Determinants of Mg^{2+}-Dependent Activities of Recombinant Human Immunodeficiency Virus Type 1 Integrase†

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ABSTRACT: The relationship between Mg^{2+}-dependent activity and the self-assembly state of HIV-1 integrase was investigated using different protein preparations. The first preparations, IN_{CHAPS} and IN_{dial}, were purified in the presence of detergent, but in the case of IN_{dial}, the detergent was removed during a final dialysis. The third preparation, IN_{zn}, was purified without any detergent. The three preparations displayed comparable Mn^{2+}-dependent activities. In contrast, the Mg^{2+}-dependent activity that reflects a more realistic view of the physiological activity strongly depended on the preparation. IN_{CHAPS} was not capable of using Mg^{2+} as a cofactor, whereas IN_{zn} was highly active under the same conditions. In the accompanying paper [Deprez, E., et al. (2000) *Biochemistry 39*, 9275−9284], we used time-resolved fluorescence anisotropy to demonstrate that IN_{CHAPS} was monomeric at the concentration of enzymatic assays. Here, we show that IN_{zn} was homogeneously tetrameric under similar conditions. Moreover, IN_{dial} that exhibited an intermediary Mg^{2+}-dependent activity existed in a monomer−multimer equilibrium. The level of Mg^{2+}- but not Mn^{2+}-dependent activity of IN_{dial} was altered by addition of detergent which plays a detrimental role in the maintenance of the oligomeric organization. Our results indicate that the ability of integrase to use Mg^{2+} as a cofactor is related to its self-assembly state in solution, whereas Mn^{2+}-dependent activity is not. Finally, the oligomeric IN_{zn} was capable of binding efficiently to DNA regardless of the cationic cofactor, whereas the monomeric IN_{CHAPS} strictly required Mn^{2+}. Thus, we propose that a specific conformation of integrase is a prerequisite for its binding to DNA in the presence of Mg^{2+}.

The integration of a proviral cDNA copy into host DNA is a critical step in the life cycle of the human immunodeficiency virus (HIV)[1] as it ensures expression and perpetuation of the viral genome (*1*). This essential reaction is catalyzed by the viral enzyme integrase that has been shown to be necessary and sufficient for the integration reaction in vitro (*2*, *3*). In vitro studies with recombinant protein have permitted the distinction between the two main reactions catalyzed by integrase, which are processing and strand transfer (*4*, *5*). Both reactions consist of a nucleophilic attack of a phosphodiester bond by a hydroxyl group and require either Mn^{2+} or Mg^{2+} as a cofactor. During the processing reaction, the enzyme removes two 3′-nucleotides from each strand of the linear viral DNA, resulting in overhanging CA ends. In the strand transfer reaction, the 3′-processed ends acting as nucleophilic agents attack phosphodiester bonds on the opposite strand of the target DNA (for a review, see ref *6*).

Although recombinant integrase is capable of performing both the strand transfer and processing reactions in vitro, it does not accurately reproduce the in vivo process. A major reason for this could be that a marked preference for Mn^{2+} over Mg^{2+} is generally observed in in vitro enzymatic assays, although Mg^{2+} is widely considered to be the biological relevant cofactor based on the physiological concentrations of these two metal ions. Accordingly, either preintegration complexes purified from HIV-infected cells (*7*, *8*) or integrase purified from virions (*9*) is capable of efficiently using Mg^{2+} to carry out the integration process. In vitro studies also suggest that the Mg^{2+}- and Mn^{2+}-dependent activities are not functionally equivalent in terms of reaction specificity (*10*−*12*). For instance, integrase displays more nonspecific nuclease activity and is less sensitive to ionic strength in the presence of Mn^{2+} than in the presence of Mg^{2+} (*10*).

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[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DMSO, dimethyl sulfoxide; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EM, electron microscopy; HIV, human immunodeficiency virus; IN, integrase; LTR, long terminal repeat; NP-40, Nonidet P-40; PEG, polyethylene glycol; RSV, rous sarcoma virus; TFA, time-resolved fluorescence anisotropy.

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Furthermore, mutational analysis of the U5-LTR sequence has demonstrated that mutations located in the integrase binding site significantly affect the activity of integrase in the presence of Mg^{2+} but not in the presence of Mn^{2+} (*11*), thus suggesting that the establishment of sequence-specific contacts between integrase and the viral DNA is different with these two cations.

Integrase functions as a multimer (*13*−*16*; for a recent review, see ref *17*). The N-terminal domain appears to play a key role in the multimerization process (*18*−*21*). In addition, it is noteworthy that the Mg^{2+}-dependent activity of integrase can be slightly stimulated by Zn^{2+} which promotes multimerization (*19*, *20*) upon binding to a HHCC motif contained in the N-terminal domain of integrase (*22*, *23*). It has been shown that the Mg^{2+}-dependent activity of integrase can be partially restored by addition of either PEG or DMSO or with a high concentration of IN, experimental conditions that favor the oligomerization process (*10*, *20*). From these observations, we hypothesized that the capacity of integrase to use Mg^{2+} as the cationic cofactor is related to its aptitude to form stable oligomers. In the accompanying paper (*44*), we demonstrated that time-resolved fluorescence anisotropy (TFA) can be used to discriminate between monomers, dimers, tetramers, and higher-order oligomers of integrase using its intrinsic tryptophan fluorescence. Furthermore, we observed that detergent-solubilized integrase (IN_{CHAPS}) is monomeric in solution at low, enzymatically active concentrations. Thus, to determine the relationship between oligomerization and the Mg^{2+}-dependent activity, we studied three distinct preparations of integrase. The first preparations, IN_{CHAPS} and IN_{dial}, were purified in the presence of detergent, but in the case of IN_{dial}, the detergent was removed during the final dialysis. The third preparation, IN_{zn}, was purified without any detergent, in the presence of zinc. Using a sensitive TFA assay, these preparations were shown to be monomeric, in equilibrium between monomer and higher-order oligomers, and homogeneously tetrameric, respectively, at low, enzymatically active concentrations. Furthermore, we show that the abilities of these preparations of integrase to use Mg^{2+} in both 3′-processing and strand transfer reactions are directly related to their oligomeric states. In contrast, Mn^{2+}-dependent activity appears to be unrelated to the self-assembly of IN. The detergent-free integrase, IN_{zn}, is tetrameric in solution at sub-micomolar concentrations and can efficiently use Mg^{2+} to perform both steps of integration without the addition of cosolvents. Addition of detergent strongly influences the activity of the integrase, IN_{dial}, in the presence of Mg^{2+} but not in the presence of Mn^{2+}, thus confirming that the Mg^{2+}-dependent activity but not the Mn^{2+}-dependent activity requires a particular self-assembly state of the protein which can be stabilized by Zn^{2+}. Finally, we demonstrate that although the detergent-free protein binds to DNA as efficiently in the presence of Mg^{2+} as in the presence of Mn^{2+}, the integration pattern obtained with these two cations is markedly different. Thus, factors that influence protein self-assembly (detergents and zinc) must be rigorously controlled during purification to allow for recovery of integrase which will more accurately reflect the in vivo enzyme activity in vitro.

