A Soluble Active Mutant of HIV-1 Integrase

IN INVOLVEMENT OF BOTH THE CORE AND CARBOXYL-TERMINAL DOMAINS IN MULTIMERIZATION*

(Received for publication, September 20, 1995, and in revised form, January 8, 1996)

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Structural studies of human immunodeficiency virus type 1 (HIV-1) integrase have been impeded by the low solubility of the protein. By systematic replacement of hydrophobic residues, we previously identified a single amino acid change (F185K) that dramatically improved the solubility of the catalytic domain of HIV-1 integrase and enabled the structure to be determined by x-ray crystallography. We have introduced the same mutation into full-length HIV-1 integrase. The resulting recombinant protein is soluble and fully active in vitro, whereas, HIV-1 carrying the mutation is replication-defective due to improper virus assembly. Analysis of the recombinant protein by gel filtration and sedimentation equilibrium demonstrate a dimer-tetramer self-association. We find that the regions involved in multimerization map to both the catalytic core and carboxyl-terminal domains. The dramatically improved solubility of this protein make it a good candidate for structural studies.

Integrase of a DNA copy of the retroviral RNA genome into a host chromosome is a crucial step in viral replication (1–3). Two specific enzymatic reactions are involved in integration. In the first reaction, 3' processing, two nucleotides are removed from each 3' end of the linear viral DNA made by reverse transcription. A subsequent DNA strand transfer reaction then splices these 3' ends into the host chromosome. The 5' ends of the viral DNA and the 3' ends of the host DNA remain unjoined in the resulting integration intermediate (4,5). Completion of integration requires only removal of the unpaired bases at the 5' ends of the viral DNA and repair of the single-strand gaps between the viral and host DNA, steps which may be completed by cellular DNA repair enzymes.

HIV-1 integrase catalyzes both the 3' processing and DNA strand transfer reactions in vitro. Short oligonucleotides that mimic the ends of HIV-1 DNA serve as the substrate for 3' processing, and a second oligonucleotide acts as a target DNA for the subsequent strand transfer reaction (6–8). Integrase will also catalyze an apparent reversal of the strand transfer reaction, termed disintegration (9). In this reaction the viral DNA segment of a branched substrate is liberated and the target DNA segment is resealed.

HIV integrase is comprised of three functional domains. Although the central core domain alone can catalyze the disintegration reaction, both the amino- and carboxyl-terminal domains are necessary for catalysis of 3' processing and DNA strand transfer (10–12). Site-directed mutagenesis has revealed that a trio of highly conserved acidic residues within the core domain is essential for all three catalytic activities of integrase (13–15). These residues, Asp-64, Asp-116, and Glu-152, comprise the D,D,E motif that is conserved in all retroviral and retrotransposon integrase proteins and is also found in some bacterial transposable elements (13, 16–18).

The limited solubility of HIV-1 integrase has impeded structural studies. However, the mutation of phenylalanine to lysine at amino acid 185 (F185K) within the catalytic core domain of HIV-1 integrase resulted in a soluble protein (19) that enabled the core domain to be crystallized and the structure solved to 2.5-Å resolution (20). We have now introduced the same mutation (F185K) into full-length HIV-1 integrase, together with a cysteine to serine substitution at position 280. The resulting protein is dramatically more soluble than full-length wild-type HIV-1 integrase and retains full activity for both the 3' processing and DNA strand transfer reactions. The mutant protein exists in an equilibrium between dimeric and tetrameric species in buffer containing 1 M NaCl. Both the core and carboxyl-terminal domains are involved in multimerization.

