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Genetics

Isolation of mutants of human immunodeficiency virus protease based on the toxicity of the enzyme in *Escherichia coli*

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ABSTRACT **The protease encoded by the *pol* gene of human immunodeficiency virus was expressed in *Escherichia coli* and found to be toxic to strain BL21(DE3). This toxicity provided a convenient selection for isolating mutants of the protease that are nontoxic and enzymatically inactive. This strong correlation between functional protease and toxicity resulted in rapid identification of several protease mutations, including mutations that exhibit temperature sensitivity. A total of 24 missense mutations and 7 nonsense mutations were identified. The described selection procedure may have wider applications for isolating mutants of other eukaryotic proteins that exhibit a toxic phenotype in *E. coli*.**

The primary translation products of the human immunodeficiency virus (HIV) include polyprotein precursors encoded by the *gag* and *pol* genes (1). The production of mature capsid proteins from *gag*, and of viral protease, reverse transcriptase, and endonuclease from *pol*, is dependent upon the cleavage of these polyproteins by the viral protease, which is generated by autodigestion of the *gag/pol* polyprotein.

As a viral-specific enzyme, the protease is an attractive target for drugs for the inhibition of viral replication and has become the focus of intensive investigation. The mature 99-amino acid protease is homologous to the aspartic protease family (2) and is inhibited by pepstatin A (3). Recombinant protease mutated at the catalytic Asp-25 residue is enzymatically inactive in bacterial expression systems and *in vitro* (4, 5), and virus mutated at this site is noninfectious (4). The protease has recently been purified from *Escherichia coli* and crystallized (6–8).

In this report, we demonstrate that the HIV protease is toxic to *E. coli*. The correlation between functional protease and toxicity was the basis of a selection procedure for isolating an extensive set of protease mutants that are nontoxic and enzymatically inactive. Because our approach only selects mutants that are enzymatically inactive, we have detected 10 mutants of HIV protease not described previously, despite extensive mutagenesis of this enzyme by other methodology (9, 10). The possible functions of the mutated loci in the wild-type HIV protease are discussed. The utility of this selection procedure for generating mutants of other eukaryotic proteins is also addressed.

MATERIALS AND METHODS

HIV Protease Subclones. The *Bgl* II–*Dra* I fragment from λBH5 (1), corresponding to nucleotides 1641–2122 of the HIV *pol* gene and encoding 18-kDa protease precursor, was subcloned with synthetic oligonucleotides into the *Nde* I–*Bam*HI sites of the T7 expression vector (11), creating plasmid pPDB16 (Fig. 1). The upstream oligonucleotides (5′-TATGTTTTTTAGGGAA-3′ and 5′-GATCTTCCCTAAAAAACA-3′) begin with an *Nde* I restriction site, providing an in-frame translational start codon, and regenerate nucleotides 1629–1640. The downstream oligonucleotides (5′-AAATTTTTAGTAAG-3′ and 5′-GATCCTTACTAAAAATTT-3′) regenerate nucleotides 2123–2129, immediately followed by two in-frame stop codons and a *Bam*HI restriction site. Plasmid pMae2 (Fig. 1) encodes the mature 10-kDa protease. Its construction is similar to pPDB16, except that the upstream oligonucleotides (5′-TATGCCTCAGATCACTCTTTGGCAACGACCCCTC-3′ and 5′-GTGACGAGGGGTCGTTGCCAAAGAGTGATCTGAGGC-3′) regenerate nucleotide 1833 to the *Mae* III restriction site.

***E. coli* Strains.** Plasmids pPDB16 and pMae2 were constructed in *E. coli* strain DH5α (New England Biolabs), prior to transformation into the expression strain, *E. coli* BL21(DE3)pLysS (12, 13).

