Identification of Temperature-sensitive Mutants of the Human Immunodeficiency Virus Type 1 Protease through Saturation Mutagenesis

AMINO ACID SIDE CHAIN REQUIREMENTS FOR TEMPERATURE SENSITIVITY*

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Human immunodeficiency virus type 1 encodes a protease whose activity is required for the production of infectious virus. An Escherichia coli expression and processing assay system was used to screen 285 protease mutants for temperature-sensitive activity. Fourteen protease mutants had a temperature-sensitive phenotype, and approximately half resulted from conservative amino acid substitutions. Of the 14 substitutions that conferred a temperature-sensitive phenotype, 11 substitutions occurred at 6 positions that represent 3 pairs of residues in the protease that contact each other in the three-dimensional structure. These mutants assist in pinpointing regions of the protease that are important for enzyme activity and stability.

The HIV-1 protease rapidly has become one of the most thoroughly characterized proteins to date. The protease now ranks with such proteins as bacteriophage T4 lysozyme, bacteriophage λ repressor and cro protein, and staphylococcal nuclease as examples of models for protein structure and function (reviewed in Refs. 1 and 2).

The structure of HIV-1 protease has been determined in its native form and in complex with numerous inhibitors (for examples see Refs. 3–8; reviewed in Ref. 9). The enzyme-inhibitor complexes yield a wealth of information regarding the side chains in the substrate and enzyme that are important for binding and catalysis. Mutagenesis studies have also contributed to the detailed picture of the HIV-1 protease (10–12). These studies, in addition to structural studies of other retroviral proteases (13–17), complement structural studies of the HIV-1 protease by identifying regions of the protease structure that are important for enzyme function.

Classical studies of temperature-sensitive (ts) mutant proteins highlight the structural and thermodynamic contributions of the substituted residue to the folded state of the protein (reviewed in Ref. 2). Studies of ts mutants of bacteriophage T4 lysozyme have localized ts substitutions to regions of the protein that are buried and held in a rigid conformation in the folded protein (18). Therefore, temperature-sensitive mutants are helpful in pinpointing specific regions of the protein that contribute to enzyme stability.

In this study 285 randomly generated mutants of the HIV-1 protease were screened for temperature sensitivity. At positions in the enzyme where a substitution was found that conferred a ts phenotype, the random mutagenesis was followed by saturation mutagenesis. These studies help define the role that particular residues play in protease structure, function, and thermostability. In addition, these studies facilitate a detailed analysis of the HIV-1 protease structure and help to define the general rules for temperature sensitivity in an enzyme.

MATERIALS AND METHODS

Bacterial Strains and Media

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The abbreviations used are: HIV-1, human immunodeficiency virus type 1; ts, temperature-sensitive.
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Identification of ts Mutants—This study used a simple screen for protease activity to evaluate the processing activity of a large number of protease mutants. Mutations were introduced by random site-directed mutagenesis using the expression clone pART2. This plasmid contains the BglII to SalI pol-containing DNA fragment from HIV-1 (strain HXB2) fused to the lac promoter in the bacterial expression plasmid pBl20 (Fig. 1, top; Ref. 1). The addition of 5 mM isopropyl-β-D-thiogalactopyranoside induces expression of a 120-kDa Pol polyprotein containing the protease. Phenotypes were assigned by assaying the ability of the protease to autoprocess the 120-kDa Pol substrate to the mature 64- and 51-kDa forms of reverse transcriptase, as determined by Western blot analysis (Fig. 1, bottom). In this study 285 protease mutants were screened, each containing a single amino acid substitution. Each mutant was expressed at 32 and 37 °C, and the extent of processing activity at either temperature was evaluated by Western blot analysis. During the initial screen nine mutants, or approximately 3% of the mutants tested, showed increased or altered processing of the Pol substrate at 32 °C compared with 37 °C (Figs. 1, bottom, and 2). An additional five mutants were identified through the subsequent saturation mutagenesis (see below) for a total of 14 ts mutants identified. These substitutions were at eight positions in the HIV-1 protease: positions 8, 29, 38, 56, 79, 85, 95, and 98 (Figs. 2 and 3). The phenotypes of these mutants covered a wide range; a number of the mutants showed no processing at the restrictive temperature and partial processing at the permissive temperature. Other mutants showed partial processing at the restrictive temperature and nearly complete processing at the permissive temperature (Fig. 1, bottom).