MATERIALS AND METHODS

Construction of Site-directed Mutations—Site-directed mutagenesis was done by overlapping PCR (21) using a two-step procedure as described (19). Plasmid DNA encoding the mutations F185K/C280S within full-length integrase, IN185K/C280S, was prepared using plNSD (13) as the PCR template DNA. After the second round of PCR, the full-length fragment was digested with NdeI and BamHI and ligated with NdeI-BamHI-digested pET-15b (Novagen, Madison, WI). This placed IN185K/C280S under the control of a T7 promoter (22) and also encoded a 20-amino acid histidine tag (HT) at the amino terminus of the protein to facilitate rapid purification on a nickel chelating column. The inclusion of a thrombin cleavage site within the HT sequence allowed the removal of 17 amino-terminal residues containing the polyhistidine motif following purification. F185K was also introduced into the pol gene of pNL4–3, an infectious plasmid DNA clone of HIV-1, using overlapping PCR. Sequential PCR with pNL4–3 as the starting DNA template produced a 1832-bp pair fragment. This fragment was digested with Agel and PstI and then ligated with Agel–PstI-digested pNL4–3 DNA. The sequences of all PCR-generated regions were confirmed by DNA sequencing (23).

Expression and Solubility of F185K/C280S Integrase from E. coli—Plasmid encoding IN185K/C280S was expressed in Escherichia coli strain BL21(DE3) (24) as described (6, 13). The solubility of the mutant protein was examined in a crude cell lysate, as follows. Cells were grown in 2 liters of Super broth (Biofluids) containing 100 μg of ampicillin per ml at 37°C until the optical density of the culture was between 0.8 and 1.0 at 600 nm. Protein expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.4 mM. After 3 h, the cells were harvested and resuspended in...
12 ml of 25 mM HEPES (pH 7.5), frozen in liquid N2, and stored at
-80°C. Cells (100 μl) were lysed by the addition, to final concentration,
of 0.15 M, 0.5 M, or 1 mM NaCl, 2 mM dithiothreitol, and 0.3 mM
lysosyme, in a final volume of 170 μl. After 30 min at 4°C, cells were
frozen in liquid N2 and then thawed at 4°C. Following ultracentrifu-
gation in a Beckman TL-100 Ultracentrifuge for 45 min at 100,000 ×
g, 100,000 rpm, the supernatant was analyzed using SDS-PAGE.

Protein Purification—Frozen resuspended cells expressing IN1–289/F185K/C280S from 24 liters of Super broth were thawed and resus-
pered in lysis buffer (1 M NaCl, 20 mM HEPES, pH 7.5, 2 mM β-mer-
captoethanol, 0.3 mM lysosyme, 5 mM (imidazole) to a final volume of
1 liter. After a 30-min incubation at 4°C, the lysed cells were homog-
enized with a DVT-100 Tissumizer (Amri Corporation). Portions of the
sonicated lysate were separated on a mini-Ti15 batch rotor for 1 h at
40,000 × g. The supernatant was filtered through a 0.25-μm filter and applied to
a nickel-affinity (Chelating Sepharose Fast Flow, Pharmacia Biotech
Inc.) column (5 × 7 cm). After loading, the column was sequentially
washed with 2 liters of 20 mM imidazole buffer containing 25 mM
HEPES, pH 7.5, 2 mM NaCl, and 2 mM β-ME, and 1 liter of 60 mM
imidazole buffer containing 25 mM HEPES, pH 7.5, 1 mM NaCl, and 2 mM
β-ME. Protein was eluted with a linear gradient of 60 mM to 1 mM
imidazole, containing 25 mM HEPES, pH 7.5, 1 mM NaCl, 2 mM β-ME, and
10% (w/v) glycerol. Fractions containing integrase were pooled, and
EDTA was added to a final concentration of 5 mM. This protein was then
dialyzed against 20 mM sodium phosphate buffer, pH 6.2, 0.3 M NaCl, 1 mM
urea, 1 mM EDTA, and 10% (w/v) glycerol. Fractions containing integrase were pooled, and
EDTA was added to a final concentration of 5 mM. This protein was then
dialyzed against 20 mM sodium phosphate buffer, pH 6.2, 0.3 M NaCl, 1 mM
urea, 1 mM EDTA, and 10% (w/v) glycerol. Fractions containing full-
length protein were pooled and diluted with an equal volume of 1 M
NaCl, 1 mM DTT, 1 mM EDTA, and 10% (w/v) glycerol. Protein was then
equilibrated with 10 mM Tris-HCl, pH 8.0, 20 mM NaCl, 0.1 mM EDTA.