EXPERIMENTAL PROCEDURES

Integrase Preparations. Detergent-solubilized integrase IN_{CHAPS} was prepared as previously described (*24*, *25*) except that 10 mM CHAPS was present throughout the purification process. The detergent-less protein IN_{zn} was purified according to the following procedure. pET-15b-IN plasmid, which contains the cDNA encoding the HBX2 HIV integrase, was a generous gift from R. Craigie. His-tagged integrase protein was overexpressed in *Escherichia coli* BL21(DE3) and purified under native conditions. Briefly, at an OD of 0.8, fusion protein expression was induced in bacterial cultures by the addition of IPTG (1 mM). Cultures were incubated for 3 h at 37 °C, after which cells were centrifuged. The cell pellet was resuspended in ice-cold buffer A [20 mM Tris-HCl (pH 8), 1 M NaCl, 4 mM β-mercaptoethanol, and 5 mM imidazole], treated with lysozyme for 1 h on ice, and sonicated. After centrifugation (30 min at 10 000 rpm), the supernatant was filtered (0.45 μm) and incubated for at least 2 h with Ni−NTA agarose beads (Pharmacia). The beads were washed twice with 10 volumes of buffer A, 10 volumes of buffer A with 50 mM imidazole, and 10 volumes of buffer A with 100 mM imidazole. His-tagged integrase was then eluted with buffer A supplemented with 50 μM $ZnSO_4$ and 1 M imidazole. The integrase concentration was adjusted to 0.1 mg/mL in buffer A. The fusion protein was cleaved using thrombin and dialyzed overnight against 20 mM Tris-HCl (pH 8), 1 M NaCl, and 4 mM β-mercaptoethanol. After removal of biotinylated thrombin by incubation with streptavidin−agarose magnetic beads (Novagen, Madison, WI), a second dialysis was performed for 2 h against 20 mM Tris-HCl (pH 8), 1 M NaCl, 4 mM β-mercaptoethanol, and 20% (v/v) ethylene glycol. Fractions were aliquoted and rapidly frozen at −80 °C. IN_{dial} was purified according to the same procedure except that no zinc was added and 10 mM CHAPS was present throughout the purification. The detergent was removed during the final dialysis.

Nucleic Acid Substrates. Oligonucleotides U5B (5′-GTGTGGAAAATCTCTAGCAGT-3′), U5B-2 (5′-GTGTGGAAAATCTCTAGCA-3′), U5A (5′-ACTGCTAGAGATTTTCCACAC-3′), and D (5′-TGCTAGTTCTAGCAGGCCCTTGGGCCGGCGCTTGCGCC-3′) were purchased from Eurogentec and further purified on an 18% denaturing acrylamide/urea gel. For processing, strand transfer, and disintegration assays, 100 pmol of U5B, U5B-2, and D oligonucleotides, respectively, were radiolabeled using T4 polynucleotide kinase and 50 μCi of [γ-^{32}P]ATP (3000 Ci/mmol). The T4 kinase was heat inactivated, and unincorporated nucleotides were removed using a Sephadex G-10 column (Pharmacia). NaCl was added to a final concentration of 0.1 M, and complementary unlabeled strand U5A was added to either U5B or U5B-2. The mixture was heated to 90 °C for 3 min, and the DNA was annealed by slow cooling. Preprocessed U3−U5 DNA substrate consists of a 492 bp DNA fragment generated by *Nde*I restriction of the pU3U5 vector as previously described (*26*). The blunt-ended U3−U5 fragment was obtained by PCR amplification of pU3U5 with addition of *Sca*I restriction sites at the ends (primers, U3-Sca, 5′-AGTACTGGAAGGGCTAATTCA-3′; U5-Sca, 5′-agtACTGCTAGAGATTTTCCA-3′). The 496 bp PCR product was subcloned into the pGEM-T vector (Promega).

LTR Processing, Strand Transfer, and Disintegration Assays. Processing, strand transfer, and disintegration reactions were performed using U5A−U5B, U5A−U5B-2, and D oligonucleotide substrates (*27*), respectively, in buffer containing 20 mM Tris (pH 7.2), 50 mM NaCl, and 10 mM DTT with or without 0.05% (v/v) NP-40 supplemented with $MnCl_2$ or $MgCl_2$. The reaction was initiated by addition of substrate DNA, and the mixture was incubated for up to 1 h at 37 °C. The reactions were stopped by phenol/chloroform extraction, and DNA products were precipitated with ethanol. The products were dissolved in TE containing 7 M urea and electrophoresed on an 18% denaturing acrylamide/urea gel. Long fragment integration assays were carried out using 10 ng of the [^{32}P]U3−U5 DNA fragment and 40 ng of pSP70 vector as a heterologous integration target. The products were separated on a SDS/agarose gel. Gels were analyzed using a STORM Molecular Dynamics phosphorimager.

DNA Binding Assay. The binding buffer for the DNA binding studies contained 20 mM HEPES (pH 7), 10% (v/v) glycerol, 50 mM NaCl, and divalent cation as indicated. The U5B−U5A oligonucleotide duplex (30 nM) and IN were incubated for 10 min at room temperature and 10 min on ice, and then diluted in 1 mL of the same buffer. Proteins and DNA−protein complexes were bound to nitrocellulose filters (NC45, Schleicher and Schuell). Prior to filtration, the filters were soaked in binding buffer. After binding, they were washed with 1 mL of the same buffer. The amount of radioactivity was measured in a liquid scintillation counter. The total amount of radioactivity was obtained by dot blotting the DNA substrate on filter without filtration, and the DNA retention was expressed as a percentage of radioactivity retained during protein−DNA interaction assays over total radioactivity.

Time-Resolved Fluorescence. Anisotropy fluorescence polarized decays were measured and analyzed according to the method of Deprez et al. (*44*).

Electron Microscopy. Integrase (5 μg/mL) was deposited onto hydrophilic carbon-coated grids obtained by a glow discharge in air (in a MED010 Balzers apparatus) and negatively stained with 2% aqueous uranyl acetate. The observation was carried out in a Zeiss 902 EM in bright-field, with a filtering out of the inelastic electrons to achieve a better contrast.

RESULTS

Detergent-Solubilized IN Is Inactive in the Presence of Mg^{2+}. Detergents such as CHAPS (10 mM) are widely used in the purification procedure of IN as this enzyme displays a strong tendency to aggregate. This type of protein preparation was used as a detergent-solubilized protein in our study and is further termed IN_{CHAPS}. In the accompanying paper (*44*), a time-resolved fluorescence approach was used to determine the oligomeric states of IN_{CHAPS}. We found that, at low concentrations (≤200 nM), IN_{CHAPS} displays a homogeneous monomer or a monomer−dimer equilibrium in the absence or in the presence of Zn^{2+}, respectively. The 3′-processing activity of this preparation was assayed in the presence of either Mn^{2+} or Mg^{2+} using a short 21-mer oligonucleotidic substrate that mimics the extremity of the U5 LTR (*2*, *28*). Results are shown in Figure 1A. In the presence of Mn^{2+}, IN_{CHAPS} efficiently processed the oligonucleotidic substrate at both 100 and 200 nM (see Figure 1, lanes 2 and 3). In contrast, at the same integrase concentrations, only a marginal activity could be detected in the presence of Mg^{2+} (lanes 4 and 5). Since we have previously observed that IN_{CHAPS} is fully dissociated into a monomer under these conditions (*44*), it is clear that the monomeric form of IN in solution is capable of forming an active integrase−DNA complex in the presence of Mn^{2+} but not in the presence of Mg^{2+}. The Mg^{2+}-dependent 3′-processing activity was also tested in the presence of zinc. As previously reported (*18*, *19*), we observed that IN activity was slightly stimulated by 10 μM Zn^{2+} (see Figure 1B). This result parallels the Zn^{2+}-dependent oligomerization of IN_{CHAPS} by TFA that was observed (*44*).