Mutagenesis of HIV Protease. To introduce mutations into the HIV protease, plasmid pPDB16 was introduced into *E. coli* strain LE30 *mut*D. This strain has a 10^3–10^4 higher frequency of spontaneous mutation compared to wild-type *E. coli* (14). Plasmid was prepared from 50,000 pooled LE30 *mut*D transformants, and this plasmid population was used for the transformation of BL21(DE3). Approximately 150 colonies were obtained, and 100 of these putative mutants were tested for synthesis of HIV protease by labeling with [^{35}S]methionine. A subset of these clones was subjected to DNA sequence analysis.

Protein Analysis. *E. coli* harboring HIV protease plasmids were tested for protease synthesis by labeling of protein with [^{35}S]methionine in the presence of isopropyl β-D-thiogalactopyranoside (IPTG), followed by electrophoresis and autoradiography as described (11). Rifampicin was added to these cultures 30 min after addition of IPTG (11) to suppress transcription and subsequent translation from *E. coli* (but not T7) promoters. Antiserum was raised to amino acids 34–46 of the mature protease (15), and immunoprecipitation was carried out (11) as described.

RESULTS AND DISCUSSION

HIV Protease Is Toxic to *E. coli*. To study the HIV protease, a 500-base-pair (bp) DNA fragment encoding the protease was placed under the control of the phage T7 promoter (12, 13), creating plasmid pPDB16 (Fig. 1) in *E. coli* strain DH5α. *E. coli* does not normally contain T7 RNA polymerase and cannot express genes from a T7 promoter. The expression strain, *E. coli* BL21(DE3)pLysS, contains a chromosomal copy of T7 RNA polymerase under the control of an inducible lacUV5 promoter and a chloramphenicol-resistant plasmid encoding T7 lysozyme, which inhibits transcription by T7 RNA polymerase in the absence of induction (12, 13). Protease synthesis from cells harboring pPDB16 was examined by labeling with [^{35}S]methionine, followed by electrophoresis and autoradiography (Fig. 2). The HIV DNA fragment in

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Abbreviations: HIV, human immunodeficiency virus; IPTG, isopropyl β-D-thiogalactopyranoside.

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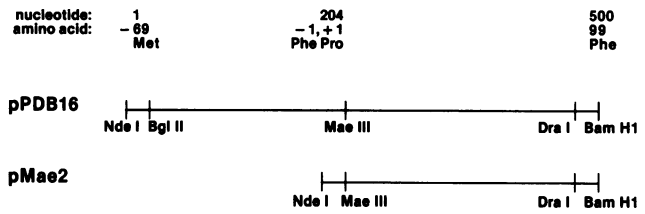

FIG. 1. HIV protease constructs. pPDB16 encodes the 18-kDa protease precursor under the control of the phage T7 promoter. For clarity, base A of the initiation codon within the *Nde* I restriction site is defined as nucleotide 1. Amino acids +1 to +99 represent the mature 10-kDa protease liberated by autodigestion at the indicated Phe-Pro bond (15, 16). pMae2 encodes the mature 10-kDa protease.

pPDB16 should encode a primary translation product of 18 kDa. Instead, a prominent band of 10 kDa is observed upon induction with IPTG (Fig. 2*A*, lane 2). This observation is consistent with efficient autodigestion of the 18-kDa precursor into the mature 10-kDa protease at the Phe-Pro bond (Fig. 1) and is in agreement with the results of several other laboratories (15–17).

The 10-kDa HIV protease band detected by autoradiography is not visible by Coomassie blue staining (data not shown), suggesting that only low levels of this protein are made. It has been previously determined that the absolute level of expression of a cloned gene under T7 promoter control is lower in BL21(DE3)pLysS than in the corresponding strain lacking lysozyme, due to the inactivation of T7 RNA polymerase by lysozyme (12). However, genes under T7 promoter control that are toxic to *E. coli* will not produce stable transformants in BL21(DE3), which lacks the lysozyme plasmid, pLysS (13). In an attempt to increase the level of expression of the HIV protease, we investigated the use of the expression strain BL21(DE3). The results of a typical transformation experiment are shown in Table 1. Whereas several plasmids used as controls transform strains BL21(DE3) and BL21(DE3)pLysS equally well, plasmid pPDB16 encoding HIV protease did not yield transformants in BL21(DE3). These data strongly suggest that expression from pPDB16 is toxic to *E. coli* BL21(DE3).

