Retroviral integrase, one of only three enzymes encoded by the virus, catalyzes the essential step of inserting a DNA copy of the viral genome into the host during infection. Using the avian sarcoma virus integrase, we demonstrate that the enzyme functions as a tetramer. In presteady-state active site titrations, four integrase monomers were required for a single catalytic turnover. Volumetric determination of integrase-DNA complexes imaged by atomic force microscopy during the initial turnover additionally revealed substrate-induced assembly of a tetramer. These results suggest that tetramer formation may be a requisite step in assembly of a tetramer. These results suggest that tetramer formation may be a requisite step in assembly of a tetramer.

Integrase catalyzes two consecutive transesterification reactions during its in vivo function (1, 2). In the “processing” reaction, the reverse transcriptase-generated DNA copy of the viral genome is trimmed by the endonuclease activity of the 3’-5’-dineotides from its ends. The two processed 3’-ends are then inserted into opposing strands of the host DNA in the “joining” reaction via a concerted cleavage-ligation reaction (3–6). Purified integrase catalyzes both reactions on synthetic oligodeoxynucleotide substrates containing viral DNA DNA and Proteins—Oligodeoxyribonucleotides were synthesized by the Center for Gene Research and Biotechnology Central Services Laboratory (Oregon State University). Purification by denaturing PAGE, spectrophotometric determination of concentrations, and 5’-radiolabeling were as described previously (9). Integrase was overexpressed in Escherichia coli BL21(DE3), purified as described (9), and stored at −80 °C in 50 mM HEPES, pH 7.5, 500 mM NaCl, and 40% glycerol.

Presteady-state Assays and Product Analysis by Denaturing Acrylamide Gel Electrophoresis—Standard reactions were carried out at 37 °C in 20 mM Tris, pH 8.0, 10 mM Na-HEPES, pH 7.5, 4% glycerol, 10 mM 2-mercaptoethanol, 0.050 mg ml⁻¹ acetylated bovine serum albumin, 250 or 400 mM NaCl, and with or without 5 mM MnCl₂. Preincubations were carried out in the absence of MnCl₂ for 30 min and the reaction subsequently initiated by the addition of 37 °C MnCl₂ to 5 mM. A complete range of preincubation times and temperatures were tested to ensure that equilibrium had been achieved. Gel analysis, image quantitation, and non-linear least squares fittings were performed according to Bao et al. (9, 10). Imaging by Atomic Force Microscopy (AFM)—Imaging was performed with a Nanoscope IIIa instrument (Digital Instrument, Santa Barbara, CA) using the tapping mode in air. Nanosensor Pointprobe non-contact/tapping mode sensors with a nominal spring constant of 48 newton/m and resonance frequencies of 190 kHz were used for all images. The protein and DNA molecules were deposited onto freshly cleaved mica (Spruce Pine Mica Co., Spruce Pine, NC), immediately washed with deionized distilled water, and dried with a stream of N₂ (gas). Depositions of disintegration reactions during the first catalytic turnover were obtained by preincubating 4 μM integrase with 5 μM substrate DNA in 20 mM Tris, pH 8.0, 10 mM HEPES, pH 7.5, and 250 mM NaCl for a minimum of 30 min on ice, diluting the reaction to 64 mM integrase and 150 mM NaCl immediately prior to initiating the reaction

**Functional Oligomeric State of Avian Sarcoma Virus Integrase***

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Kogan K. Bao‡, Hong Wang§, Jamie K. Miller‡, Dorothy A. Erie§, Anna Marie Skalka¶, and Isaac Wong‡化工

From the ‡Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331, the §Department of Chemistry, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, and the ¶Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

