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Human Immunodeficiency Virus Type 1 and Type 2 Protease Monomers Are Functionally Interchangeable in the Dimeric Enzymes

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Human immunodeficiency virus type 1 (HIV-1) and HIV-2 proteases are dimers of identical subunits. We made a construct for the expression of recombinant one-chain HIV-2 protease dimer, which, like the previously described one-chain HIV-1 protease dimer, is fully active. The constructs for the one-chain dimers of HIV-1 and HIV-2 proteases were modified to produce hybrid one-chain dimers consisting of both HIV-1 and HIV-2 protease monomers. Although the monomers share only 47.5% sequence identity, the hybrid one-chain dimers are fully active, suggesting that the folding of both HIV-1 and HIV-2 protease monomers is functionally similar.

Both types 1 and 2 of the human immunodeficiency virus (HIV) are etiological agents for AIDS. Considerable efforts are devoted to the study of HIV proteases because of their potential as therapeutic targets in the treatment of AIDS. In addition to the extensive biochemical and genetic studies of HIV-1 protease (5, 6, 12, 13), its crystal structure was also solved (10, 17). The sequences of HIV-1 and HIV-2 proteases are less than 50% identical; nevertheless, their enzymatic properties are generally very similar (11, 15, 16). It has been proposed that the folding of the two proteases is similar (15). However, in the absence of a crystal structure for HIV-2 protease, the above hypothesis has not been verified.

We and others have recently shown that the one-chain HIV-1 protease dimers, consisting of two copies of the HIV-1 protease monomer linked via 2 to 5 amino acids in a head-to-tail fashion, function equivalently to the natural two-chain HIV-1 protease dimer (4, 7). In this study, we used a similar strategy to construct an active one-chain HIV-2 protease dimer. We then modified the HIV-1 and HIV-2 one-chain dimer constructs to produce polypeptides consisting of linked HIV-1 and HIV-2 monomeric subunits. These one-chain hybrid dimer proteases are fully active, suggesting that the folding of HIV-1 and HIV-2 protease monomers is functionally similar, so that subunit exchanges have no apparent effect on enzymatic activities.

Constructions for the expression of one-chain HIV protease dimers. The constructs for the HIV-1 and HIV-2 protease genes and the one-chain HIV-1 protease dimer were reported previously (2–4). We will use the same nomenclature as described previously for the HIV protease genes and their protein products. B represents the HIV protease N-terminal flanking sequences, and A represents the HIV protease coding sequences (2–4). The subscripts 1 and 2 represent sequences from HIV-1 (HXB2) and HIV-2 (ROD), respectively. For example, gene B_2A_2 (Fig. 1) has a DNA sequence spanning nucleotides 1931 to 2380 of the HIV-2 (ROD) genome, containing 51 codons of the N-terminal flanking sequence and 99 codons of the HIV-2 protease (3, 9). Gene B_2A_2 was modified to replace the 51 codons of the N-terminal flanking sequence with ACTTCGAGCTCAGGC, which has a unique *Sst*I site. The modification was done by the polymerase chain reaction, using 2 oligonucleotides: ACT TCGAGCTCAGGCCCTCAATTCTCTCTTTGGAAAAG ACCAGTA and GGTGAAGCTTACTATAGATTTAAT GACATGCCTAAG. The resulting modified DNA was called *Sst*I-A_2. Gene B_2A_2 was also modified by the polymerase chain reaction with 2 oligonucleotides (TCGAATTCAT GTCCAGCAGTGGATCTACTGG and TAATGAGCTCC CTCCTAGATTTAATGACATGCCTAAGGCTGTCAG) to append the 3′ end of gene B_2A_2 with GGAGGGAGCTCA TTA, which also carries a unique *Sst*I site. The modified DNA was called B_2A_2-*Sst*I. These two intermediates, B_2A_2-*Sst*I and *Sst*I-A_2, were digested with *Sst*I and ligated, resulting in a gene called $B_2A_2A_2$. Gene $B_2A_2A_2$ has 783 nucleotides, starting with GAATTCATG and the 51 codons for the N-terminal flanking sequence of HIV-2 protease shown as a thin line in Fig. 1. The above sequence is followed with 99 HIV-2 protease codons (nucleotides 2084 to 2381), a 5-codon linker (GGAGGGAGCTCAGGC [for Gly-Gly-Ser-Ser-Gly]), another copy of the 99 HIV-2 protease codons, 2 stop codons, and a sequence for a *Hin*dIII site. The gene $B_2A_2A_2$ was expressed in *Escherichia coli* as a 258-amino-acid precursor protein, which was quickly processed to the mature 203-amino-acid one-chain HIV-2 protease dimer (data not shown).