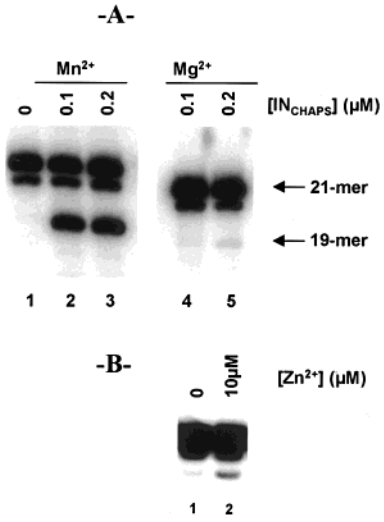

FIGURE 1: 3′-Processing activity of detergent-solubilized IN_{CHAPS}. (A) IN_{CHAPS} was incubated for 1 h at 37 °C with 10 nM 21-mer U5A−[^{32}P]U5B duplex in a buffer containing 20 mM Hepes (pH 7.2), 1 mM DTT, 1 mM CHAPS, and either 10 mM $MnCl_2$ (lanes 1−3) or 10 mM $MgCl_2$ (lanes 4 and 5): lane 1, no IN_{CHAPS}; lanes 2 and 4, 100 nM IN_{CHAPS}; and lanes 3 and 5, 200 nM IN_{CHAPS}. Arrows indicate the 21-mer DNA substrate and the 19-mer product. (B) Effect of Zn^{2+} on Mg^{2+}-dependent 3′-processing. The 3′-processing was performed as described above in the presence of 200 nM IN and 10 mM $MgCl_2$: lane 1, no Zn^{2+}; and lane 2, 10 μM Zn^{2+}.

A Sub-Micromolar Detergent-Free Integrase Is Highly Active in the Presence of Mg^{2+}. The experiments described above as well as previous reports (*10*, *19*, *20*) provided hints that the Mg^{2+} activity of integrase could be related to its ability to form stable oligomers at low concentrations. To address this hypothesis, we purified recombinant wild-type HIV-1 integrase in the absence of detergent and in the presence of zinc, since detergents and zinc have been shown to play a negative and a positive role in the self-assembly of IN, respectively (*44*). To accomplish this, a new protocol for purification of His-tagged integrase was developed that permitted us to suppress CHAPS or other detergents. The protocol was designed to allow integrase to form oligomers while eliminating nonspecific aggregation. To limit the nonspecific aggregation, 50 μM Zn^{2+} was added prior to the elution from the Ni−NTA agarose column and was present throughout the remainder of the purification. In addition, the purified protein was diluted down to about 0.1 mg/mL prior to the final dialysis (see Experimental Procedures). We observed that the presence of zinc during the purification led to a marked decrease in the level of macroscopic

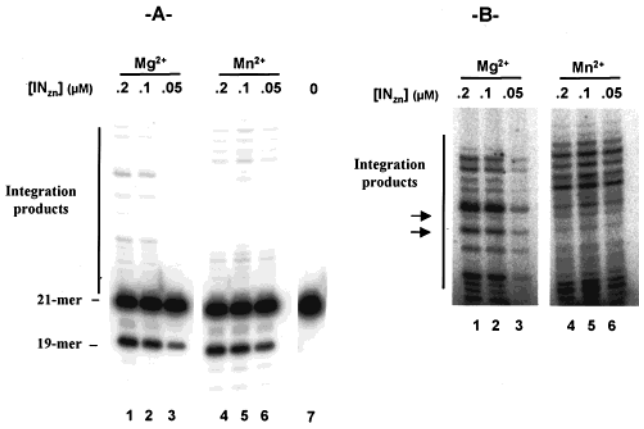

FIGURE 2: 3′-Processing and strand transfer activities of detergent-free IN_{zn}. (A) IN_{zn} was incubated for 1 h at 37 °C with 10 nM 21-mer U5A−[^{32}P]U5B duplex in a buffer containing 20 mM Hepes (pH 7.2), 1 mM DTT, and either 10 mM $MgCl_2$ (lanes 1−3) or 10 mM $MnCl_2$ (lanes 4−7): lanes 1 and 4, 200 nM IN_{zn}; lanes 2 and 5, 100 nM IN_{zn}; lanes 3 and 6, 50 nM IN_{zn}; and lane 7, no IN_{zn}. (B) Overexposure of the integration pattern shown in panel A. Arrows on the left indicate the strand transfer events that occur in the presence of Mg^{2+} but not in the presence of Mn^{2+}.

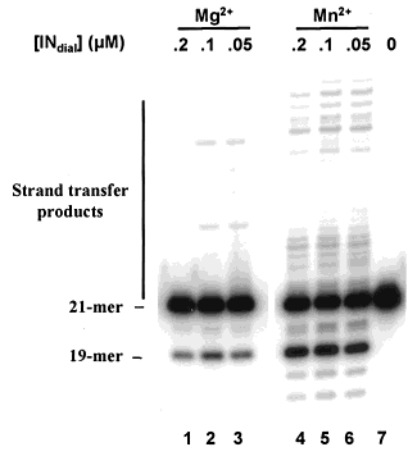

FIGURE 3: 3′-Processing and strand transfer activities of IN_{dial}. IN_{dial} was incubated for 1 h at 37 °C with 10 nM 21-mer U5A−[^{32}P]-U5B duplex in a buffer containing 20 mM Hepes (pH 7.2) and 1 mM DTT in the presence of 10 mM $MgCl_2$ (lanes 1−3) or 10 mM $MnCl_2$ (lanes 4−7): lanes 1 and 4, 200 nM IN_{dial}; lanes 2 and 5, 100 nM IN_{dial}; lanes 3 and 6, 50 nM IN_{dial}; and lane 7, no IN_{dial}. The 21-mer DNA substrate and the 19-mer product are indicated.

aggregates that could be observed visually. In contrast, when HIV integrase was purified in the absence of divalent cations, it rapidly aggregated during the final dialysis. Zn^{2+} was a more efficient cation at solubilizing the integrase than Mg^{2+}, suggesting that Zn^{2+} has a specific effect on protein conformation that increases protein solubility. Moreover, the protein purified in the presence of Mg^{2+} displayed no activity when assayed in the presence of Mg^{2+} as a cofactor (data not shown). Integrase purified in the absence of detergent and in the presence of Zn^{2+} (see Experimental Procedures) is termed IN_{zn}. The activity of IN_{zn} was investigated in a 3′-processing assay using either Mn^{2+} or Mg^{2+} as the divalent cationic cofactor. As shown in Figure 2 (lanes 1−3), IN_{zn} from 50 to 200 nM can efficiently use Mg^{2+} to process LTR oligonucleotides without addition of a cosolvent such as PEG or DMSO. Thus, IN_{zn} was able nondiscriminately to use either Mg^{2+} or Mn^{2+} for the 3′-processing reaction (compare lanes 1−3 to lanes 4−6 in Figure 2A). This is in sharp contrast to the results observed with IN_{CHAPS}. The 3′-processing efficiency peaked for 10 mM Mg^{2+} and remained high from 10 to 40 mM Mg^{2+} (not shown). Finally, integration products were obtained, indicating that IN_{zn} was capable of efficiently carrying out the strand transfer reaction as well. Interestingly, we also noted that the integration pattern was qualitatively different depending on whether Mg^{2+} or Mn^{2+} was used as a cofactor (for instance, compare lanes 1 and 4).

Differential Effects of Mn^{2+} and Mg^{2+} on the Nature of the IN_{zn}−DNA Complex. Figure 2B shows the pattern of strand transfer obtained in the experiment shown in Figure 2A after a longer exposure of the gel. It can be readily observed that the presence of either Mg^{2+} or Mn^{2+} led to dramatically different integration patterns. In the presence of Mn^{2+}, the distal positions were more favored with a clear footprint spanning the central region of the oligonucleotide target. In contrast, the Mg^{2+}-dependent activity gave rise to a set of intense bands located in the middle of the target. This strongly suggested that the specificity of the IN_{zn}−DNA complexes formed upon binding of integrase to the oligonucleotides is dependent upon the cation added to the reaction buffer.