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RESULTS AND DISCUSSION

Single-turnover Disintegration Assays—For single-turnover experiments, the substrate was 5'-radiolabeled on the 19-nt target fragment, denoted by the asterisk in Fig. 1, which is converted to a 44-nt product during disintegration (inset, Fig. 2). Substrate (0.25 μM) was preincubated with excess ASV integrase (1 μM monomers) for 30 min in the absence of Mn²⁺ to allow equilibrium formation of productive complexes (9, 10). Reactions were initiated by the addition of Mn²⁺ to 5 mM. Fig. 2 (closed circles) shows biphase conversion of the radiolabeled 19-nt substrate to the expected 44-nt product. Non-linear least squares fit of the data to a double-exponential function yielded observed rate constants of 0.05 and 0.002 s⁻¹ with respective amplitudes of 17 and 60%. When the reaction was initiated by mixing integrase and substrate without preincubation but in the presence of Mn²⁺, we observed a single 0.002 s⁻¹ exponential phase (Fig. 2, open circles) with an amplitude (75 ± 0.9%) that was, within error, equal to the sum of the amplitudes (77 ± 1.4%) observed with preincubation. The fast phase reflected 17% productive integrase-substrate complexes formed during the preincubation period in the absence of Mn²⁺ that was rapidly converted to product at 0.05 s⁻¹ upon addition of the metal cofactor. In contrast, the 0.002 s⁻¹ phase observed in both experiments reflected slower assembly of productive complexes in the presence of Mn²⁺. The significant amplitude of the slower exponential phase in the preincubated experiment, therefore, indicated that 60% more productive complexes were formed following the addition of the metal cofactor, suggesting that the formation of these complexes is facilitated by the presence of the metal cofactor (26).

Active Site Titrations—When experiments were performed at lower concentrations of integrase with preincubation, the amplitudes of the two exponential phases became reduced and an additional linear phase became apparent (Fig. 3A, inset). The apparent “burst” kinetics behavior is characteristic of enzymatic mechanisms where products are formed at the active site faster than they dissociate, resulting in rapid turnover of the first equivalent of substrates bound while subsequent steady-state turnovers are rate-limited by slow product release to regenerate the free enzyme. As in the single-turnover experiments, the first turnover burst consisted of two exponential phases reflecting the rapid 0.05 s⁻¹ conversion of productive complexes preformed in the absence of Mn²⁺ followed by a slower rate of assembling additional complexes in the presence of the Mn²⁺.

To determine the number of integrase protomers required to catalyze the disintegration of a single substrate, we performed active site titrations by measuring the sum of the two exponential amplitudes at fixed DNA concentrations as a function of increasing integrase to substrate DNA ratio. Fig. 3A shows two such titrations carried out at 250 mM NaCl with 0.25 μM DNA and at 400 mM NaCl with 0.5 μM DNA. In both cases, the total first turnover amplitude increased linearly with added integrase up to a ratio of four integrase protomers per substrate DNA. At integrase:substrate ratios above 4:1, a plateau was reached with no further apparent change in the burst amplitude, indicating that the substrate binding capacity of the added integrase exceeded the concentration of substrate. These results defined a reaction stoichiometry of four protomers per substrate molecule. Additionally, the same reaction stoichiometry was obtained at different DNA and NaCl concentrations, and titrations performed using integrase purified by different protocols also yielded identical results (data not shown).

The maximum burst amplitude observed at both salt concentrations represented less than complete binding of substrate DNA added albeit to different extents. If the missing 15–25% reflected a subpopulation of substrate that could not be bound by integrase, then the measured reaction stoichiometry of four should be divided by this plateau value to obtain the true reaction stoichiometry. On the other hand, if the missing fraction represented a subpopulation of integrase-substrate complex that was silent in the disintegration assay, i.e. did not generate the monitored product, then an adjustment may not be necessary. When we monitored the release of the viral-end fragment from the Y-shaped substrate rather than the regeneration of the 44-nt target fragment under identical conditions, we observed an additional exponential phase in which the missing 15–25% of the substrate was slowly cleaved (2 × 10⁻⁵ s⁻¹) to completion at the viral-target joining junction (data shown in Fig. 3B for 250 mM NaCl). This reaction was integrase-catalyzed yet could not have arisen from 3'-hydroxyl attack by the target 19-nt fragment as the corresponding target 44-mer product was not generated concurrently. We therefore concluded that the missing 15–25% amplitude represented a competing but silent subpopulation of the integrase-substrate complex that underwent hydrolytic attack at the joining junc-