Saturation Mutagenesis at Positions Encoding ts Mutants—To examine the side chain requirements for temperature sensitivity, each codon in the protease where a ts-conferring mutation had been found was saturated with mutations. A mutagenic oligonucleotide was designed to randomize the codon corresponding to that position. For the single position mutant libraries generated, mutation frequency averaged 80%. Phenotypes of new mutants generated by saturation mutagenesis were determined at 37 and 32 °C. Mutants were grouped according to their phenotype in the E. coli processing assay (Fig. 2). Temperature-sensitive phenotypes were generally as-

Temperature-dependent Processing Activity of HIV-1 Protease Mutants in E. coli. Top, composition of the HIV-1 Pol substrate expressed in the bacterial expression vector pART2. lacPO, E. coli β-galactosidase promoter/operator sequence; PR, protease; RT/RN, reverse transcriptase/RNase H; IN, integrase. Bottom, Temperature-dependent autoprocessing of the HIV-1 Pol precursor by mutant proteases in E. coli. Each pair of lanes depicts a particular mutant; the mutant designations are expressed in single-letter code, with the wild-type residue, position number, and encoded substitution indicated. All mutants were expressed at 37 and 32 °C (see "Materials and Methods"). Viruses (lanes 1), lysate of pelleted HIV-1 virions; JM101 (lanes 2), lysate of pelleted E. coli JM101. E. coli lysates were separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose filters. The filters were probed with a monoclonal antibody to HIV-1 reverse transcriptase.

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Table 1. Mutant HIV-1 Protease Phenotypes

| Phenotype | Arg8 | Asp29 | Leu38 | Val56 | Pro79 | Ile85 | Cys95 | Asn98 |
|-----------|------|-------|-------|-------|-------|-------|-------|-------|
| ts        | lys  | gin   | glu   | val   | phe   | thr   | leu   | phe   |
| wild-type | thr   | cys   | ser   | asp   | lys   | arg   | asn   | thr   |
| intermediate | met   | val   | leu   | pro   | ser   | pro   | ser   |
| negative  | asn   | leu   | gln   | ser   | lys   | arg   | leu   | pro   |

Fig. 2. Side chain requirement for function and temperature sensitivity at eight positions in the HIV-1 protease. The eight positions where substitutions conferring temperature sensitivity were observed are listed across the top. Substitutions at each position were grouped according to their ability to process correctly the HIV-1 Pol substrate at both 37 and 32 °C (as described under “Materials and Methods”). Mutants with a substitution that allowed complete processing at both temperatures are listed as wild-type. Those that showed an intermediate level of processing at both temperatures are listed as intermediate. Those that did not process at either temperature are listed as negative. The substitutions in the ts group are those that showed increased processing activity at 32 °C when compared with their phenotype at 37 °C.

associated with conservative amino acid substitutions, with either the approximate size or chemical character of the wild-type residue retained (Fig. 2).

Characterization of Temperature-sensitive Mutants—Substitutions conferring a ts phenotype were distributed throughout the length of the protease (Fig. 3). Most of these substitutions were found within regions that previously have been shown to be conserved at the level of structure and to be mutationally sensitive (4, 11, 13, 28, 29). The ts mutants are discussed below, grouped according to the location of the mutated amino acid in the HIV-1 protease structure.

Dimer Interface—Two of the mutants were found in the dimer interface region at positions 95 and 98 in the protease. The structure of this region is highly conserved among the retroviral proteases (4, 5, 13, 15–17, and 28). The amino and carboxyl termini of each monomer subunit interact in this region to form a four-stranded, antiparallel β-sheet structure (Fig. 4). Substitutions in this region of the HIV-1 protease may weaken intersubunit interactions at the restrictive temperature. Alternatively the substitutions might affect the hinge motions, centered in this region, that occur during substrate binding (6).