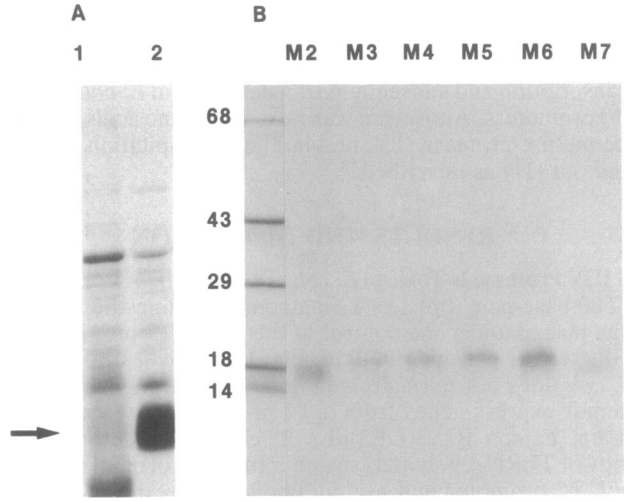

FIG. 2. Expression of HIV protease in *E. coli*. (*A*) Protease synthesis in BL21(DE3)pLysS cells harboring pPDB16. Proteins were labeled with [^{35}S]methionine as described (11), followed by electrophoresis and autoradiography. Lane 1, no IPTG; lane 2, 1 mM IPTG. The arrow denotes the mature 10-kDa protease. (*B*) Protease synthesis in BL21(DE3) survivors harboring mutagenized pPDB16. The numbers above each lane correspond to the clone designations in Tables 2 and 3. The sizes (in kDa) of molecular mass markers are shown on the left.

Table 1. Transformation of BL21(DE3) strains by HIV protease constructs

Plasmid	No. of transformants	
	BL21(DE3)	BL21(DE3)pLysS
Exp. 1		
pPDB16	0	400
pPol1	400	400
pPol2	350	300
pZM1	200	200
pZM2	200	200
Exp. 2		
pMae2	0	960
pP48Mae6	330	840
pP46Mae10	0	800
pP12Mae14	250	800
pPol1	600	2400

BL21(DE3) cells containing or lacking the pLysS plasmid were made competent by the standard calcium chloride procedure (18). Fifty nanograms of protease or control plasmid was used for each transformation. Plasmids pPDB16 and pMae2 encode the wild-type 18-kDa protease precursor and 10-kDa mature protease, respectively (see Fig. 1). Plasmids pP48Mae6, pP46Mae10, and pP12Mae14 encode 10-kDa protease containing the identical mutation present in the 18-kDa mutants P48, P46, and P12, respectively (Table 3). Plasmids pPol1 and pPol2 encode the polio polymerase (11), and pZM1 and pZM2 encode fibroblast growth factor.

Isolation of Protease Mutants. The apparent toxicity of HIV protease could be due to its inherent enzymatic activity or to some other effect of this protein. To address this question, we attempted to isolate mutants of the HIV protease by screening for viable transformants of BL21(DE3). To introduce mutations into the HIV protease, plasmid pPDB16 was first introduced into *E. coli* strain LE30 *mut*D, which has a 10^3–10^4 higher frequency of spontaneous mutation compared to wild-type *E. coli* (14). Plasmid isolated from 50,000 pooled LE30 *mut*D transformants was used for the transformation of BL21(DE3), and ≈150 colonies were obtained. These clones were examined for HIV protease production by labeling of cells with [^{35}S]methionine in the presence of IPTG, followed by gel electrophoresis and autoradiography. As shown in Fig. 2*B*, these putative mutants do not produce the 10-kDa protein observed for the wild-type clone. Instead, they produce protein of either 18 kDa (lanes M3, M4, and M5) or protein smaller than 18 kDa (lanes M2, M6, and M7). These proteins are only observed upon induction with IPTG, indicating that they are under the control of the T7 promoter.