Addition of Detergent Is Detrimental to Mg^{2+}-Dependent Activity. To investigate the effect of detergent on integrase activity, we purified another protein in the presence of the detergent CHAPS. However, the detergent was subsequently removed by a final dialysis. The protein is termed IN_{dial}. Its activity was assayed in either Mn^{2+}- or Mg^{2+}-containing buffers (Figure 3). In contrast to IN_{zn}, IN_{dial} exhibited marked quantitative differences in 3′-processing activity depending on whether the activity was assayed in the presence of Mn^{2+} or Mg^{2+}. In the presence of Mg^{2+}, the activity was notably lower. This result indicates that detergent present during protein purification has a detrimental role in Mg^{2+}-dependent activity. On the other hand, as shown in lanes 1−3, IN_{dial} retained some activity in the presence of Mg^{2+}, demonstrating that the negative effect of detergent on IN activity can be partially reversed upon removal.

To further address the effect of detergent, IN activity was assayed in the presence of increasing concentrations of NP-40, a detergent widely used in enzymatic assays of IN activity (Figure 4). The presence of NP-40 had no effect on the Mn^{2+}-dependent activity of either IN_{dial} (lanes 7−9) or IN_{zn} (lanes 10−12). However, addition of NP-40 strongly inhibited the Mg^{2+}-dependent activity of IN_{dial}, in a dose-dependent manner (lanes 1−3). Interestingly, the Mg^{2+}-dependent activity of IN_{zn} was not affected by the addition of NP-40. Thus, although the Mg^{2+}-dependent activity did not strictly require Zn^{2+} (compare lanes 3 and 6), the presence of Zn^{2+} was able to prevent the disruptive effect of detergent (compare lanes 1 and 4).

Multimerization Defective Mutants Do Not Carry Out Disintegration in the Presence of Mg^{2+}. When purified in the presence of low concentrations of zinc, the detergent-free protein, IN_{zn}, was more active in the Mg^{2+}-containing buffer than the detergent-solubilized proteins IN_{CHAPS} and IN_{dial} which were purified in the absence of zinc. Thus, one could argue that the stimulation of the Mg^{2+}-dependent activity of integrase by zinc was due to the free zinc ions playing a direct role in catalysis. To address this possibility,

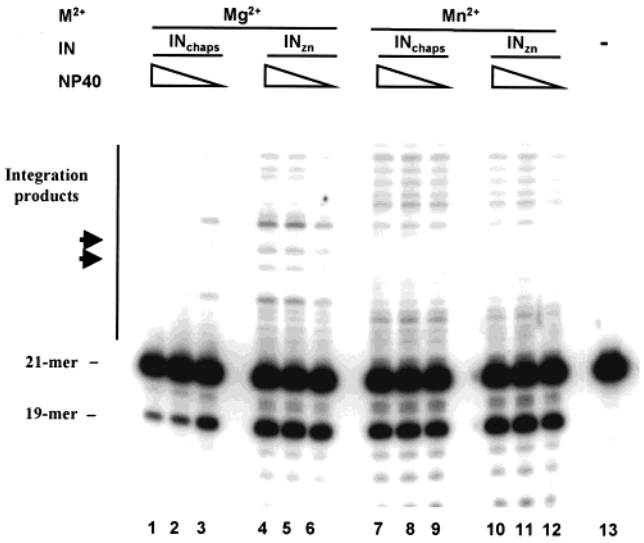

FIGURE 4: Detergent effect on Mg^{2+}- and Mn^{2+}-dependent activities of IN_{zn} and IN_{dial}. IN_{dial} or IN_{zn} (200 nM) was incubated for 1 h at 37 °C with 10 nM 21-mer U5A−[^{32}P]U5B duplex in a buffer containing 20 mM Hepes (pH 7.2), 1 mM DTT, and either 10 mM $MgCl_2$ (lanes 1−6) or 10 mM $MnCl_2$ (lanes 7−13) in the presence of various concentrations of NP-40: lanes 1, 4, 7, and 10, 0.1% NP-40; lanes 2, 5, 8, and 11, 0.05% (v/v) NP-40; lanes 3, 6, 9, and 12, without NP-40; and lane 13, no integrase and no NP-40. The 21-mer DNA substrate, the 19-mer 3′-processed product, and the strand transfer products are indicated on the left.

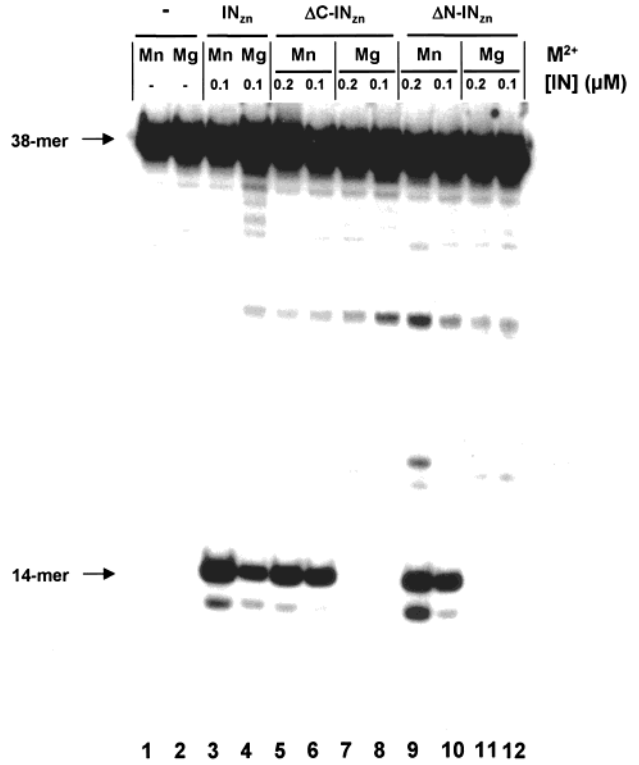

FIGURE 5: Mg^{2+}- and Mn^{2+}-dependent disintegration activity of full-length and truncated IN_{zn}. IN_{zn}, ΔC-IN_{zn}, and ΔN-IN_{zn} were incubated for 1 h at 37 °C with 10 nM 38-mer [^{32}P]D substrate in a buffer containing 20 mM Hepes (pH 7.2) and 1 mM DTT in the presence of 10 mM $MgCl_2$ (lanes 2, 4, 7, 8, 11, and 12) or 10 mM $MnCl_2$ (lanes 1, 3, 5, 6, 9, and 10): lanes 1 and 2, no integrase; lanes 3 and 4, 100 nM IN_{zn}; lanes 5 and 7, 200 nM ΔC-IN_{zn}; lanes 6 and 8, 100 nM ΔC-IN_{zn}; lanes 9 and 11, 200 nM ΔN-IN_{zn}; and lanes 10 and 12, 100 nM ΔN-IN_{zn}. The 38-mer DNA substrate and the disintegration product are indicated.

the effect of two deletions which have been shown to impair the multimerization capability was examined (*20*, *29*). We prepared two truncated proteins in which the N-terminal domain and the C-terminal domain were deleted. The truncated proteins were purified under the same conditions as IN_{zn}, i.e., in the absence of detergent and in the presence of zinc. These mutants were not able to carry out either the processing or strand transfer reaction (not shown). However, they efficiently performed the disintegration reaction using a dumbbell substrate (*27*). The disintegration activity of the mutants was investigated in the presence of either Mn^{2+} or Mg^{2+} and compared to that one of the wild-type IN_{zn}. As shown in Figure 5, IN_{zn} efficiently performed the disintegration reaction regardless of the cationic cofactor (Figure 5, lanes 3 and 4). In sharp contrast, both deletion mutants were active only when Mn^{2+} was present. Since 10 μM Zn^{2+} was present in all cases, it can be concluded that Zn^{2+} was not sufficient to elicit a noticeable disintegration activity of integrase in the presence of Mg^{2+}. Consequently, this result provides additional evidence that the enhancement of Mg^{2+}-dependent activity by Zn^{2+} is related to a particular conformation of the full-length IN_{zn}.

