Chemical Rescue of I-site Cleavage in Living Cells and in Vitro Discriminates between the Cytomegalovirus Protease, Assemblin, and Its Precursor, pUL80a

Chemical rescue is an established approach that offers a directed strategy for designing mutant enzymes in which activity can be restored by supplying an appropriate exogenous compound. This method has been used successfully to study a broad range of enzymes in vitro, but its application to living systems has received less attention. We have investigated the feasibility of using chemical rescue to make a conditional-lethal mutant of the cytomegalovirus (CMV) maturational protease. The 28-kDa CMV serine protease, assemblin, has a Ser-His-His catalytic triad and an internal (I) cleavage site near its midpoint. We found that imidazole can restore I-site cleavage to mutants inactivated by replacing the critical active site His with Ala or with Gly, which rescued better. Comparable rescue was observed for counterpart mutants of the human and simian CMV assemblin homologs and occurred in both living cells and in vitro. Cleavage was established to be at the correct site by amino acid sequencing and proceeded at ~11%/h in bacteria and ~30%/h in vitro. The same mutations were unresponsive to chemical rescue in the context of the assemblin precursor, pUL80a. This catalytic difference distinguishes the two forms of the CMV protease.

As an essential step in their maturation, herpes group viruses package their DNA genome into preformed capsids (1–7). For this to occur, internal proteins of the nascent capsid must be eliminated through a process catalyzed by a virus-encoded maturational protease (8–10). In the absence of this enzyme, the capsids produced are devoid of viral DNA and unable to mature into infectious virus. The enzyme required for this step is a serine protease but is distinguished from other members of its family by an atypical catalytic triad (Ser-His-His instead of Ser-His-His/Asp/Glu) (11–14), an unusually slow cleavage rate (15–18), and a requirement to dimerize for activity (19–22). These deviations from the more typical serine proteases increase interest in the relationships between its enzymatic mechanism and biological function.

Like its homologs in other herpesviruses, the cytomegalovirus (CMV)* protease is autoproteolytically derived from a precursor by sequential cleavage at its maturational (M) site and then its release (R) site to yield a mature form, called assemblin (10, 23, 24). Among the CMV-type herpesviruses (members of the β-herpesvirus family, Ref. 25), the assemblin homolog is additionally cleaved at an internal (I) site (15, 26) (e.g. see Fig. 1D), converting it to a two-chain form that remains active (27–29). Although these self-processing steps are coupled with capsid maturation, little is understood about their regulation.

To further investigate this enzyme and the relationship of its self-processing to capsid assembly and maturation, we considered creating a mutant controlled by a rationally designed small-molecule switch. Such “chemical rescue” mutants have an inactivating amino acid substitution that can be functionally compensated by an exogenous compound (30, 31). Given that two catalytic triad members of the herpesvirus protease are histidines and that their imidazole group is well tolerated by cells as a free compound (32), we targeted these residues for chemical rescue. Because the goal of creating a virus carrying this mutation requires its growth and chemical rescue in live cells, we began our study using a cell-based transfection assay system.

Our approach was to express specific mutants of assemblin and its precursor in transfected mammalian cells and in transformed bacteria, establish whether their proteolytic activity could be restored by imidazole added to the culture medium, and reproduce the effect in vitro. Our results show that imidazole enabled I-site cleavage of mutant assemblin in living cells and in vitro but that the effect was not seen when the same mutations were tested in the assemblin precursor. Although this finding challenges the feasibility of creating an imidazole-regulated protease mutant of CMV, it reveals interesting and potentially important structural and/or enzymatic differences between assemblin and its precursor.

Initial and progress reports of this work have been presented (see Refs. 52–55).

EXPERIMENTAL PROCEDURES

Cells, Plasmids, and Expression—Human endothelial kidney (HEK, line 293; American Type Culture Collection, Manassas, VA) carcinoma cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (HyClone, Logan, UT) and 100 units each of penicillin and streptomycin per ml. HEK cells growing at ~70% confluence in 24-well plates (Falcon Multiwell, BD Biosciences) were transfected using FuGENE 6 (Roche Applied Science) with RSV.5(neo) expression plasmids according to the manufacturer’s instructions. Transfected cells were collected into 75 μl of protein sample buffer and frozen at ~80 °C until analyzed.

