 20 000 rpm and at 20 °C. Sample volumes were 0.18 mL, giving column heights of approximately 5 mm. The buffer was 25 mM HEPES, pH 7.5, 0.5 mM DTT, 0.5 M NaCl, 1 mM EDTA, and 10% glycerol. Figure 10 illustrates the simultaneous fit of a monomer−dimer equilibrium model to the data. The mathematical model for distribution of mutant integrase (50−212,F185K) is given by

$$A_r = A_{b,1}\exp[AM_1(r^2 - r_b^2)] + A_{b,1}{}^2\exp[\ln K_{12} - \ln(E_{280}/2) + 2AM_1(r^2 - r_b^2)] + \epsilon \quad (2)$$

where A_r is the total absorbance at a given radius (r), $A_{b,1}$ is the absorbance of monomer at the radial position from the cell bottom (r_b), M_1 is the compositional molecular mass of the monomer (M_1 = 20 005.7 Da), E_{280} is the molar extinction coefficient of mutant integrase monomer at 280 nm (E_{280} = 28 010 M^{-1} cm^{-1}), and ϵ is a small baseline error correction term.

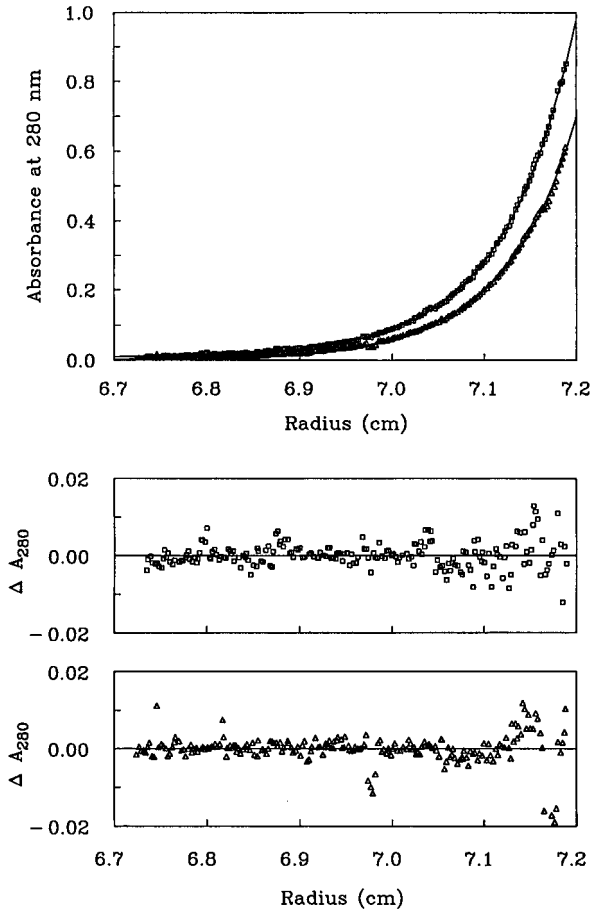

FIGURE 10: Distributions of concentration and residuals of mutant HIV-1 integrase (50−212,F185K) at centrifugal equilibrium at 20 000 rpm. Sedimentation equilibrium studies of mutant integrase (0.1 and 0.2 mg/mL) were performed in 25 mM HEPES, pH 7.5, 0.5 M NaCl, 1 mM EDTA, and 10% glycerol at 20 °C. The top panel presents the concentration distribution fit for a monomer−dimer equilibrium model, and the bottom panel displays the distribution of the residuals for this fit.

When attempts were made to include tetrameric or higher species in the model, none were detected under the experimental conditions. The recovered ln K_{1-2} was 16.4 ± 0.14, which corresponds to an apparent dissociation constant of 7.54×10^{-8} M for dimer dissociation. As mentioned earlier, recent sedimentation studies of truncated mutant integrase (50−212) indicated that this protein undergoes a monomer−dimer transition with a dissociation constant of 8.0×10^{-5} M (Hickman et al., 1994). Therefore, these results suggest that the mutation of phenylalanine to lysine at amino acid 185 (F185K) within the catalytic core domain of HIV-1 integrase not only increased the solubility of the protein (Jenkins et al., 1995) but also has a significant enhancing effect on dimerization. As previously demonstrated, Zn^{2+} binding was neither expected nor detected with this mutant. As expected, our sedimentation equilibrium and gel filtration studies did not find Zn^{2+}-induced changes in colligative properties. Thus, the core domain is capable of forming dimeric species, but appears incompetent to form tetrameric or higher oligomeric species.

CONCLUSIONS

The requirement for Zn^{2+} as a structural ion maintaining subunit interactions has been well documented in other systems. Aspartate transcarbamoylase (ATCase) contains a

single structural Zn^{2+}, important in maintaining the quaternary structure of ATCase. This is exemplified by the separation of quaternary structure into regulatory and catalytic subunits upon loss of the structural Zn^{2+} (Hunt et al., 1984). The gene 32 protein from T4 is another example of Zn^{2+} maintaining subunit interactions (Giedroc et al., 1986). NMR studies demonstrated that apo-g32P is a monomer, while Zn^{2+}-bound g32P is a dimer (Pan et al., 1989).

In summary, we present evidence that Zn^{2+} binding does not influence Mn^{2+}-dependent integrase-mediated activities, while it does play important structural and functional roles in Mg^{2+}-dependent activity. It seems that the latter better predicts the functional events which occur *in vivo* (Cannon et al., 1994; Wiskerchen & Muesing, 1995; Leavitt et al., 1996; Vora et al., 1994, 1995; Goodarzi et al., 1995). We have shown that the apoprotein adopts a conformation that yields mostly monomers (seen in both size-exclusion and fluorescence anisotropy experiments). In contrast, the holoenzyme yields multimeric (tetrameric and octameric) species as determined by both fluorescence and sedimentation equilibrium studies. *In vitro* assay systems may be critical in precisely defining and understanding the mechanism of retroviral integration, and their accuracy is essential for the screening of potential inhibitors. Confidence that the *in vitro* assay faithfully reflects *in vivo* processes of HIV-1 integrase is crucial. In this light, the contribution of divalent cations such as Mg^{2+} and Zn^{2+} to integrase structure and function is important. More thorough thermodynamic studies will be required to better understand the self-association of integrase. Since free integrase should be subject to enormous dilution in the host cell, we recognize that the measured association is probably just a "signature" for other, more significant physiological interactions. Hence, we expect that the colligative picture will be heavily influenced by other proteins and the presence of DNA. Ultimately, the active conformers promoted by Zn^{2+} must be evaluated in that more realistic light.

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