IN_{zn} Is Active with a Long DNA Substrate. It has been previously shown that integrase is more active in the presence of Mg^{2+} using long DNA substrates (*10*). IN_{CHAPS} and IN_{zn} were tested with a 500 bp miniviral DNA substrate (*26*), the extremities of which mimic the U5 and U3 LTR ends. Alternative digestion with either the *Nde*I or *Sca*I restriction enzyme allowed for the measurement of IN_{CHAPS} and IN_{zn} activities on both preprocessed and blunt viral substrates (Figure 6). In all cases, both homologous and heterologous integration could be observed. When Mn^{2+} was used as the cationic cofactor, both integrase preparations were active and displayed a marked preference for the preprocessed substrate (see Figure 6, lanes 7−10). Thus, once again the presence of detergent did not influence the Mn^{2+}-dependent activity. In contrast, IN_{CHAPS} exhibited a markedly lower activity using either the preprocessed or blunt-ended substrate when the integration reaction was assayed in Mg^{2+}-containing buffer (lanes 5 and 6). This result confirms the inefficiency of this protein in catalyzing both processing and strand transfer using Mg^{2+}, although some residual activity could be detected when long substrates were used. In sharp contrast, IN_{zn} was able to efficiently catalyze integration in the presence of Mg^{2+} using both substrates (lanes 3 and 4).

IN_{zn} Is a Homogeneous Oligomer at Low, Enzymatically Active Concentrations. The activity assays showed that detergents and zinc play antagonistic roles in the capacity of integrase to use Mg^{2+} as a cofactor. The presence of zinc has been reported to enhance the multimerization and catalytic activity of a soluble, mutant form of integrase (*19*). The effect of zinc on integrase self-assembly was confirmed in the accompanying paper (*44*). In contrast, we also observed that detergents have a dissociation effect on the oligomers (*44*). Accordingly, IN_{CHAPS} was found to be strictly monomeric at low, enzymatically active concentrations ($\leq$200 nM) in the absence of zinc. This protein was active only in the presence of Mn^{2+} (see Figure 1). Taken together, these results hinted that the ability of integrase to use Mn^{2+} or Mg^{2+} could be related to different conformations of the protein in solution. For these reasons, we wondered whether the oligomeric states of IN in the three preparations (IN_{CHAPS}, IN_{dial}, and IN_{zn}) were different in solution. To address this,

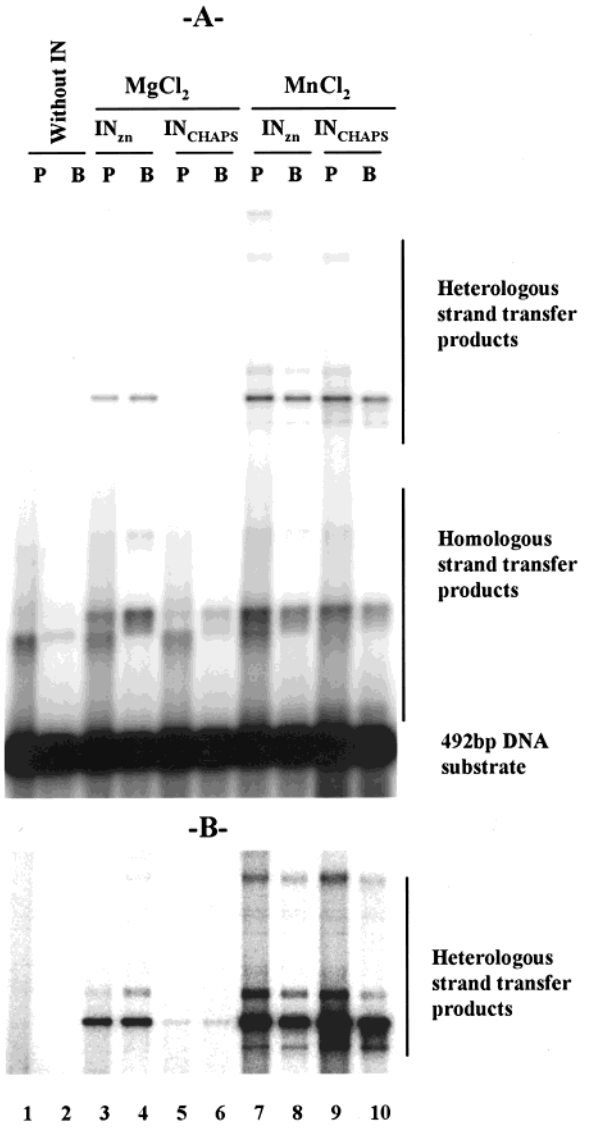

FIGURE 6: Long DNA substrate integration. IN_{zn} or IN_{CHAPS} was incubated for 1 h at 37 °C with 10 ng of either 492 bp [^{32}P]U3U5 DNA blunt (B) or 492 bp [^{32}P]U3U5 DNA 3′-processed fragment (P) and 40 ng of pSP70 vector in a buffer containing 20 mM Hepes (pH 7.2) and 1 mM DTT in the presence of 10 mM $MgCl_2$ (lanes 3−6) or 10 mM $MnCl_2$ (lanes 7−10). (A) Odd lanes, 3′-processed substrate; even lanes, 3′-blunt substrate; lanes 1 and 2, no IN and 10 mM $MnCl_2$; lanes 3, 4, 7, and 8, 100 nM IN_{zn}; and lanes 5, 6, 9, and 10, 100 nM IN_{CHAPS}. (B) Heterologous strand transfer products visualized after overexposition of the gel shown in panel A.

the oligomeric state of integrase was determined using time-resolved fluorescence anisotropy (TFA) and electron microscopy (EM). TFA yields the rotational correlation time (θ) of protein which is related in first approximation to the hydrated volume. In the accompanying paper (*44*), we have shown that TFA is a convenient tool that can discriminate between monomer ($\theta = 20$ ns), dimer ($\theta = 40$ ns), tetramer ($\theta = 80-100$ ns), and higher-order oligomeric forms of IN. At 200 nM, IN_{zn} displays rotational correlation times higher than 100 ns which correspond to a high-molecular weight oligomer of IN_{zn} (at least eight subunits) (Figure 7A, curve b). At the same concentration, IN_{CHAPS} was found as a monomer ($\theta = 20$ ns) (*44*). This result is shown as curve a. In the presence of 1 mM $MgCl_2$, the rotational correlation time distribution of IN_{zn} indicated a tetramer−high-order oligomer equilibrium (Figure 7A, curve c), and at 10 mM $MgCl_2$, the distribution was consistent with a solution consisting mostly of integrase tetramers (Figure 7A, curve d). At 35 °C, the tetramerization promoted by addition of 10 mM Mg^{2+} on zinc-bound protein was striking (see Figure 7B, curve b). The correlation time found at 70 ns corresponds to that of a tetramer following temperature and viscosity correction at 35 °C. To address the role of zinc bound to integrase, two cation-chelating agents were added to the 200 nM integrase solution. EGTA, a weak zinc-chelating agent, was added in excess (1 mM). A partial dissociation of protein oligomers was observed that resulted in an equilibrium between dimers and tetramers (Figure 7C, curve b). Next, zinc was completely removed from integrase by adding 1 mM DTPA, an efficient zinc-chelating agent which has a greater affinity for zinc than zinc fingers (*30*). The rotational correlation time under these conditions was consistent with a homogeneous solution of dimers ($\theta = 40$ ns) (Figure 7C, curve a). On the other hand, we also studied the oligomeric state of the intermediary protein, IN_{dial}, which retained some Mg^{2+}-dependent activity, although at a lower level than IN_{zn}. As shown by the very broad distribution (Figure 7D), this protein was found as a mix of multiple oligomeric forms. Addition of 10 mM Mg^{2+} led to partial dissociation but not to complete homogenization as was observed for IN_{zn} (Figure 7E). Thus, the Mg^{2+}-dependent activity of the different IN preparations can be related to the self-association state of the protein in solution. At low protein concentrations (200 nM), the Mg^{2+}-dependent activity efficiency increased as a function of the degree of oligomerization, with a homogeneous tetramer being the most efficient.