The substitution at position 95, cysteine 95 changed to phenylalanine (C95F), occurs at one of two cysteines present in the protein. Neither cysteine participates in a disulfide linkage (4). Most substitutions of this cysteine resulted in an intermediate or negative phenotype at both 37 and 32 °C (Fig. 2). The exception was phenylalanine, which showed increased processing activity at 32 °C compared with 37 °C (Fig. 1, bottom, lanes 21 and 22; Fig. 2). Cysteine 95 interacts with the side chain atoms of leucine 5 and asparagine 97 on the opposite subunit and is close (within 3.5 Å) to the backbone carbonyl oxygen of asparagine 98 on the opposite subunit (Fig. 4). Substitution with phenylalanine may satisfy some of the hydrophobic interactions required in this interface. In other retroviruses the side chain at this position is either a small or aliphatic side chain (29), and the substitutions that allowed at least some activity were of this nature (Fig. 2). A leucine substitution gave nearly wild-type activity, stressing the hydrophobic environment of this side chain, while a proline or serine substitution allowed only low levels of activity.

Mutants with substitutions at asparagine 98 had an interesting phenotype. As seen in Figs. 1 and 2, substitutions with tyrosine, alanine, isoleucine, phenylalanine, and arginine resulted in a ts phenotype. The phenotypes ranged from partial processing (N98R, N98F, N98Y, and N98I) to nearly complete processing at 32 °C (N98A; Fig. 1, bottom, lanes 24 and 26). The side chain of asparagine 98 interacts closely (2.3 Å) with arginine 98 on the opposite subunit. Asparagines 98 also makes close contacts (within 3–4 Å) with residues threonine 96 on the same subunit and glutamine 2 on the opposite subunit. As discussed above, the backbone carbonyl oxygen atoms of asparagine 98 on either subunit also interact with the cysteine 95 side chains (Fig. 4).

Asparagine 98 also serves as part of the substrate. A protease cleavage site exists between the last residue in the protease (phenylalanine 99) and the first residue of reverse transcriptase. Thus, position 98 forms the second residue to the amino-terminal side of the cleaved peptide bond in the substrate, i.e. the P2 substrate residue. The P2 amino acid in the different protease cleavage sites in the HIV-1 Pol substrate is either asparagines (gag/pol transframe region/protease, protease/reverse transcriptase), threonine (reverse transcriptase/RNase H) or valine (reverse transcriptase/integrase) (reviewed in Ref. 30–32). Alanine and isoleucine are also found in the P2 position in other protease cleavage sites in Gag. Thus, of the substitutions tested at this position, alanine or isoleucine are least likely to affect substrate binding. In contrast, other substitutions at this position (N98R, N98F, N98Y) have never been seen at the P2 position in any of the known retroviral protease cleavage sites. These substitutions might have more of an effect on substrate binding than on enzyme activity. Inhibition of cleavage at the protease/reverse transcriptase junction should result in the generation of two fusion proteins, a 75- and a 62-kDa form. Proteins of these approximate sizes are seen in the N98R mutant (Fig. 1, bottom, lane 26), but only slight amounts of these forms are visible. Thus, it seems likely that, with the possible exception of the N98R mutant, the substitutions recovered at position 98 affect enzyme activity independently of substrate binding. Of the other substitutions tested, threonine and cysteine gave essentially wild-type-like activity in this assay, each having a short polar side chain like arginine but with hydrophobic characteristics. Phenylalanine and tyrosine had intermediate activity at 37 °C, while isoleucine was negative at this temperature. The activity at this temperature with the two aromatic side chains and the increased activity at the lower temperature with all three hydrophobic amino acids may indicate that hydrophobic interactions between position 98 amino acids in each subunit with each other may substitute for the interaction between the wild-type asparagines.

Hydrophobic Core—The hydrophobic core of each subunit in
aspartic proteinases is formed by a unique structure known as the psi-loop (Fig. 3; Refs. 3–5). Loeb et al. (11) showed that the vast majority of residues forming the psi-loop structure in HIV-1 protease are mutationally sensitive. We previously reported one substitution that conferred a ts phenotype, proline 79 changed to threonine (P79T), that occurs within the psi-loop at the top of a β-turn in the core (Ref. 1; Fig. 1, bottom, lanes 17 and 18, and Fig. 5). Proline is not well conserved at this position among the retroviral proteases (29). This is consistent with the large number of substitutions allowed at this position (Fig. 2) and indicates that prolines at position 79 is not particularly important for function. In contrast, the second proline in the turn (P81) is much more highly conserved and is probably more important for forming the β-turn and as part of the substrate binding site (9, 29). Proline 79 can be substituted with an acidic, basic, or polar residue and retain full activity (Fig. 2), suggesting that the side chain of the substituted residue is solvent-exposed, an unusual situation for a ts substitution. The two hydrophobic residues tested had either intermediate or no activity (methionine and leucine, respectively).