* The abbreviations used are: CMV, cytomegalovirus; IMAC, immobilized metal affinity chromatography; MOPS, 4-morpholinepropanesulfonic acid; MES, 4-morpho-ethanesulfonic acid; SCMV, simian CMV; HCMV, human CMV; pPR, protease precursor; HEK, human endothelial kidney.
Plasmids encoding wild-type simian CMV (SCMV) assemblin, its precursor (pPR), and mutants S118A, H47A, H47Q, and H142A have been described elsewhere (10, 24). The H47G mutants of SCMV assemblin and pPR were made by site-directed mutagenesis (His-47 codon CAC → GCC; QuickChange Kit, Stratagene, La Jolla, CA) followed by subcloning a small mutation-containing fragment of the PCR product into plasmids encoding the wild-type SCMV proteins (10). The corresponding HCMV assemblin and pPR mutants were made similarly, by changing the His-63 codon from CAC to GCC (H63A) or to GGC (H63G) and subcloning the mutant fragment into plasmids encoding wild-type HCMV assemblin or pPR (28). The intended mutant sequence of all constructs was verified by the Johns Hopkins University Biosynthesis and Sequencing Facility.

Bacteria (BL21(DE3)) were transformed with the appropriate plasmid and induced for protein expression with 1 mM isopropyl 1-thio-β-D-galactopyranoside. Following overnight (~18 h) incubation at 16°C in the presence or absence of 80 mM imidazole, the bacteria were collected by centrifugation and further processed as specified.

**Recovery of Mutant Assemblin by Affinity Chromatography**—In preparation for immobilized metal affinity chromatography (IMAC), bacterial pellets collected (6,000 × g for 20 min at 4°C) from 100-ml cultures were suspended in 10 ml of lysis buffer (50 mM NaPO4, 300 mM NaCl, pH 8.0) and ruptured using a French press (~14,000 p.s.i.), and the lysates were clarified by ultracentrifugation (100,000 × g for 30 min at 4°C).

H63G mutant assemblin tagged with an amino-terminal His/Trp purification handle (MHWHWHWH-) (33) was affinity-selected from the clarified lysate onto nickel-nitrilotriacetic acid Superflow beads (Qiagen, Valencia, CA), washed with lysis buffer, and eluted using 50 mM EDTA (to avoid exposure to imidazole). The two or three 0.5-ml fractions containing the most H63G were combined and concentrated 3-fold by centrifugal filtration (catalog no. 3491.2, Orbital Biosciences, LLC., Topsfield, MA), leaving the protein in the same elution buffer or exchanging the elution buffer with cleavage buffer (500 mM Na2SO4, 10 mM dithiothreitol, 10% glycerol, 100 mM MOPS, pH 7.2), as specified.

**Chemical Rescue of CMV Protease**

In vitro cleavage assays contained IMAC-recovered protein in 1× cleavage buffer, 50 mM EDTA, and 80 mM imidazole, each adjusted to pH 8.0. Optimization experiments showed that EDTA in the range of 2–50 mM had little effect on the reaction, imidazole in the range of 20–100 mM gave ≥15% better rescue than higher or lower concentrations, and incubation at 16°C or room temperature (~24°C) gave better cleavage than at 37 or 45°C.

The following compounds provided by the Drug Synthesis and Chemistry Branch of NCI, National Institutes of Health, were also tested in vitro for chemical rescue of H63G HCMV assemblin, all at 25 mM final concentration: NSC1895 (1,2,4-triazolidine, 3,5-diimino-), NSC51143 (1H-pyrazolo[2,3-a]imidazole, 2,3-dihydro-), NSC105831 (2-nitroimidazole), and NSC113496 (5-aminoimidazole-4-hydrochloride).

**Gel Electrophoresis and Western Immunoassay**—Protein samples were solubilized by adding an equal volume of protein sample buffer 1, composed of 3 parts NuPAGE sample buffer (catalog no. NP0007, Invitrogen) and 2 parts 1 mM dithiothreitol or, where indicated, in protein sample buffer 2 (34). Proteins were electrophoretically separated in SDS containing 4–12% polyacrylamide gradient gels (catalog no. NP0032, Invitrogen) or, where indicated, in 14-cm-long 10% polyacrylamide gels, essentially as described by Laemmli (34). Following SDS-PAGE, the proteins were either stained with SYPRO Ruby (SYPRO-R, catalog no. 179-3125, Bio-Rad) or electrotransferred to a polyvinylidene difluoride membrane (e.g. 0.2 μm, catalog no. LC2002, Invitrogen) for Western immunoassays.

Western immunoassays were performed as described previously (28). Anti-peptide antiserum was to synthetic peptide mimics of (i) the 21-amino acid amino terminus (N2) and 14-amino acid carboxyl terminus (C2) of SCMV assemblin (anti-N2 and anti-C2) (24), (ii) the 13-amino acid amino terminus (N1) of the SCMV assembly protein (anti-N1) (35), and (iii) an antiserum to IMAC/SDS-PAGE-purified HCMV assemblin (anti-assemblin) (36). 125I-Protein A (NEX-46L, PerkinElmer Life Sciences) was used as the secondary reagent. Processed membranes were phosphorimagined with a BAS1000 and ImageQuant Version 3.3 (Fuji Photo Film Co., Tokyo, Japan).