To determine if a specific genetic lesion could be correlated with the altered size of the inducible protein, the entire 500-bp HIV DNA insert of each putative mutant was sequenced. The first group of 18 BL21(DE3) survivors was composed of 8 clones producing 18-kDa protein (M3, M4, M5, M8, M9, M12, P2, and P5), 7 clones producing protein smaller than 18 kDa (M2, M6, M7, M10, M11, P49, and P74), and 3 clones that did not produce protein upon induction with IPTG (M1, P3, and P26). The sequencing results are presented in Tables 2 and 3. Each clone that produced protein smaller than 18 kDa contained a premature translation termination codon (Table 2). The premature termination codons of clones M6, M7, and P49 result from a C → U transition. For clones M2, M10, M11, and P74, the insertion or deletion of a G residue within a stretch of 5 or 6 guanines results in a translational frameshift that should lead to termination as indicated (Table 2). In each case, whether a transition or frameshift occurred, the size of the protease predicted from the DNA sequence is in excellent agreement with the size of the protein observed upon induction (Fig. 2*B* and data not shown).

Clone P26, which apparently did not produce labeled protein upon induction with IPTG, was shown to contain a

Table 2. Nonsense mutants of HIV protease

Clone	Mutation
P26	CAG → UAG at nt 60
M7	CAA → UAA at nt 225
P49	CGA → UGA at nt 228
M10, M11	G insertion at nt 259, UGA at nt 293
M2	G deletion at nt 352, UAA at nt 373
P74	G insertion at nt 353, UGA at nt 383
M6, P52	CAG → UAG at nt 387

The entire 500-bp protease insert of pPDB16 from BL21(DE3) survivors (see text) was subjected to DNA sequence analysis, and a premature stop codon was identified. Clone P26 encodes a truncated protease of 2 kDa that was not detected upon labeling of *E. coli* proteins, probably due to its small size. Each other clone encodes a protein smaller than 18 kDa that was detected by labeling (see Fig. 2*B*, lanes M2, M6, and M7). nt, Nucleotide.

premature translation termination codon at nucleotide 61 (Table 2). The truncated protein expected upon induction of this construct would be ≈2 kDa and is too small to be resolved by our gel system. Clones M1 and P3 were also subjected to DNA sequence analysis and were found to encode wild-type protease. Since whole plasmid was subjected to mutagenesis in strain LE30 *mut*D, these clones may contain mutations outside of the HIV protease region that would prevent transcription or translation from genes under T7 promoter control, such as mutations in the promoter region itself or in the ribosome binding site (13).

For each clone that produces 18-kDa protein, a single nucleotide mutation leading to a single amino acid substitution was detected by DNA sequencing (Table 3). After these initial results with the first group of 18 clones were obtained, only those clones that produced 18-kDa protein upon induction were examined in detail. The specific amino acid change determined for each mutant is summarized in Table 3. In all, 24 different point mutations were identified. Six of these mutations, at amino acids Ile-3, Ile-47, Ile-50, Gly-73, Thr-74, and Val-77, were identified in two independent clones. The mutations at Leu-38 and Ile-66 were each identified in three independent clones. Because we reisolated mutations without obtaining some other known mutations (e.g., mutation at Asp-25; refs. 3 and 4), it is possible that we are detecting sister plasmids from the mutagenized plasmid pool.