Characterization of a Soluble Mutant of HIV-1 Integrase

Determination of the Multimeric State of IN1–288/F185K/C280S—
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gel filtration and analytical ultracentrifugation. For gel filtration, pu-

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Cells and Analysis of HIV-1 Proteins—HeLa cells were grown in Dulbecco’s modified Eagle’s medium containing 5% fetal calf serum, 100
units of penicillin G sodium, and 0.1 mg/ml streptomycin sulfate as
described (30). To prepare virus stocks, 3.6 × 109 HeLa cells were trans-

ta-nincluding the extended nucleotide sequence described above, was
measured on a Perkin Elmer 550 spectrophotometer (Ant攀登an d
density meter) and corrected using standard tables. Sedimentation

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Labeling and Analysis of HIV-1—Integrase—The plasmids used in
this study were generated by site-directed mutagenesis as described
previously (31). \(\frac{(~23P)TTP}{3000\text{Ci/mmol}}\) was used as a

The model describing a reversible dimer-tetramer self-association
yielded excellent fits for both IN1–289/F185K/C280S and IN1–289/
F185K/C280S. Data analysis in terms of a single ideal solute gave poor
fits with residuals typical for aggregating species and weight
average molecular weights corresponding to values between those ex-
pended for integrase dimers and tetramers.

Integrase Activity Assays—Double-stranded oligonucleotides that
mimic the sequence at the 5’ end of HIV-1 DNA were used as sub-
strates for both the 3’ processing and strand transfer reactions. Oligo-

nucleotides AE 117 (5’-ACTCTAGAGATTTCACACG), AE 138 (5’-GTTGGAACATCTTGACGGT), and AE 350 (5’-GTTGGAAAT

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RESULTS

The F185K Mutation Increases the Solubility of HIV-1 Integrase—Expression of IN\textsuperscript{1–288}/F185K and subsequent purification by Ni\textsuperscript{2+}-affinity chromatography yielded a protein that was considerably more soluble than wild-type integrase in buffer containing 1 M NaCl (data not shown). However, this protein displayed time-dependent aggregation in the absence of a reducing agent. This aggregation was readily reversed in the presence of 5 mM DTT. Preliminary studies with a carboxyl-terminal deletion mutant and the soluble catalytic core domain of integrase revealed that these proteins did not aggregate in the absence of DTT. We therefore suspected that the aggregation was caused by disulfide linkages involving cysteine 280, the only cysteine present in the carboxyl-terminal region of the protein. Serine was substituted for cysteine at this position. The resulting protein IN\textsuperscript{1–288}/F185K/C280S retained the markedly improved solubility properties compared with wild-type integrase but, unlike IN\textsuperscript{1–288}/F185K, remained soluble even in the absence of reducing agent.

Purification of IN\textsuperscript{1–288}/F185K/C280S Forms Both Dimers and Tetramers in Solution—We used both gel filtration and sedimentation equilibrium studies to determine the multimeric state of IN\textsuperscript{1–288}/F185K/C280S in solution. Gel filtration using a Superdex 200 column, with the protein loaded at high concentration (>4 mg/ml), resulted in a single eluted peak that migrated at the predicted position of a tetramer relative to globular protein standards (Fig. 3). When IN\textsuperscript{1–288}/F185K/C280S was loaded at a lower concentration (<0.4 mg/ml), a single peak was again eluted; however, this corresponded to the position expected for a dimer of integrase. Nevertheless, both the dimer and tetramer peaks contained elements of tetramer and dimer, respectively (Fig. 3).

Although qualitatively informative, the gel filtration data represent the average behavior of reassociating molecules as they migrate through the column. To more rigorously determine the multimeric state of the protein, a series of protein dilutions were examined by sedimentation equilibrium at 40 °C and different rotor speeds. A single ideal solute fit gave molecular masses which indicated the presence of at least a dimer, even at integrase concentrations as low as 0.1 mg/ml. At higher concentrations, evidence for the self-association of the integrase dimers was observed. The sedimentation equilibrium data for each loading concentration were fitted using a dimer-tetramer association model (see "Materials and Methods," Equation 1). Protein concentrations of 0.3, 1.0, and 1.25 mg/ml yielded an excellent fit to this model (Fig. 4A) with residuals normally distributed about zero. For all three concentrations, similar values of K\textsubscript{d} were obtained, averaged at 10.7 ± 0.2, leading to an apparent dissociation constant, K\textsubscript{d}, of 44,000 M\textsuperscript{−1}. This corresponds to an effective dissociation constant, K\textsubscript{eff}, of 2.2 × 10\textsuperscript{−5} M. The experimental data are thus consistent with a reversible dimer-tetramer self-association (Fig. 4B).