**RESULTS**

Experiments to test the toxicity of imidazole showed that HEK cells grow for at least 3 days in medium containing 40 mM imidazole and remain adherent for 5 h in 80 mM imidazole and that isopropyl 1-thio-β-D-galactopyranoside-induced bacteria tolerate 80 mM imidazole for at least 24 h at 16°C.

**I-site Cleavage by His-47 Mutants of Simian CMV Assemblin Rescued by Imidazole**—As an initial screen for the effect of imidazole on assemblin activity, wild-type simian CMV assemblin was compared with previously described mutants having an Ala substitution for one of the catalytic triad residues, Ser-118, His-47, or His-142 (24). One day after transfection, the maintenance medium was replaced with fresh medium or with fresh medium containing imidazole. Two days later the medium was removed, and the cells were solubilized in protein sample buffer 2 and subjected to SDS-PAGE followed by Western immunoassay to detect assemblin and its cleavage products.

In the absence of imidazole the phenotypes were as expected (24): (i) wild-type assemblin (PRn) was partially cleaved to its products An and D (Fig. 1A, lane 1, and D); (ii) mutation of the essential serine nucleophile (S118A) or the critical histidine (H47A) prevented I-site cleavage (Fig. 1A, lanes 3 and 5); and (iii) mutation of His-142 (H142A) had comparatively little effect on I-site cleavage (Fig. 1A, lane 7). The presence of imidazole had essentially no qualitative effect on the I-site cleavage of wild-type assemblin or the S118A and H142A mutants (Fig. 1A, lanes 2, 4, and 8). Encouragingly, however, when H47A was expressed with imidazole, the An and A0 fragments diagnostic of I-site cleavage were detected (Fig. 1A, lane 6, and C, lane 4), consistent with chemical rescue of that mutation.

Because the nature of the substituting amino acid can influence the extent to which activity is restored (37), two additional substitutions for His-47 were tested for comparison. The bulkier glutaminate (H47Q) partially restored I-site cleavage but with apparently reduced specificity. In the absence of imidazole it enabled I-site cleavage (i.e. presence of An and A0) and additional cleavages resulting in a group of proteins less evident in the other preparations (Fig. 1A, lane 9, indicated by brace). This pattern was similar in the presence of imidazole (Fig. 1A, lane 10),
and the H47Q mutant was not analyzed further. The smaller glycine substitution (H47G), like H47A, was inactive without imidazole (Fig. 1B, lane 1). However, in the presence of imidazole, I-site cleavage by the H47G mutant dramatically exceeded that of the H47A mutant (Fig. 1B, lanes 2 and 3, and C, compare lane 4 with lane 5). Calculations based on phosphorimaging measurements indicate that 36% of wild-type assemblin, 3% of the H47A mutant, and 28% of the H47G mutant was cleaved to A_n + A_C in the presence of imidazole. Thus, the percentage of assemblin cleaved by the H47G mutant was nearly 10-fold greater than that of the H47A mutant and 78% greater than that of the wild-type SCMV enzyme.

I-site Cleavage by His-63 Mutant of Human CMV Assemblin Rescued by Imidazole—To extend this finding to the human CMV (HCMV) homolog, with which most structural and enzymatic characterizations have been performed, we tested the counterpart mutants of HCMV assemblin. Because HCMV assemblin expresses less well than its SCMV homolog in transfected mammalian cells (28), it was expressed in HEK cells with 20 mM imidazole. The cells were treated with no (0 mM) or 20 mM imidazole (Imid). Shown here is a phosphorimage of a Western immunoassay performed, as described for A, on lysates of HEK cells transfected with a plasmid encoding mutant H47G-assemblin. The smaller glycine (G) and carboxyl (C) amino (N2) and carboxyl (C2) peptide sequences used to prepare antisera are indicated as shaded boxes. Positions of the catalytic triad amino acids and the internal and release (R) cleavage sites (arrows) are also indicated.

I-site cleavage products An and Ac. Protein molecular weights (×10^3) are in parentheses; the amino (N2) and carboxyl (C2) peptide sequences used to prepare antisera are indicated as shaded boxes. Positions of the catalytic triad amino acids and the internal and release (R) cleavage sites (arrows) are also indicated.
ent with the transfection experiments performed with SCMV assemblin, neither of the His-63 mutants showed I-site cleavage in the absence of imidazole (Fig. 2A, lanes 2 and 3), and there was little cleavage by the H63A mutant even with imidazole added (Fig. 2A, lane 5). In contrast, the H63G mutant expressed with imidazole yielded readily detected An and Ac bands, consistent with restored I-site cleavage (Fig. 2A, lane 4). Quantification of the stained proteins (Fig. 2A, lanes 4 and 6) indicated that 34% of the H63G mutant and 72% of the wild-type assemblin was cleaved to An + Ac with imidazole present.