Finally, using negative staining of the protein, EM confirmed the relationship between the oligomerization state and divalent cations. In fact, IN_{zn} appeared also to be oligomeric at low, enzymatically active concentrations (Figure 8, panel 1). However, the sizes of oligomers appeared to be heterogeneous and included small aggregates. Addition of 10 mM Mg^{2+} promoted the dissociation of the aggregates leading to the homogenization of the IN solution. Oligomers appeared well-ordered, and some structures could be distinguished (Figure 8, panel 2). To assess the role of zinc in the Mg^{2+}-dependent ordering, IN_{zn} was treated with DTPA. Virtually no oligomers could be observed following this treatment (Figure 8, panel 3). Since the size of monomeric integrase is below the detection threshold of EM (≈100 kDa), we can reasonably estimate that IN_{zn} was converted to a dimeric−monomeric form. Consequently, the EM and TFA results are consistent and confirm that both Mg^{2+} and Zn^{2+} ions are required to maintain an ordered and homogeneous tetramer solution that is enzymatically active.

IN_{zn} but Not IN_{CHAPS} Efficiently Binds to DNA in the Presence of Mg^{2+}. Finally, although the capability of using Mg^{2+} was clearly connected to the oligomeric state of IN in solution, it remained to be determined whether monomeric and tetrameric proteins were different in terms of DNA binding ability. Thus, we used a filter binding assay to determine the DNA binding properties of the different integrase preparations under different cationic conditions (Figure 9). First, it can be noted that IN_{CHAPS} did not bind DNA in the absence of cations whereas IN_{zn} exhibited a high-level DNA binding ability under the same conditions. Moreover, whereas DNA binding of IN_{zn} did not seem to

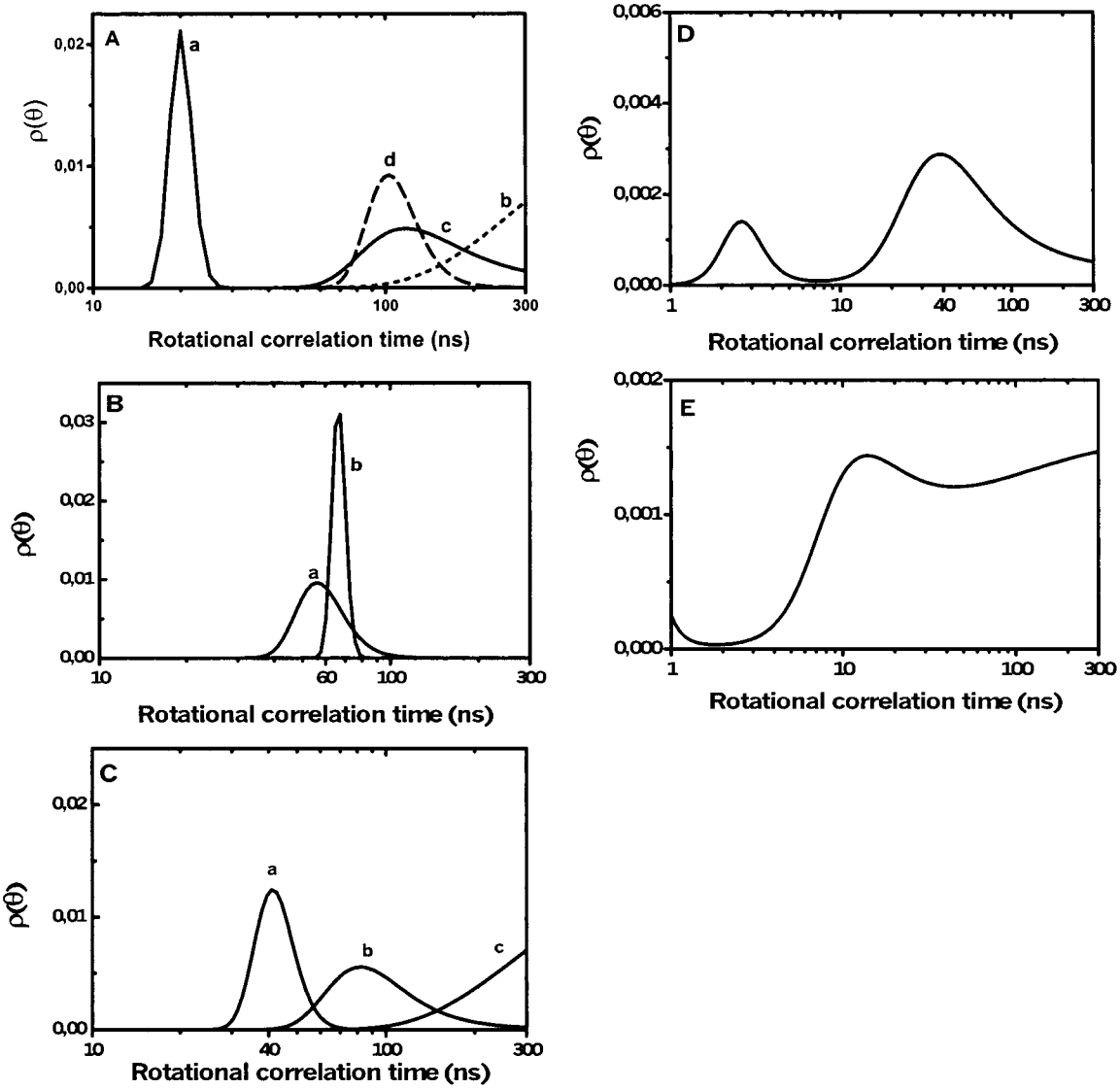

FIGURE 7: Distribution of rotational correlation times of IN as recorded by time-resolved fluorescence anisotropy. Fluorescence decay experiments were performed according to the method of Deprez et al. (*44*) with 0.2 μM integrase in a buffer containing 20 mM Tris-HCl (pH 7.2), 100 mM NaCl, 1% (v/v) glycerol, and 0.4 mM β-mercaptoethanol. (A) At 25 °C: (a) IN_{chaps} (from ref *44*), (b) IN_{zn} and no $MgCl_2$, (c) IN_{zn} and 1 mM $MgCl_2$, and (d) IN_{zn} and 10 mM $MgCl_2$. (B) Effect of chelating agent: (a) 200 nM IN_{zn} and 1 mM DTPA, (b) 200 nM IN_{zn} and 1 mM EGTA, and (c) 200 nM IN_{zn}. (C) At 35 °C: (a) 200 nM IN_{zn} and (b) 200 nM IN_{zn} and 10 mM $MgCl_2$. (D) At 25 °C with 200 nM IN_{dial}. (E) At 25 °C with 200 nM IN_{dial} and 10 mM $MgCl_2$.