The basic construction of $B_2A_2A_2$ for the one-chain HIV-2 dimer is the same as that of the one-chain HIV-1 dimer (4). In addition, identical linker sequences, joining the two protease monomeric sequences, were built in both one-chain dimer genes. Within the linker sequence, there is a unique *Sst*I restriction site which was used to construct hybrid one-chain dimer genes. The larger fragment of the *Eco*RI-*Sst*I-digested $B_1A_1A_1$ was ligated with the smaller fragment of the *Sst*I-*Hin*dIII-digested $B_2A_2A_2$ to generate the gene $B_1A_1A_2$, which was used to produce a mature hybrid one-chain dimer consisting of an HIV-1 protease at the N-terminal half and an HIV-2 protease at the C-terminal half (Fig. 1). Similarly, a ligation of the larger fragment of the *Eco*RI-*Sst*I-digested $B_2A_2A_2$ and the smaller fragment of the *Sst*I-*Hin*dIII-digested $B_1A_1A_1$ resulted in gene $B_2A_2A_1$ (Fig. 1). All constructs were verified by DNA sequence analyses. Later, the *Eco*RI-*Hin*dIII fragments of these genes were

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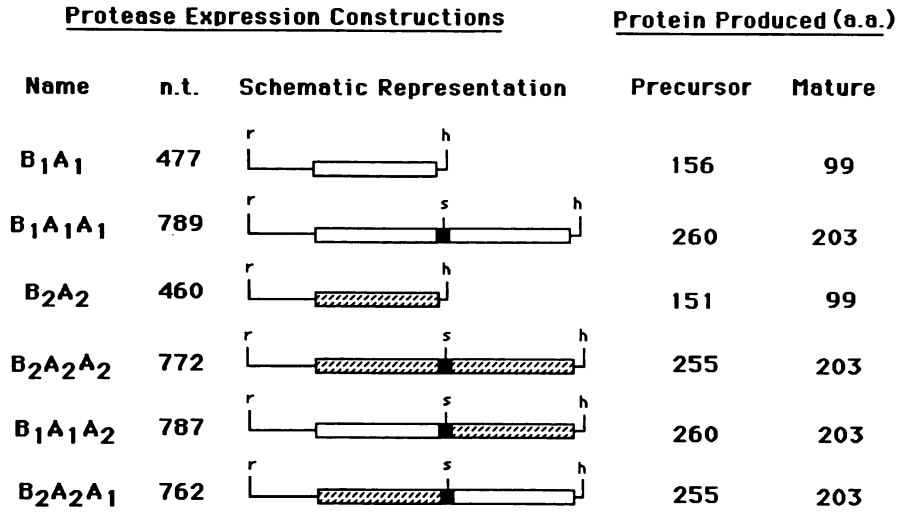

FIG. 1. Schematic representation of constructs for the expression of various forms of HIV-1 and HIV-2 one-chain dimeric proteases. The HIV-1 and HIV-2 protease coding sequences are represented with open and striped bars, respectively. The lines to the left of the open and striped bars represent the N-terminal flanking sequences of HIV-1 and HIV-2 proteases, respectively. Sequence GGAGGGAGCTCAGGC (small filled bar), encoding a pentapeptide (Gly-Gly-Ser-Ser-Gly), was used to join the two protease coding sequences. The lines to the right of the open or striped bars contain two stop codons: TAG and TAA. Restriction sites: r, *Eco*RI; s, *Sst*I; h, *Hin*dIII. These constructs were expressed in a T7 promoter expression vector, pET3AM (2). The sizes (in amino acids [a.a.]) of expressed precursor proteins and mature autoproteolytic products are shown in the two right columns.

inserted into the expression vector pET3AM, regulated by a T7 promoter, as previously described (2, 14).