Some of these mutations can be grouped according to the proposed function of the amino acid within HIV protease. The triplet Asp, Thr, and Gly is conserved throughout the family of aspartyl proteases (2) and helps to locate the active site of HIV protease at residues 25, 26, and 27. Mutations at or near these residues should render the protease enyzmatically inactive by disruption of the catalytic site. The three-dimensional structure of HIV protease has been recently solved (6–8), and it appears that our mutations at Ala-22, Gly-27, Asp-29, Thr-31, Ile-85, Gly-86, and Arg-87 are within the cleft of the active site. We also found mutations in amino acids that are proposed to participate in hydrogen bonding within the protease, including Ile-15, Ala-22, Thr-31, Lys-45, Ile-47, Ile-50, Ile-66, Gly-73, Leu-76, Val-77, Ile-85, Gly-86, Arg-87, and Ile-93 (8).

The active form of HIV protease is a dimer (6–8, 19), with an interface between the amino and carboxyl termini of two protease monomers (8). Our mutations at Gln-2 and Ile-3 (P46 and P12, Table 3) could interfere with dimer formation. Alternatively, since Gln-2 and Ile-3 are very close to the Phe-Pro cleavage junction (Fig. 1, residues −1, +1), these mutations could also interfere with autodigestion of the 18-kDa protease precursor. To distinguish between these possibilities, several plasmids were constructed that encode only the 10-kDa protease, bypassing the need for autodigestion of precursor (Fig. 1). If these mutations interfere only with autodigestion, they should be silent in the 10-kDa protease. Wild-type 10-kDa protease plasmid (pMae2, Table 1) is toxic to *E. coli* strain BL21(DE3) but not BL21(DE3)-pLysS, consistent with results for the 18-kDa precursor plasmid (pPDB16, Table 1). Plasmid pP46Mae10 (Gln-2 → Arg) is now toxic to *E. coli* strain BL21(DE3), suggesting that this mutation interferes with autodigestion of the 18-kDa precursor. Conversely, plasmid pP12Mae14 (Ile-3 → Asn) remains nontoxic, suggesting that this mutation may interfere with dimerization and not with autodigestion of the 18-kDa precursor. Plasmid pP48Mae6 encoding 10-kDa protease

Table 3. Missense mutants of HIV protease

Clone	Position: Nucleotide	Position: Amino acid	Mutation
P57	196	−4	UCC → UUC Ser Phe
P46	211	2	CAG → CGG Gln Arg
P12, P37	214	3	AUC → AAC Ile Asn
P32	250	15	AUA → ACA Ile Thr
P29	270	22	GCU → ACU Ala Thr
M12	285	27	GGA → AGA Gly Arg
P48	292	29	GAU → GGU Asp Gly
P15	297	31	ACA → GCA Thr Ala
P1, P5, P80	319	38	UUG → UCG Leu Ser
P66	339	45	AAA → GAA Lys Glu
M3, M5	346	47	AUA → ACA Ile Thr
P31, P44	355	50	AUU → ACU Ile Thr
M9	381	59	UAU → CAU Tyr His
P24	385	60	GAU → GUU Asp Val
P13, P47, P68	403	66	AUC → ACC Ile Thr
M4, P73	424	73	GGU → GAU Gly His
P45, P79	427	74	ACA → AUA Thr Ile
M8	433	76	UUA → UCA Leu Ser
P2, P8	436	77	GUA → GCA Val Ala
P22	442	79	CCU → CUU Pro Leu
P38	460	85	AUU → ACU Ile Thr
P9	463	86	GGA → GAA Gly Glu
P50	465	87	AGA → GGA Arg Gly
P27	484	93	AUU → ACU Ile Thr

The entire 500-bp protease insert of pPDB16 from BL21(DE3) survivors producing 18-kDa protease precursor (see text and Fig. 2*B*) was subjected to DNA sequence analysis, and a single point mutation was identified in each clone. Nucleotide and amino acid numbering are described in the legend to Fig. 1.

with a point mutation at amino acid 29 (Table 3) was used as a control. This construct, like its parent 18-kDa precursor (P48) was able to transform both *E. coli* strains. Extracts prepared from BL21(DE3)pLysS harboring p48Mae6 failed to cleave a *gag* substrate, whereas extracts of wild-type 10-kDa protease did cleave *gag* (data not shown), further demonstrating that this mutant encodes inactive protease.