At one other position in the hydrophobic core, isoleucine at position 85, substitution with leucine (I85L) results in a ts phenotype (Fig. 1, bottom, lanes 19 and 20). Position 85 is at the top of the conserved α-helix in the hydrophobic core (Fig. 5; Refs. 4 and 5). This α-helical region is extremely sensitive to mutation (11). Isoleucine 85 passes through a loop formed by residues 22–33, contacting the side chains of alanine 22 on one side and both threonine 31 and valine 33 on the other (Fig. 5). In addition, interactions between isoleucine 85 and leucine 89 connect the top and bottom of the α-helix. This position is conserved as a large hydrophobic side chain in other retroviral proteases (29). Most substitutions at isoleucine 85 were negative for activity, with the leucine substitution having ts activity but still only intermediate activity at the lower temperature of 32 °C (Figs. 1, bottom, and 2). This conservative leucine substitution at position 85 is probably able to satisfy hydrophobic contacts at the low temperature, confirming that the hydrophobic interactions in this region are crucial for enzyme function. By using this reasoning, the intermediate activity of the valine substitution can also be understood. The wild-type activity of the asparagine is more difficult to rationalize, although a new polar interaction with the threonine 33 side chain is a possibility (Fig. 5).

Intersubunit Salt Bridge—Studies of enzyme-inhibitor complexes reveal an important role for the interaction between arginine 8 of one subunit and aspartic acid 29 on the other subunit (Fig. 6; Refs. 4–9). These side chains participate in an ionic interaction between the two subunits of the enzyme and in substrate binding.

A change of arginine at position 8 to lysine or glutamine (R8K, R8Q) gave similar ts phenotypes (Fig. 1, bottom, lanes 7 and 8). None of the other substitutions tested gave activity (Fig. 2). A change of aspartic acid at position 29 to glutamic acid (D29E) also gave a ts phenotype (Fig. 1, bottom, lanes 9 and 10), but again none of the other substitutions tested at this position permitted activity (Fig. 2). The D29E substitution extends the length of the side chain at this position, which resulted in a
temperature-dependent cleavage of the reverse transcriptase/integrase boundary. At 32 °C, cleavage of the reverse transcriptase/integrase cleavage site was complete, resulting in the formation of an 86-kDa intermediate. At both 32 and 37 °C, however, cleavage at protease/reverse transcriptase and reverse transcriptase/RNase H was not complete (Fig. 1, bottom, lanes 9 and 10). The double mutant R8K/D29E was unable to rescue the ts phenotype (not shown), indicating that a simple adjustment of chain length at both positions is not sufficient to confer complete activity.

Miller et al. (6) have shown that aspartic acid 29 also interacts with arginine 87. Of 11 different substitutions tested at position 87, including both conservative and nonconservative substitutions, none showed processing activity at either 37 or 32 °C.2 Louis et al. (33) also have shown that substitution of arginine 87 with lysine or glutamine completely abolishes protease activity. In addition to its role in intersubunit interactions, this salt bridge is also involved in contact with certain substrates (reviewed in Ref. 34). The pattern of allowed substitutions at these positions confirms an important role for this salt bridge in enzyme function. Residues capable of making ionic interactions are present at these positions in all known retroviral proteases (29), and, based on modeling, this ionic interaction between the subunits is believed to be conserved at the level of structure (34).

The Flap—In retroviral proteases the flap structure is highly conserved (29, 35). Saturation mutagenesis of the flap region has demonstrated that the conserved residues in the flap are sensitive to substitution.3 A 7A shift in flap position has been observed in comparing structures of native protease with a protease bound to a substrate analog (4, 6). Gustchina and Weber (35) have speculated that for substrate to enter the active-site cleft, the ends of the flaps must open still further, moving as much as 13A. Given such a large range of motion it is not surprising that temperature-dependent phenotypes result from substitutions in the flap.