Isolation and purification of HIV proteases. All six constructs were expressed under identical conditions for the production of precursor proteins as initial translation products. As shown previously, mature HIV proteases are generated by the removal of the N-terminal flanking peptide from the protease precursors by autoproteolysis (2–4). The expected protein sizes for the precursor and the mature forms are shown in Fig. 1. Both the precursor and the mature forms were detectable in cell extracts of every one of the six constructs (data not shown). Only the mature forms were isolated for further studies. To simplify the names for these mature proteases, the natural HIV-1 and HIV-2 proteases expressed by genes B_1A_1 and B_2A_2 are called PR-A_1 and PR-A_2, respectively. Similarly, their one-chain dimers are called PR-A_1A_1 and PR-A_2A_2, respectively. The hybrid one-chain protease dimer with HIV-1 protease at the N terminus and HIV-2 protease at the C terminus is called PR-A_1A_2, whereas the one with HIV-2 protease at the N terminus and HIV-1 protease at C terminus is called PR-A_2A_1. As described previously, both the natural HIV-1 protease (PR-A_1) and its one-chain dimer (PR-A_1A_1) were produced exclusively as insoluble inclusion bodies (2, 4). In contrast, both the HIV-2 natural protease (PR-A_2) and its one-chain dimeric form (PR-A_2A_2) were totally soluble. It is interesting to note that the hybrid dimers produced in *E. coli* cells were found as both soluble and insoluble forms, at a roughly one-to-one ratio. The predicted pI values for HIV-1 and HIV-2 one-chain dimers are 9.30 and 5.10, respectively. The predicted pI values for the hybrid one-chain proteases PR-A_1A_2 and PR-A_2A_1 are 8.39 and 8.08, respectively. Our observation suggests that perhaps the solubility of the recombinant HIV proteases in *E. coli* is determined by their overall protein charges.

We showed previously (4) that PR-A_1 and PR-A_1A_1 can be successively purified from *E. coli* inclusion bodies by acetic acid extraction, refolding at neutral pH, and gel permeation

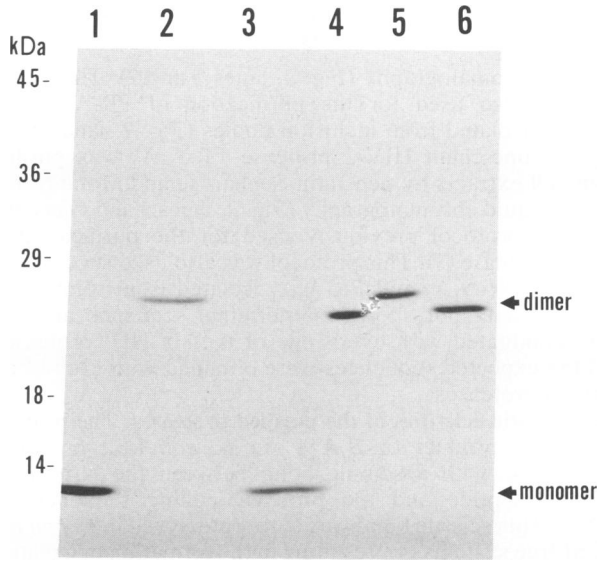

FIG. 2. Purification of the novel recombinant HIV proteases. HIV-1 protease PR-A_1 (lane 1), and its one-chain dimer, PR-A_1A_1 (lane 2), were isolated from cell inclusion bodies, refolded, and further purified with a gel permeation column as described previously (2). The hybrid one-chain dimers PR-A_1A_2 (lane 5) and PR-A_2A_1 (lane 6) were also purified by the same method. HIV-2 protease PR-A_2 (lane 3) and its one-chain dimer, PR-A_2A_2 (lane 4), were purified with a pepstatin affinity column followed by Mono-S chromatography as described elsewhere (3). Arrows point at the positions of the one-chain dimers and the natural monomeric HIV proteases in the Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel. The protein size standards are shown at the left.