Loeb *et al.* (9) performed extensive site-directed mutagenesis between amino acids 21 and 46 of the HIV protease and then determined processing activity. Most missense mutations within the region that is highly conserved among retroviral proteases abolished processing. These mutations include Asp-29 → Gly and Thr-31 → Ala, in agreement with our results. In contrast to their approach, our method of mutant isolation appears to select only for nonfunctional protease. Consistent with this are 31 mutations in amino acids 21–46, which Loeb *et al.* determined had little or no effect on processing. None of these mutations came through our screen. Our results also indicate that point mutations throughout the protease, outside of the conserved regions, can destroy the activity of the enzyme (Table 3). More recently, Loeb *et al.* (10) have carried out complete mutagenesis of the 10-kDa protease, by constructing mutations at every position of the 99-amino acid protein followed by assay of proteolytic activity *in vitro*. Our approach to obtaining mutant protease is fundamentally different, since those authors isolated many mutants that are silent with respect to the activity of the enzyme. Our 24 point mutations, without exception, are inactive by the criterion of autoproteolysis. Fourteen of our point mutations overlap with those of Loeb *et al.* (9, 10). Eleven of these were shown by Loeb *et al.* (10) to be enzymatically inactive, and 2 of these display a low level of activity. These results further support the strong correlation between the enzymatic activity of HIV protease and its toxicity in *E. coli*. The single difference between our results and those of Loeb *et al.* is the mutation of Ile-3 → Asn, which twice came through our screen as the 18-kDa precursor and is still mutant (nontoxic) as the 10-kDa mature protease (Table 1) but was shown by Loeb *et al.* to possess wild-type activity. We cannot explain this discrepancy.

Ten of our mutations, at amino acid positions −4, 2, 38, 45, 59, 73, 76, 77, 85, and 87, are substitutions that were not detected in previous studies (9, 10). However, substitution of other amino acids at most of these positions was shown to decrease or abolish proteolysis (9, 10), consistent with our results. A notable exception is substitution at Lys-45, where Loeb *et al.* (9, 10) determined wild-type activity for Arg, Gln, and Thr substitutions. We found that Glu at this position (mutant P66) apparently inactivates the protease.

Interestingly, mutant P66 exhibits temperature sensitivity, in that these cells failed to grow at 30°C compared to the 37°C control. Mutant P22 likewise failed to grow at 30°C, which supports the finding by Loeb *et al.* (10) that this mutant can process *gag* precursors at low temperature but not at 37°C. Our selection procedure of BL21(DE3) survival at 37°C vs. death at 30°C is an advantage in screening large numbers of clones for temperature sensitivity compared to performing conventional protease assays *in vitro* (10).

Krausslich *et al.* (20) have shown that deletion of the carboxyl-terminal 17 amino acids from the 18-kDa HIV protease precursor prevents autodigestion to liberate the mature 10-kDa protease and, furthermore, that the precursor is unable to cleave a *gag* substrate in trans. Similar results were obtained by Hansen *et al.* (21). Four of our point mutations, at Ile-85, Gly-86, Arg-87, and Ile-93, are within these 17 carboxyl-terminal amino acids.