FIG. 1. SDS-PAGE of whole cell extracts and soluble fractions of cells expressing wild-type IN and IN\textsuperscript{1–288}/F185K/C280S. Whole cell extracts of E. coli cells expressing wild-type and IN\textsuperscript{1–288}/F185K/C280S show induced proteins of the predicted size that migrate just above the 31-kDa molecular mass marker (see arrow). IN\textsuperscript{1–288}/F185K/C280S migrates slightly slower as this protein contains a HT, whereas wild-type IN does not. Supernatants, from cells lysed in 0.15 M, 0.5 M, or 1.0 M NaCl and subsequently ultracentrifuged, are shown in adjacent lanes.

FIG. 2. Purification of IN\textsuperscript{1–288}/F185K/C280S. Purified protein fractions were analyzed by SDS-PAGE and stained with Coomassie Blue. A 5-µg loading of IN\textsuperscript{1–288}/F185K/C280S eluted from the Ni\textsuperscript{2+}-affinity column revealed minor contaminating species. These were removed by chromatography on a Mono S cation ion exchange column. Loading of 2–12 µg of the pooled Mono S fractions demonstrates the purity of the protein.

FIG. 3. Gel filtration profiles of IN\textsuperscript{1–288}/F185K/C280S. Two separate gel filtration chromatograms are superimposed. 30 µl of purified protein was loaded onto the column at either 0.4 mg/ml (solid line) or at 4 mg/ml (dashed line). The multimeric state, estimated from the mobility relative to globular protein standards, is indicated. The A\textsubscript{280} scale on the left is for the 4 mg/ml injection, and the A\textsubscript{280} scale on the right is for the 0.4 mg/ml injection.
The Carboxyl-terminal Domain of Integrase Is Required for Tetramerization—To further investigate the requirements for tetramerization, two deletion mutant proteins were constructed. The first contained the core and carboxyl-terminal domains, IN50–288/F185K/C280S, and was purified as described for the full-length counterpart. The second comprised the core and amino-terminal domains, IN1–212/F185K, and was purified as described for IN50–212/F185K (19). Gel filtration experiments revealed that IN1–288/F185K/C280S is exclusively a dimer in solution (data not shown). In contrast, sedimentation equilibrium data showed that IN50–288/F185K/C280S, like IN1–288/F185K/C280S, exists in an equilibrium between dimers and tetramers. Data were collected at 0.4 mg/ml and fitted using a dimer-tetramer association model (Equation 1). The data yielded an excellent fit to this model (Fig. 5) with residuals normally distributed about zero. An $K_a$ value of 10.8 ± 0.4 was obtained, leading to an apparent association constant, $K_a$, of 51,400 M$^{-1}$. This corresponds to an effective dissociation constant, $K_d$, of 2.0 × 10$^{-5}$ M.

Catalytic Activities of IN1–288/F185K/C280S—Integrase activity was analyzed using double-stranded oligonucleotide substrates that mimic the sequences found at the 5' end of HIV-1 DNA (6, 7). It has been shown that 3' processing by wild-type integrase in the presence of Mn$^{2+}$ generates three specific products of nucleophilic attack on the phosphodiester bond at the site of cleavage (28, 33). When water acts as the nucleophile, a simple dinucleotide (D) is generated, attack by glycerol produces a glycerol adduct (G), and attack by the 3’-OH end of the DNA strand yields a cyclic dinucleotide product (C). However, in the presence of Mg$^{2+}$, the simple dinucleotide (D) is the sole product (33). IN1–288/F185K/C280S was compared with wild-type integrase for the preference of nucleophile utilization in the cleavage reaction with either Mn$^{2+}$ or Mg$^{2+}$. No difference could be observed between the two enzymes with regard to the selection of nucleophile or the extent of cleavage under either assay condition (Fig. 6). The strand transfer activities of
IN1–288/F185K/C280S and wild-type integrase were also compared, and no significant differences were observed (Fig. 7). Disintegration activities were also found to be identical. We conclude that the combined mutations of F185K and C280S do not significantly alter in vitro activities of integrase.