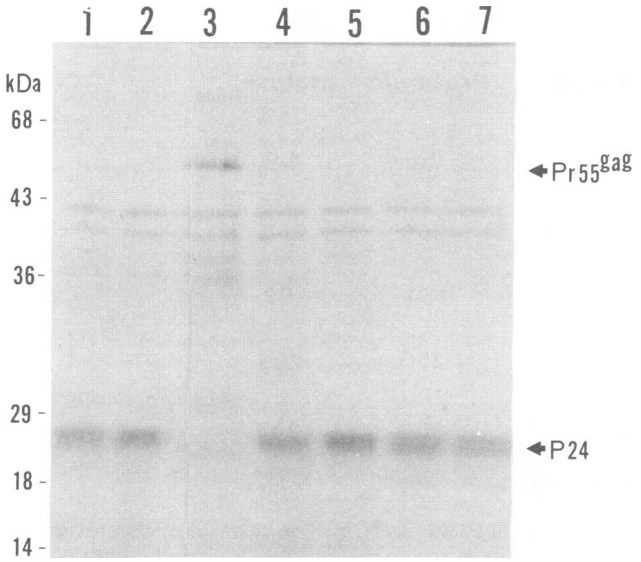

FIG. 3. Proteolysis of radiolabelled HIV-1 Pr55^{gag} substrate by various recombinant HIV proteases. The preparation of the radiolabelled Pr55^{gag} and its digestion by HIV protease were described previously (8). Equal counts of the labelled Pr55^{gag} were incubated with PR-A_1 (lane 1), PR-A_1A_1 (lane 2), buffer only (lane 3), PR-A_2 (lane 4), PR-A_2A_2 (lane 5), PR-A_1A_2 (lane 6), and PR-A_2A_1 (lane 7) at 30°C for 20 min. At the end of the incubation, the digestion products were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Arrows point at the positions of the Pr55^{gag} and its proteolytic product, P24, in the autoradiogram. Protein size standards are shown at the left.

column chromatography (Fig. 2, lanes 1 and 2). This protocol was also used for the purification of PR-A_1A_2 and Pr-A_2A_1 isolated from inclusion bodies (Fig. 2, lanes 5 and 6). The one-chain HIV-2 protease PR-A_2A_2 was purified from cell extracts by pepstatin-Sepharose and Mono-S fast-protein liquid chromatography (Fig. 2, lanes 3 and 4) according to a protocol previously used for the purification of HIV-2 protease (3). This protocol was also used successfully to purify PR-A_1A_2 and PR-A_2A_1 isolated from soluble cell extracts (data not shown). N-terminal sequence analyses were conducted with every one of the six HIV proteases, and the expected sequences were obtained with each of the purified proteases.

Enzymatic activities of the purified proteases. The primary translation product of $B_1A_1A_1$ in *E. coli* is a precursor polypeptide with a scissile bond between the N-terminal flanking peptide and the protease coding sequences of HIV-1. This scissile bond was autoproteolyzed in *E. coli* and in cell-free systems (4), resulting in the formation of a mature one-chain dimer, PR-A_1A_1. Similarly, mature hybrid one-chain dimers PR-A_1A_2 and PR-A_2A_1 were made from $B_1A_1A_2$ and $B_2A_2A_1$ constructs, which synthesized precursors with scissile bonds of HIV-1 and HIV-2 sequences, respectively. Therefore, both hybrid dimers can efficiently autoproteolyze the scissile bonds with either HIV-1 or HIV-2 sequences.

We also tested the cleavage of the HIV-1 Pr55^{gag} by these recombinant proteases. Figure 3 shows that the HIV-2 one-chain dimer (lane 5) and the hybrid HIV-1 and HIV-2 one-chain dimers (lanes 6 and 7) all effectively proteolyzed HIV-1 Pr55^{gag} to P24 in a manner qualitatively identical to the proteolysis by HIV-1 protease, its one-chain dimer, and HIV-2 protease (Fig. 3, lanes 1, 2, and 4).