Our results indicate that the mutant 18-kDa protease precursor is incapable of autodigestion and is apparently not toxic to BL21(DE3). We hypothesize that the 10-kDa mature protease exerts its toxic effect by digesting an unidentified essential *E. coli* protein and that the 18-kDa precursor is unable to carry out this digestion. Failure to generate the mature 10-kDa protease could result from mutating an amino acid required for catalytic activity or for proper folding of the protein or from disruption of the scissile Phe-Pro bond, such that autodigestion of the 18-kDa precursor could not occur. Our mutations at Ser-(−4) and Gln-2 (Table 3) probably render the Phe-Pro bond uncleavable. Loeb *et al.* (9) used site-directed mutagenesis of the *pol* polyprotein, encoding the protease, reverse transcriptase, and endonuclease, to introduce substitutions at the upstream Phe-Pro bond. One class of mutants is defective at autodigestion but can still cleave at the nonmutated reverse transcriptase and endonuclease borders. This processing results either from a small amount of undetected mature protease or from the precursor that retained activity. Another class of mutants that fails to liberate the mature protease is also inefficient at cleavage at the reverse transcriptase and endonuclease borders. It is likely that our method of mutant detection, the requirement for stable transformation of BL21(DE3), selects only those 18-kDa precursors that are enzymatically inactive.

Further evidence that the HIV protease is toxic to BL21(DE3) is provided by an examination of the survival of cultures in the presence of IPTG. In this experiment, cultures of BL21(DE3)pLysS cells harboring wild-type protease (pPDB16) or mutant protease (pP48) were grown to midlogarithmic phase, and protease synthesis was induced with IPTG. The number of viable cells was determined by plating aliquots of cultures for colony-forming units. After 30 min of exposure to 1 mM or 0.1 mM IPTG, cells harboring wild-type protease experienced ≈4 logarithms of killing compared to untreated cells (Fig. 3). In contrast, cells harboring the mutant protease are unaffected by the addition of IPTG. These data indicate that HIV protease is extremely toxic to BL21(DE3). Similarly, cells harboring plasmid encoding wild-type 10-kDa protease (pMae2) were significantly more sensitive to IPTG than cells harboring plasmid encoding the corresponding mutant protease (p48Mae6) (data not shown).

The amount of protein produced upon IPTG induction by cells harboring plasmids encoding wild-type or mutant versions of the 18-kDa or 10-kDa protease was examined by [^{35}S]methionine labeling and immunoprecipitation, followed

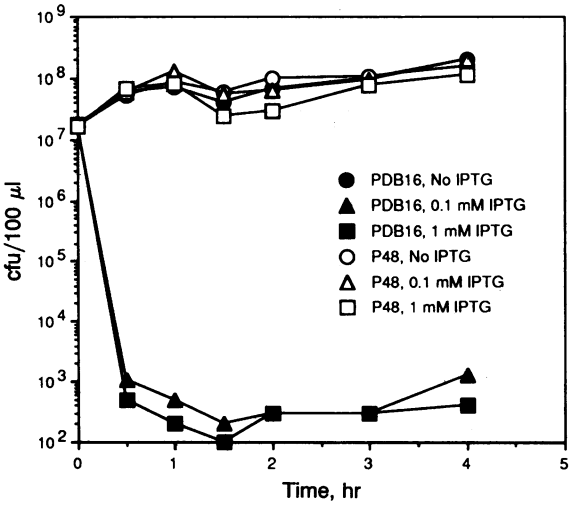

FIG. 3. Killing of BL21(DE3)pLysS upon induction of HIV protease synthesis with IPTG. Cells harboring plasmid encoding wild-type (pPDB16) or mutant (pP48) protease precursor were grown to 0.5 absorbance unit (600 nm), and IPTG was added to the indicated concentrations. At the indicated times, an aliquot of each culture was removed, and dilutions were plated on LB plates containing kanamycin and chloramphenicol. After overnight incubation, colony-forming units (cfu)/100 μl of culture were determined.