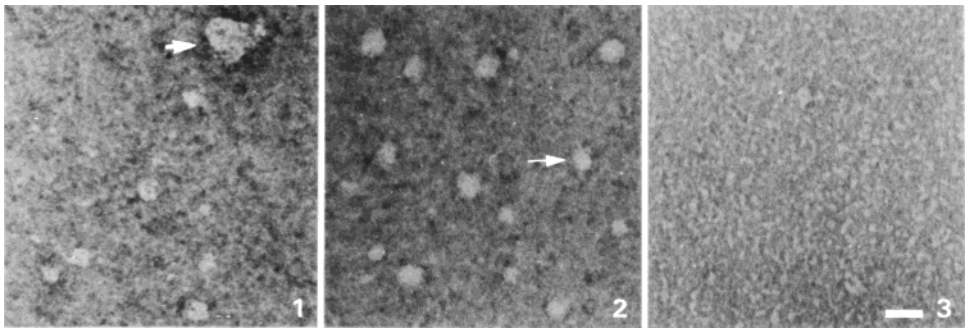

FIGURE 8: Electronic microscopy study of IN_{zn} oligomeric state. IN_{zn} (5 μg/mL) was diluted in 20 mM Tris-HCl (pH 8) and 50 mM NaCl and deposited onto hydrophilic carbon-coated grids. Grids were obtained by a glow discharge in air (in a MED010 Balzers appartus) and negatively stained with 2% aqueous uranyl acetate. The observation was carried out in a Zeiss 902 EM in bright-field, with a filtering out of the inelastic electrons to achieve a better contrast. Arrows point to the integrase oligomers. The scale bar represents 20 nm: (1) IN_{zn}, (2) IN_{zn} and 10 mM $MgCl_2$, and (3) IN_{zn} and 5 mM DTPA.

be influenced by the nature of the added cation, IN_{CHAPS} strictly requires Mn^{2+} to bind DNA. For both proteins, an excess of cations was accompanied by a lowered level of DNA binding (Figure 9A). To prevent the possibility of free or weakly bound cations in IN_{zn} becoming involved in IN−DNA interactions, EGTA, a weak chelating agent, was added to the solution. As can be seen in Figure 9B, IN_{zn} binding was only slightly affected by EGTA at a concentration of

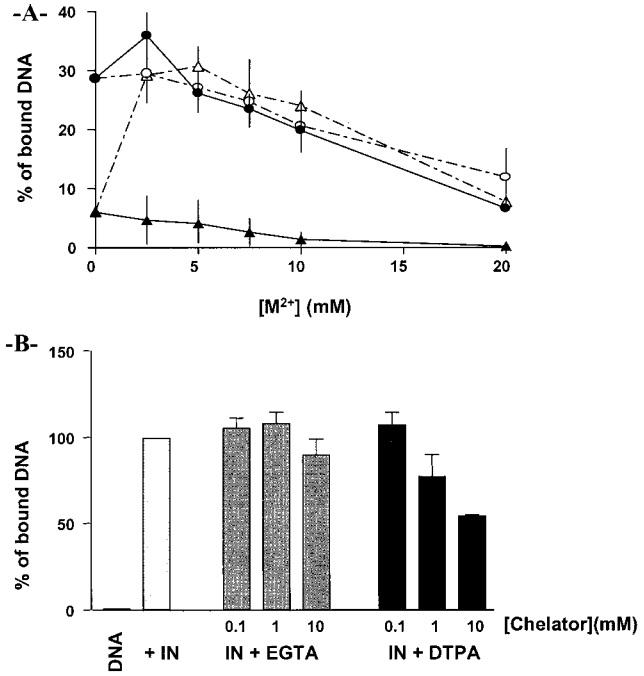

FIGURE 9: DNA binding assay of IN_{zn} and IN_{CHAPS}. [^{32}P]U5B−U5A oligonucleotide duplex (30 nM) and IN (80 nM) were incubated for 10 min at room temperature followed by 10 min one ice. The DNA binding buffer contained 20 mM HEPES (pH 7), 10% (v/v) glycerol, 50 mM NaCl, and increasing divalent cation concentrations; the mixture was diluted in 1 mL of the same buffer. Proteins and DNA−protein complexes were bound on a nitrocellulose filter (NC45, Schleicher and Schuell): (A) (○) IN_{zn} and $MnCl_2$, (●) IN_{zn} and $MgCl_2$, (△) IN_{CHAPS} and $MnCl_2$, and (▲) IN_{CHAPS} and $MgCl_2$ and (B) effect of chelating agents.

≤10 mM, thus showing that IN_{zn} does not require weakly bound cations for DNA binding. In contrast, withdrawal of Zn^{2+} from the protein by DTPA treatment resulted in a decrease in the level of DNA binding, which paralleled the dissociation of tetramers. However, more than 50% of the DNA remained bound, demonstrating that tetramers are not required for efficient DNA binding.

DISCUSSION

On the basis of in vivo observations (*16*) and in vitro subdomain transcomplementation experiments (*13*, *23*), integrase is strongly believed to function as an oligomer. Indeed, dimers as well as tetramers and octamers of recombinant integrase have been observed in solution on several occasions (*19*, *20*, *29*, *31*). Nevertheless, the precise relationship between the oligomeric state of IN and its enzymatic activity remains unclear. By analogy with other polynucleotidyl transferases and on the basis of the physiological concentration of divalent cations, IN is believed to use Mg^{2+} as a cofactor. It is thus surprising that recombinant integrase requires Mn^{2+} for efficient activity in vitro, whereas little or no activity can be detected in the presence of Mg^{2+} under standard assay conditions. Mg^{2+}-dependent activity can be partially restored by adding agents such as PEG or DMSO or by increasing the integrase concentration in the activity assay (*10*). In both cases, a possible explanation for this stimulatory effect may be ascribed to a change in integrase oligomerization. On the other hand, the propensity of IN to aggregate has resulted in the development of enzyme purification procedures that involve the addition of detergents. Furthermore, 0.05% NP-40 is often added to the reaction buffers for similar reasons. In the accompanying paper (*44*), we observed that although a detergent-solubilized protein, IN_{CHAPS}, in micromolar concentrations is present as high-order oligomers, it becomes mostly monomeric at submicromolar concentrations in the presence of 1 mM CHAPS and 0.05% (v/v) NP-40, which represents standard assay conditions. Therefore, the presence of both detergents can induce the dissociation of integrase oligomers. Furthermore, we showed that IN_{CHAPS} was unable to use Mg^{2+} as a cofactor in vitro. Thus, from our work and a previous report (*31*), it appears that although the monomeric form of IN in solution is catalytically active in the presence of Mn^{2+}, this protein cannot efficiently use Mg^{2+}. It is thus tantalizing to hypothesize that the activity of IN in the presence of Mg^{2+} is related to its aptitude to adopt an oligomeric conformation that is not required in the presence of Mn^{2+}. Therefore, we sought to determine the precise oligomeric state of integrase under conditions where it was active in Mg^{2+}-containing buffer. Our results demonstrate that (i) IN purified and assayed in the absence of detergent displays functional properties similar to those that are expected for the physiological enzyme, in contrast with a protein purified in the presence of detergent and, in particular, with IN_{zn} that can efficiently use Mg^{2+} as a cationic cofactor; (ii) this capability is related to the presence of a homogeneous tetramer at low, enzymatically active concentrations; and (iii) the presence of zinc bound to IN stabilizes the enzyme in a Mg^{2+}-active conformation which is not altered upon addition of detergent.