TABLE 1. Kinetic constants of HIV proteases[a]

Protease	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (10^3 M^{-1} s^{-1})
PR-A_2[b]	0.25 ± 0.040	1.8 ± 0.60	7 ± 1.4
PR-A_2A_2[b]	0.12 ± 0.001	1.2 ± 0.20	10 ± 1
PR-A_1A_1[c]	0.14 ± 0.004	4.2 ± 0.14	29.7 ± 1.8
PR-A_2A_1[c]	0.06 ± 0.004	1.5 ± 0.10	24 ± 0.2
PR-A_1A_2[c]	0.08 ± 0.004	1.0 ± 0.10	12 ± 1.4
PR-A_1A_2[b]	0.07 ± 0.020	1.2 ± 0.02	18 ± 4.1

[a] Protease activity measurements were made at 25°C in 50 mM morpholineethanesulfonic acid (MES) buffer (pH 5.5)–1.0 mM EDTA–1.0 mM dithiothreitol–1 M NaCl–10% glycerol.
[b] Purified from *E. coli* soluble extracts.
[c] Purified from *E. coli* inclusion bodies.

For a quantitative comparison of these proteases, we measured the kinetic constants of these proteases on a peptide substrate, Ala-Thr-His-Gln-Val-Tyr-Phe-(NO_2)-Val-Arg-Lys-Ala. The results are summarized in Table 1. Kinetic constants were determined at pH 5.5 and 1.0 M NaCl by the procedure described previously (4), except that a substrate range of 0.025 to 0.15 mM rather than 0.20 to 0.22 mM was used. Reported values are the averages of at least two separate determinations, and the standard deviations are shown. k_{cat}/K_m values for PR-A_2A_2 and natural HIV-2 protease are comparable, indicating that the one-chain HIV-2 protease dimer has a catalytic activity similar to that of the natural HIV-2 protease. The hybrid one-chain dimers PR-A_1A_2 and PR-A_2A_1 are all very active in the digestion of this peptide substrate, with k_{cat}/K_m values between those for PR-A_1A_1 and PR-A_2A_2 (Table 1).

We have previously established that the biochemical properties of the one-chain HIV-1 protease dimer are very similar to those of the natural HIV-1 protease (4). We show in this report that the HIV-2 protease one-chain dimer can also be produced; its enzymatic activity is also very similar to that of the natural form.

Previous study indicated that the function of both linked monomers is needed to constitute an active enzyme. When single amino alterations were made at the active site of one of the two monomers of the one-chain dimer, completely inactive proteases resulted (4). Nevertheless, the hybrid one-chain dimer proteases consisting of tethered HIV-1 and HIV-2 protease monomers, which are less than 50% identical in sequence, are fully active. This result suggests that in spite of their obvious sequence differences, the HIV-1 and the HIV-2 protease monomers are folded in a functionally similar manner. It is interesting to note that the k_{cat}/K_m values of the hybrid dimers are roughly the mean of those of the HIV-1 and HIV-2 one-chain dimeric proteases, suggesting that both linked monomers contribute the activity of the hybrid forms. To our knowledge, this is the first report comparing the folding properties of two dimeric proteins by functional comparison of the hybrid one-chain dimers of their constituent monomers.

Babe et al. recently observed a reduction of expected protease activity in a mixture of HIV-1 and HIV-2 proteases (1). This was interpreted as an inhibition of HIV protease activity by the formation of a noncovalent heterodimer which may display a disordered dimer interface, thereby affecting the catalytic potential of the enzymes (1). This interpretation, however, is complicated by their observation

that heterogeneous protein species were found in the mixtures of the incubated HIV-1 and HIV-2 proteases. The natures of these heterogeneous protein species were not known, nor were their contributions to the overall protease activity assessed. In contrast, our hybrid one-chain dimers, which are single-species and biochemically defined proteins, are highly active. The high specific activities of these hybrid one-chain dimers suggest that they are unlikely to have a disordered dimer interface as proposed by Babe et al. (1). The covalent linkage of our one-chain dimers may contribute to the alignment and stability of dimer formation (4). Nevertheless, the distance between the covalent dimer linkage and the major dimer interface suggests that the covalent stabilization effect is unlikely to be a major factor for the ordered nature of the dimer interface.

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