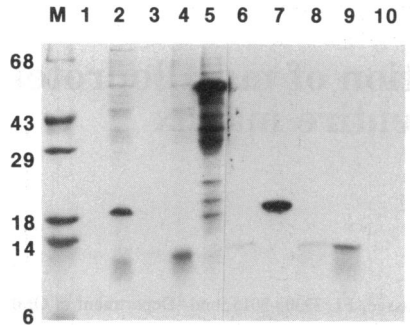

FIG. 4. Comparison of amounts of wild-type and mutant protease production. BL21(DE3)pLysS cells harboring the indicated plasmids were induced with 1 mM IPTG, and proteins were labeled with [^{35}S]methionine as described (21). Whole cell extracts (lanes 1–5) and immunoprecipitates (lanes 6–10) were subjected to electrophoresis and autoradiography. Lanes 1 and 6, pPDB16; lanes 2 and 7, pP48; lanes 3 and 8, pMae2; lanes 4 and 9, pP48Mae6; lanes 5 and 10, pPol, encoding the polio polymerase (11). Lane M, molecular mass markers (sizes given in kDa).

by gel electrophoresis and autoradiography (Fig. 4). We estimate that mutants P48 and P48Mae6, which encode the 18-kDa precursor and the 10-kDa protease, respectively (lanes 2 and 4), produce as much as 10-fold more protease than the corresponding wild-type clones pPDB16 (lane 1) and pMae2 (lane 3). The decreased amount of the wild-type protease produced compared to the mutant is probably due to the killing of cells by wild-type protease upon induction with IPTG (Fig. 3). Unexpectedly, however, the mutants still produce very low levels of the protease or precursor, despite their lack of toxicity. The mutant proteins cannot be detected by Coomassie blue staining of the corresponding gels, in contrast to several other unrelated proteins that we and others have successfully expressed in this same vector/host system (refs. 11, 13; data not shown). Different genes are expressed at different efficiencies in the T7 system (13), and there may be limitations for overproducing HIV protease, even when its toxicity is apparently alleviated.

The HIV protease has been successfully expressed in a variety of *E. coli* K-12 strains (3–5, 15–17, 21), although, at least in HB101, inhibition of growth upon induction of protease synthesis has been reported (22). The HIV protease may be especially toxic to BL21(DE3), which is an *E. coli* B strain. In contrast to our results, Krausslich *et al.* (23) report expression of HIV protease in strain BL21(DE3) lacking the lysozyme plasmid. Differences between their protease construct and ours may account for our consistent observation that BL21(DE3) cannot be transformed by pPDB16. The amino terminus of our 18-kDa protease is codon 1 of the *pol* gene preceded by a single methionine residue, whereas that of Krausslich *et al.* is codon 5 of the *pol* gene preceded by 12 amino acids from phage T7 gene 10 (20). In addition, our protease construct is on a plasmid encoding resistance to kanamycin, not ampicillin. However, the protease is also toxic to BL21(DE3) when introduced on a vector specifying ampicillin resistance (data not shown), indicating that the putative target of the HIV protease is probably not the phosphotransferase that confers kanamycin resistance.

Recent studies with peptide substrates have identified minimal sequence requirements for cleavage by the HIV protease, and as few as seven amino acids are sufficient (24–26). Thus, the bacterial protein that is the putative target of the protease need bear little homology to the natural *gag* and *pol* substrates. The toxicity of the HIV protease to *E. coli* strain BL21(DE3) provides a method to isolate mutants of this important viral enzyme and, more importantly, the potential to screen for protease inhibitors useful for therapy of HIV infection.

It is worth noting that the mutagenesis and selection procedures that we describe may be of general utility for isolating mutants of eukaryotic proteins in *E. coli*. The method requires that the biological activity of the protein of interest is toxic to the *E. coli* host and that mutant protein is both inactive and nontoxic. The mutagenesis is then performed in a host that is incapable of expressing the toxic gene. This is easily done by placing the toxic gene under T7 promoter control and performing the mutagenesis in a strain that lacks T7 RNA polymerase. Alternatively, mutagenesis can be performed in a strain of *E. coli* that is resistant to the toxic protein.

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