Two substitutions in the flap, at residues 38 and 56, resulted in a ts phenotype. Leucine 38 is at the base of the flap, in a region that interacts with a number of nonconserved residues. Substitution of this leucine with phenylalanine (L38F) or valine (L38V) resulted in a ts phenotype (Fig. 1, bottom, lanes 11–14; Fig. 2). The local environment of the leucine is such that it occupies a predominantly hydrophobic pocket, interacting primarily with methionine at position 36. The hydrophobic side chain of valine apparently can satisfy at least some of the hydrophobic contact requirements at the permissive temperature on the basis of its size. Substitution with the even larger phenylalanine side chain resulted in a ts phenotype with more activity at the permissive temperature than the valine substitution (Fig. 1, bottom, lanes 11–14). All other substitutions tested were negative for activity.

The second ts mutant with a substitution within the flap is valine at position 56 changed to glycine (V56G). Valine 56 participates in a hydrophobic interaction with the side chains of the flap residues isoleucine 47 and isoleucine 54 (Fig. 7). Valine 56 is also near proline 79 (4–5Å) in structures of enzyme, substrate complexes (Fig. 3). Replacement of the valine side chain with glycine may create a cavity in the hydrophobic network between residues 47, 54, and 56 or may cause alterations in the positions of these residues and/or rearrangement of hydrophobic contacts. Substitution of valine 56 with the hydrophobic side chains cysteine and threonine gave full activity (Fig. 2). Both of these amino acids have the same chain length as valine and are somewhat hydrophobic, again suggesting that both the size and chemical nature of the residue at this position are important for function.

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2 L. Everitt, M. Manchester, and R. Swanstrom, unpublished observation.

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CONCLUSIONS

In this study extensive mutagenesis of the HIV-1 protease has yielded a varied group of mutants with temperature-sensitive phenotypes, each of which contributes to an understanding of the activity of this important enzyme. Alber et al. (18) showed that for bacteriophage T4 lysozyme, substitutions that confer a temperature-sensitive phenotype are generally localized to residues that are buried in the hydrophobic core and are held in a rigid conformation. For the HIV-1 protease, most of the ts substitutions were localized to hydrophobic regions. There were, however, several exceptions. Although it is within the hydrophobic core, P79T appears to be solvent-exposed in the crystal structure (8), and charged residues are tolerated at this position (Figs. 2 and 5). Substitutions that conferred a temperature-sensitive phenotype were also found at the hydrophilic residues arginine 8 and aspartic acid 29 in the intersubunit salt bridge. Generally, the amino acid substitutions resulting in temperature sensitivity retain the approximate size or chemical character of the wild-type residue (Fig. 2). A number of the ts mutants resulted from substitutions at residues that interact with or are nearby each other: 98 and 95, 8 and 29, and 56 and 79. This observation suggests that these regions of the protein are particularly important for enzyme activity and thermal stability. Characterization of the R8Q protease mutant has shown that the enzyme has a reduced catalytic efficiency.4

Two of the protease mutants (V56G and P79T) have been tested within the context of protein processing during virion assembly and both show temperature-dependent processing of the Gag polyprotein precursor.5

Two other groups have reported isolating ts mutants of the HIV-1 protease using different types of genetic assays. Baum et al. (12) reported two ts mutants, lysine 45 changed to glutamic acid (K45D) and proline 79 changed to leucine (P79L), that were recovered in a system that used cytotoxicity of the protease as an indicator in E. coli. In our study (Fig. 2), it was recovered as a ts mutant during selection for cytotoxicity at 32 °C in the study by Baum et al. (12). The differences in temperatures used may account for this conflicting result; alternatively, these results may reflect differences in the selection criteria between the two types of assays. Nevertheless, the discovery of two ts mutants with substitutions at the same position (P79T from this study and P79L from Baum et al. (12)) using two different approaches confirms the importance of this residue for enzyme activity. Neither K45D nor V111 was tested in our study.

Structural studies comparing ts lysozyme mutants with wild-type enzyme have shown that amino acid substitutions resulting in temperature sensitivity cause only very minor alterations in protein structure (37–39). The data in Fig. 2, showing that ts phenotypes often result from conservative amino acid substitutions, are consistent with those observations. It seems likely that temperature sensitivity in an enzyme occurs only over a small range of structural perturbation, beyond which the enzyme no longer functions. These types of mutagenesis studies of the HIV-1 protease, combined with structural and enzymatic data, will assist in understanding the nuances of protein structure and the requirements for enzyme activity and thermostability.

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