Mutation F185K Is Deleterious to Virion Assembly in Vivo—Following our in vitro characterization of the soluble mutant IN/F185K, but before introduction of the additional mutation C280S, we investigated the in vivo effects of the F185K mutation on the replication of HIV-1 in cell culture. The mutation was introduced into the infectious molecular clone HIV-1NL4–3 and virus particles were generated by transfection of HeLa cells. Supernatants from cells transfected with wild-type or F185K clones were normalized for reverse transcriptase activity and used to infect the human T-cell line, CEM-12D7. To assess infection kinetics, CEM-12D7 culture supernatants were monitored for reverse transcriptase activity for 60 days. Wild-type virus production, as measured by reverse transcriptase activity, peaked at day 8. No replication of the mutant (F185K)-HIV-1NL4–3 was detected (Fig. 8A). After failure of the mutant virus to infect CEM-12D7 cells, the virus generated by transfection of the HeLa cells was examined. Transfected cells were radiolabeled, and cell- and virus-associated HIV-1 proteins were analyzed by immunoprecipitation and SDS-PAGE. Lysates of cells transfected with the wild-type clone contained prominent precursor and mature forms of envelope, gp160 and gp120(SU), respectively, and capsid, Pr55gag, and p24(CA), respectively, as indicated on the left. The migration position of the p66 subunit of reverse transcriptase and p32(IN) are included on the right. Gels for analyzing particle-associated HIV-1 proteins were exposed for approximately 5 weeks. The migration positions of molecular mass standards are indicated in kilodaltons in the center.
of reverse transcriptase and integrase (p32). However, when normalized for gp120 and p24 content, both p66 and p32 were dramatically reduced in lysates of virions from cells transfected with the F185K mutant done. Electron microscopy of cross-sections of transfected cells revealed that the mutant virus appeared to consist of immature rings devoid of nucleoid material or aberrant particles that contained nucleoid material located near the membrane of the particle (data not shown). The F185K change is apparently another example of an inte-grase mutation which can affect virion protein composition and morphology (30, 34, 35).

DISCUSSION

Structural and biophysical studies of HIV integrase have been impeded by the poor solubility of the protein. We find that a single amino acid substitution that dramatically improves the solubility of the core domain (19) has a similar effect on the solubility of the full-length protein. This protein required the presence of reducing agent to prevent slow time-dependent aggregation, a need which was alleviated by introducing the additional mutation C280S. The double mutant provides a protein that should facilitate structural studies of full-length integrase.

Solubility Studies—Wild-type HIV-1 integrase is poorly soluble except in the presence of reagents that are likely to cause at least partial denaturation. Maximum solubility is approximately 1 mg/ml, even under the most favorable conditions of high ionic strength. The F185K mutation considerably increases the solubility of the full-length integrase, provided high ionic strength is maintained. However, like the wild-type integrase, the solubility is greatly reduced at low ionic strength. The requirement for the presence of a reducing agent to maintain the protein in a nonaggregated state is abrogated by the additional mutation C280S. We speculate that intermolecular disulfide cross-links involving Cys-280 may also contribute to the propensity of wild-type integrase to aggregate, but the presence of reducing agents does not significantly reduce aggregation because other intermolecular interactions dominate the aggregation process.