![Fig. 1. Schematic depicts integrase-catalyzed disintegration and joining reactions of the Y-substrate superimposed on a generic tetramer model for integrase (left). The sequences of the component single-stranded synthetic oligonucleotides are: 5'-ggttggttggtagctatggcagcatagtgtcttatgcaatagc (a), ttcgggagccg (b), aatgtgtcttcatgagaatcacaacctaggattgatadcagc (c), and gtagttgcatgagcttcgagcttcgaggaa (d). The asterisk denotes the position of the 5'-32P-radiolabel.

![Fig. 2. Single-turnover time course of the disintegration reaction at 1 μM integrase and 0.25 μM substrate DNA was obtained by quantifying the amount of 44-mer formed following separation by denaturing PAGE (inset). Experiments were conducted with (closed circles) and without (open circles) preincubating enzyme and substrate prior to initiating the reaction. Lines represent the best fit of the data to sum of exponential terms with A₈ preinc = 0.17 ± 0.01, A₉ preinc = 0.60 ± 0.01, A₁₀ preinc = 0.05 s⁻¹ ± 0.006, A₁ preinc = 0.002 s⁻¹ ± 0.0001, A₉ nonpreinc = 0.74 ± 0.009, and A₁₁ nonpreinc = 0.002 s⁻¹ ± 0.00004.](image-url)
A typical 1-µm² AFM image of integrase alone showed primarily particles with volumes consistent with monomers and dimers (Fig. 4A, top). By comparison, increased numbers of tetramer-sized particles were observed in images deposited in the presence of the Y-shaped substrate (Fig. 4A, bottom). Analysis of the integrated volumes for all particles imaged confirmed that in the absence of DNA, integrase appeared predominantly as monomers and dimers (Fig. 4B, top). In contrast, a new peak with a mean molecular volume of 154 ± 7.5 nm³, corresponding to a calculated molecular weight of 138 kDa, became apparent and accounted for ~20% of the particles in the presence of DNA (Fig. 4B, bottom). When these population distributions were adjusted to more accurately reflect the number of integrase protomers subsumed within each volumetric subpopulation by taking into account the mass of each particle (Fig. 4C), we observed that the presence of substrate DNA induced nearly half of the integrase protomers analyzed to assemble into tetramers. Aggregates larger than tetramers, though visible, did not accumulate in sufficient numbers to segregate into discrete populations of a defined size, e.g. an octamer, and were therefore excluded from the statistical analysis. Additionally, the small number of these larger aggregates is inconsistent with the magnitude of productive complexes observed in the presteady-state burst.

The 138-kDa apparent mass observed was larger than expected for a tetramer of four 32-kDa subunits presumably due to the additional presence of the bound DNA. However, the apparent difference of 10 kDa underestimated the actual mass of the DNA. It is plausible that part of the volume contributed by the DNA may be topologically obscured within the concavity of the DNA binding site. Alternatively, the linear calibration curve determined for globular proteins is unlikely to yield accurate volume to mass conversion for DNA whose partial specific volume in aqueous solution can be considerably smaller than for proteins.

**Hypothetical Structural Models of Integrase Tetramers**—The structure of the full-length 32-kDa integrase protein is unknown. However, the central catalytic core domain (15–17) and all two-domain fragments consisting of the core plus either the C (18–20) or N terminus (21), from a variety of retroviral sources, form dimers in the crystal structures. Based on these structures, several dimer-of-dimers models have been proposed (20–22). Fig. 5A shows a recent model proposed based on the structure of the core plus N-terminal two-domain structure of HIV-1 integrase (21). In this model, a pair of inward-facing functional active sites is contributed by the two inner, or “proximal,” protomers at the dimer-dimer interface. The two remaining outward-facing active sites of the two “distal” protomers are not used for catalysis (20–22). Coordinated processing of both ends of the viral DNA would therefore require concurrent binding of the two viral ends in active sites on both sides of the dimer-dimer interface (Fig. 5A).