Relationship between the Oligomeric State and Mg^{2+}-Dependent Activity of IN. We have studied three distinct preparations of integrase. Their 3′-processing activities were assayed at low enzyme concentrations (0.2 μM). Of these three preparations, the first one, IN_{CHAPS}, strictly required Mn^{2+} for activity, the second one, IN_{dial}, was more active with Mn^{2+} than with Mg^{2+}, whereas the third one, IN_{zn}, used both cations with equal efficiency. Using a sensitive TFA assay, these preparations were shown to be monomeric, in equilibrium between monomer and higher-order oligomers, and homogeneously tetrameric, respectively, at the protein concentration used in the enzyme assays. Furthermore, addition of detergent to the protein preparation, IN_{dial}, which has a low level of Mg^{2+}-dependent activity, strongly reduced this activity, whereas its Mn^{2+}-dependent activity was unaffected. Since addition of detergent decreases the oligomeric state of the protein (*44*), these results show that the Mg^{2+}-dependent but not the Mn^{2+}-dependent activity is largely related to the ability of the protein to maintain stable oligomers at low concentrations. This conclusion is reinforced by the fact that C-terminally and N-terminally truncated proteins impaired in their ability to form oligomers but not their catalytic disintegration activity were inactive in the presence of Mg^{2+} whereas their activity in the presence of Mn^{2+} was similar to that of the full-length protein.

We also examined the role of zinc in the activity of IN. We observed like others that zinc enhances both the oligomerization (*19*, *44*) and the Mg^{2+}-dependent activity of integrase (refs *17*, *19*, and *20* and this report). This is thought to occur via its binding to an HHCC motif in the N-terminal domain of integrase. In support of this hypothesis, HHCC mutants of the integrase from RSV, another retrovirus, were reported to be inactive in the presence of Mg^{2+}

but retained their Mn^{2+}-dependent activity (*32*). As discussed above, a protein that was purified in the absence of detergent and in the presence of zinc, IN_{zn}, forms stable oligomers and is capable of using Mg^{2+} as a cofactor. Moreover, this Mg^{2+}-dependent activity of IN_{zn} was shown to be insensitive to the addition of detergent, thus confirming the possible role of Zn^{2+} in stabilizing the oligomers. However, we have observed that zinc is not strictly required for either oligomerization or Mg^{2+}-dependent activity. For instance, we showed that a zinc-free integrase, IN_{dial}, was found as a complex mixture of protein oligomers. The protein was slightly active in the presence of Mg^{2+}, but this activity remained sensitive to detergent and was abolished by the addition of 0.1% (v/v) NP-40 to the reaction buffer. Consequently, we conclude that zinc is not critical for the protein to adopt a conformation active in the presence of Mg^{2+} but is instead necessary to stabilize the active conformation. Accordingly, IN_{zn}, which was purified in the presence of zinc, was found as a tetramer in solution at low, enzymatically active concentrations. This multimerization effect of Zn^{2+} is reversible since the protein undergoes a tetramer−dimer transition upon addition of DTPA, a strong zinc-chelating agent. Finally, we observed that the protein adopted a homogeneous tetrameric arrangement only upon addition of Mg^{2+}. Numerous reports have shown that divalent cations play important roles as catalytic and structural cofactors for integrase (*33*−*37*). Apart from its catalytic role, Mg^{2+} induces a conformational change in IN that has been monitored by antibody probing (*34*) and mass spectrometry studies (*35*). This result was confirmed in our TFA assay. Together, these results support a model in which Zn^{2+} and Mg^{2+} cooperate to stabilize a particular conformation of integrase. Since a significant activity of the tetrameric IN_{zn} in either processing or strand transfer was observed in Mg^{2+}-containing buffer, it can be concluded that tetrameric IN_{zn} represents a correct association with an active conformation.

Mg^{2+}-Dependent Activity and DNA Binding. The first event of the integration process is the binding of integrase to its cognate DNA sequence. We compared the DNA binding capacity of fully active integrase in the presence of Mg^{2+}, IN_{zn}, with that of IN_{CHAPS}, which is inactive under the same conditions. IN_{zn} was able to bind DNA in the absence of either Mn^{2+} or Mg^{2+}, whereas IN_{CHAPS} strictly required Mn^{2+} to reach the same level of binding. In the presence of Mg^{2+}, IN_{CHAPS} did not bind to DNA. Thus, the difference between both enzymes can be clearly ascribed to contrasting DNA binding properties. This result raises the question of which form is the actual active form of the enzyme, i.e., the one that binds to the substrate DNA and catalyzes both processing and strand transfer reactions. We observed that both monomeric IN_{CHAPS} and oligomeric IN_{zn} bind efficiently to DNA in the presence of Mn^{2+}. Thus, IN is capable of binding to DNA regardless of its oligomeric state under this condition. In contrast, in the presence of Mg^{2+}, oligomeric IN_{zn} efficiently binds to DNA whereas the monomeric IN_{CHAPS} does not. Therefore, our results suggest that a higher self-assembly state, i.e., dimer or tetramer, is a prerequisite for DNA binding in the presence of Mg^{2+}. Furthermore, tetramers, although not strictly required, seem to be preferred over dimers for DNA binding since IN−DNA complexes are quantitatively reduced but not abolished by DTPA treatment that triggers a complete tetramer−dimer transition in solution. We note that even if IN_{CHAPS}, which exists mostly as a monomer in the presence of Mn^{2+}, binds efficiently to DNA, it does not necessarily mean that the monomer is the active form under this condition. Indeed, a strong cooperative binding to DNA of a similar detergent-solubilized integrase has been observed in the presence of Mn^{2+} but to a much lesser extent in the presence of Mg^{2+} (*33*), thus suggesting that the multimerization process can occur on DNA in the presence of Mn^{2+}. In light of these results, our data support two different mechanisms of DNA recognition depending on the nature of the divalent cation. As found in the accompanying paper (*44*), IN is more rigid in the presence of Mg^{2+}, thus suggesting that distinct conformations of the monomer are responsible for these two different binding modes. The increased rigidity of the protein in the presence of Mg^{2+} may also give rise to more specificity in binding DNA as has been previously demonstrated for the restriction enzyme. The activity of the restriction enzyme necessitates the establishment of narrower contacts with DNA in the presence of Mg^{2+} than in the presence of Mn^{2+}, leading to a more specific activity (*38*−*41*). This has effectively been shown to be the case with integrase since DNA−protein contacts are more important in the presence of Mg^{2+} than in the presence of Mn^{2+}. Where only five LTR nucleic acid residues are important for the Mn^{2+}-dependent activity, almost 10 of them are critical for Mg^{2+}-dependent 3′-processing (*11*). The rigidity of IN−DNA complexes in the presence of Mg^{2+} is reinforced by the fact that in our hands, IN_{zn} can use a blunt DNA substrate that more closely mimics the viral substrate than the preprocessed substrate whereas IN_{CHAPS} could not. Thus, IN_{zn} may represent a protein closer to the physiological one than previously purified integrases. We also observed a different integration pattern depending on whether Mg^{2+} or Mn^{2+} was used as a cofactor. Although in our experiments the bands corresponding to strand transfer events cannot be unambiguously attributed to a single site of integration (*42*), it remains that in the presence of manganese, the bands corresponding to integration in the central part of the oligonucleotide target are missing. This pattern may be due to the formation of a nonspecific complex between integrase and the oligonucleotide which prevents the strand transfer from taking place, yielding a footprint spanning the central sequence. In contrast, in the presence of Mg^{2+}, the central sequence was efficiently used as a target, thus providing compelling evidence for a different IN−DNA complex. Moreover, in an attempt to localize the preferred sites of insertion, we observed that the strand transfer pattern comes close to the one described by Engelman and co-worker (*43*) when they used the preintegration complex as a source of integration activity. Thus, this pattern may reinforce the idea that this IN−DNA complex is closer to the physiological complex that the one obtained in the presence of manganese.

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