Multimerization of Integrase—Our results demonstrate that IN1–288/F185K/C280S exists in a reversible dimer-tetramer self-association in solution (Figs. 3 and 4). Gel filtration results demonstrate the absence of higher molecular weight aggregates, and that at protein concentrations approaching 10 mg/ml the predominant form of the protein is tetrameric. Dimers, but not tetramers, are observed with either the HIV-1 integrase core domain alone, IN50–212/F185K; a carboxyl-terminal truncation mutant, IN1–212/F185K; or with the carboxyl-terminal domain, IN213–288. In fact, NMR studies of a truncated carboxyl-terminal domain of HIV-1 integrase, IN200–270, show a dimer in solution (36, 37). However, a deletion mutant containing the core and carboxyl-terminal domains, IN50–288/F185K/C280S, exists in a dimer-tetramer self-association in solution (Fig. 5). Thus, multimerization interfaces appear to be located in both the core and carboxyl-terminal domains of HIV-1 integrase.

Complementation studies with HIV-1 and HIV-2 integrases (12, 38) have demonstrated that multimerization is required for the 3' processing and strand transfer activities, but these experiments cannot discriminate whether the active multimer is dimeric, tetrameric, or higher order. Several retroviral integrases have been reported to exist as dimers (6, 39–41). Rous sarcoma virus integrase has been reported to form a reversible monomer-dimer-tetramer association in solution, and it was also suggested that the protein functioned as a multimer (41). Collectively, these studies point to a propensity of integrases to form both dimers and tetramers, but do not directly address which multimer is the active species for catalysis.

Effects of the F185K/C280S Mutations on Enzymatic Activity and Multimerization—A major concern when using mutagenesis as a tool to improve the behavior of a protein for structural studies is fortuitous alteration of other properties, especially catalytic activity. We therefore examined IN1–288/F185K/C280S to determine if there were any measurable perturbations in catalytic activity. Previous studies with F185K in the context of the catalytic core domain revealed that the mutant protein was in fact slightly more active than the unmutagenized core (19). However, no significant differences could be observed between the unmutagenized and mutant full-length proteins in assays for 3'-processing, strand transfer, or disintegration activities. We conclude that the combined F185K and C280S mutations do not adversely affect the catalytic properties of HIV-1 integrase in vitro.

We were unable to detect any difference in the multimerization properties between IN1–288/F185K and IN1–288/F185K/C280S (data not shown) and conclude that this amino acid substitution has little, if any, effect on multimerization. It was not possible to compare directly the multimerization properties of IN1–288/F185K/C280S with wild-type protein because the latter exhibits extensive aggregation, except at relatively low concentrations in the presence of detergent. The F185K mutation was previously reported to stabilize dimerization of the isolated core domain (19), and this mutation appears to have the similar effect of stabilizing dimerization of the full-length integrase; at a protein concentration of 0.2 mg/ml, wild-type protein exhibits a monomer-dimer equilibrium in the presence of the detergent CHAPS, whereas IN1–288/F185K/C280S was exclusively dimeric (data not shown).

We also wished to determine the effect of the F185K mutation on HIV replication in cell culture. Unfortunately, this mutation disrupted proper virion assembly and blocked replication at a step prior to reverse transcription; its potential effect on integration, therefore, could not be determined. This phenotype has been observed with several other mutations in integrase (30, 34, 35) and probably reflects effects on protein-protein interactions involving integrase at earlier stages of the replication cycle.

Utility for Structural Studies—The soluble integrase mutant IN1–288/F185K/C280S is an attractive candidate for structural studies since it is well behaved and soluble up to at least 20 mg/ml in a buffer containing 1 M NaCl. Soluble derivatives of integrases have been reported to exist as dimers (6, 39–41). Rous sarcoma virus integrase is now available that include all three domains. Structures of the core (20) and carboxyl-terminal domains (36, 37) have already been determined for HIV-1 integrase, and the structure of the core domain of the closely related Rous sarcoma virus integrase has also been solved (42). These structures should eventually prove invaluable in determining the mechanism of action of inhibitors to HIV integrase and ultimately to guiding the design of therapeutically useful compounds. A major remaining objective is determination of the structure of the intact HIV integrase in a complex with its DNA substrates. The soluble mutant described here may help accomplish this goal.

Acknowledgments—We thank A. Hickman for initial purification attempts and for careful reading of the manuscript, and M. Martin (LMM, NIAID) for use of the Bldg. 4 B2 AIDS laboratory.

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J. Biol. Chem. 1996, 271:7712-7718.
doi: 10.1074/jbc.271.13.7712

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