Our results suggest that concerted integration may further require binding of the target DNA bridging the dimer-dimer interface (Fig. 5B). Although we cannot be certain of the molecular details of DNA binding in the absence of cocystal structure, the observation that the Y-substrate, with only a single viral-end mimic, was able to induce active tetramer assembly would suggest significant contribution by the target DNA in mediating the dimer-dimer interaction. The calculated electrostatic potential at the surface of the proposed tetramer (Fig. 5B) shows that a ~30 Å wide, positively charged groove extends along this interface, suggesting that the assembly of the tetramer would require the juxtaposition of two positively charged surfaces. The neutralization of this like-charge repulsion by the binding of the negatively charged target DNA into this groove would greatly enhance the stability of the tetramer and may form the molecular basis for the observed DNA-induced assembly.
Relevance of the Disintegration Reaction—Despite being widely employed for assaying the activity of integrase and integrase mutants (8, 12–14), including that of the catalytic core domain constructs used in crystallographic studies (15–17, 27, 28), concerns persist that the disintegration reaction may not be mechanistically relevant. The skepticism arises in part from the observation that the catalytic core domain can catalyze the disintegration reaction but not the forward joining reaction, leading to the notion that the disintegration reaction might represent a less “stringent” measure of activity. While it may be more compelling to determine the reaction stoichiometry for the more obviously relevant processing or joining reactions, the results reported here with the disintegration reaction represent the first successful quantitative measure of a reaction stoichiometry using any integrase assay, because the propensity of the enzyme to aggregate has precluded the use of both the processing and the joining reaction in active site titrations (9, 10). Additionally, the reported weak disintegration activity of the ASV catalytic core (16) is 100-fold slower than the competing, nonspecific hydrolysis reaction shown in Fig. 3B and at least 5 orders of magnitude slower than the disintegration activity reported here for the full-length enzyme (data not shown). This result, therefore, suggests that the disintegration reaction may not be as permissive as previously thought, at least for the ASV enzyme.

A more substantive concern regarding the use of the disintegration assay stems from the inclusion of only one viral-end mimic in the structural design of the Y-shaped substrate. As a result, our data do not directly rule out an octameric (dimer of tetramers) model (20–22) for the binding of two viral ends. However, as the minimum requisite set of active sites are present in a tetramer to bind both viral ends plus the target DNA, the available data likewise do not dictate any direct need to recruit additional active sites. In the absence of direct evidence for a higher order aggregate, we therefore favor the minimally sufficient tetrameric model. We note further that while the tetramer with a single Y-shaped substrate bound can accommodate the additional binding of a second viral end, the 30-Å wide groove of the tetramer is too narrow to permit the binding of a second Y-shaped substrate. Additionally, the complementary relation-
ship between the structure of this substrate and its ability to mediate functional tetramer assembly lends compelling support for the structural and functional relevance of the disintegration substrate in modeling the active-site architecture.

Implications for Structural Studies and Antiviral Design—

Our ability to complete active site titrations suggests that the presence of target DNA in the disintegration substrate mitigated protein aggregation problems observed with other DNA substrates. Alternatively, the disintegration substrate conferred sufficient binding stability to maintain active complexes at the higher NaCl concentrations required to prevent aggregation. These results suggest potential benefits from using disintegration-like substrates, containing both target and viral DNA, in structural studies of the active integrase unit to promote tetramer formation as well as to minimize non-productive aggregation.

The observed substrate DNA- and metal cofactor-dependent oligomerization further suggests that the assembly of the active tetramer may be an integral and dynamic component of the catalytic pathway. The dynamic nature of the dimer-dimer interface should make it an ideal target for inhibitor design. The diketo acid family of inhibitors targeting the active site of integrase has recently been shown to inhibit the V(D)J recombination. These results suggest potential benefits from using disintegration-like substrates, containing both target and viral DNA, in structural studies of the active integrase unit to promote tetramer formation as well as to minimize non-productive aggregation.

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