The band just below wild-type assemblin (Fig. 2A, lanes 1 and 6) is likely to be the Cn product of cleavage at the cryptic (C) site (Fig. 2C). Assemblin cleaved to An was displaced from the beads with EDTA in lysis buffer, which was replaced with cleavage buffer by centrifugal filtration, as described under "Experimental Procedures." A discernible band at the same position in the preparation of H63G expressed with imidazole suggests that there may also be some rescue of C-site cleavage (e.g. Fig. 2A, lane 4, white dots). The original of the band migrating close to the 6-kDa marker in both preparations of wild-type assemblin is unknown; however, sequence analysis established that it has the same amino-terminal sequence as Ac (data not shown), compatible with it being the 7.2-kDa Ac fragment predicted if assemblin were cleaved at both its I and C sites (Fig. 2C). It was not detected in any of the H63A or H63G preparations (Fig. 2A, lanes 2–5).

Sequence Confirmation of I-site Cleavage—Bacterial expression of the HCMV protease gave sufficient amounts to confirm the cleavage site by direct sequence analysis. Lysates of bacteria expressing wild-type HCMV assemblin or the H63G mutant in the presence of imidazole were subjected to SDS-PAGE in five adjacent lanes each. The Ac bands were located and subjected to amino acid sequence analysis, all as described under "Experimental Procedures." Both the wild-type and H63G A, fragments gave the predicted, unambiguous N'-ATSLSG sequence, establishing that cleavage resulting from imidazole rescue is at the I site, VEA ↓ ATSLSG.

Imidazole Rescues I-site Cleavage by H63G Mutant of Human CMV Assemblin In Vitro—Rescue of the HCMV H63G mutation by imidazole was also tested in vitro. Bacterially expressed H63G assemblin was affinity-selected from a clarified cell lysate by IMAC, and the bound protein was displaced from the beads with EDTA in lysis buffer, which was replaced with cleavage buffer by centrifugal filtration, as described under "Experimental Procedures.” Half of the sample was incubated with 98 mM imidazole and half without for ~20 h at room temperature, and the samples were subjected to SDS-PAGE followed by protein staining. For comparison, bacteria expressing the H63G or H63A mutants, in the presence or absence of imidazole, were directly solubilized in protein sample buffer 2 and analyzed in parallel.

An image of the resulting stained gel showed little, if any, I-site cleavage of affinity-selected H63G in the absence of imidazole (Fig. 2B, lane 6) but readily detected An and Ac cleavage products with imidazole added (Fig. 2B, lane 5). The bacterial lysates included for reference gave results similar to those in the preceding experiment: no I-site cleavage for either mutant in the absence of imidazole, little if any cleavage for the H63A mutant even with imidazole, and obvious I-site cleavage by the H63G mutant in the presence of imidazole (Fig. 2B, lanes 1, 2, 4, and 3, respectively). Calculations based on measurements made from this gel indicate that, in the presence of imidazole, 46% of H63G assemblin was cleaved to An + Ac in bacteria and 30% in vitro.

Time Course of Chemical Rescue in Bacteria and in Vitro—The rate of I-site cleavage resulting from imidazole rescue in bacteria was determined by measuring the appearance of the An and Ac fragments after adding imidazole to an isopropyl-β-D-galactopyranoside-induced culture that had been grown overnight at 16 °C. At the indicated times after adding imidazole, samples were removed from the culture and volumetrically adjusted, according to their A590, to have the same amount of bacteria as the t = 0 sample. The bacteria were then collected by centrifugation (16,000 × g, 2 min, room temperature), solubilized in protein sample buffer 1, heated in a boiling water bath for 3 min, and subjected to SDS-PAGE followed by staining with SYPRO-R. Quantification of the progressive decrease in assemblin and increase in An + Ac, evident over the time course (Fig. 3A), showed that cleavage was fastest (~11%/h) and approximately linear from 0 to 60 min and then slowed to ~1%/h between 2 and 24 h, at which point ~47% of the assemblin had been cleaved (Fig. 3C).

We also determined the time course of chemical rescue in vitro. H63G assemblin was prepared by IMAC and exchanged into cleavage buffer, as described under "Experimental Procedures.” 200 μl of the resulting H63G preparation was made 98 mM in imidazole by adding 4 μl of a 5 M stock, and the reaction was continued at room temperature for 3 days. At the times indicated in Fig. 3B, 20-μl samples were removed from the incubation mixture, combined with 20 μl of protein sample buffer 1 and frozen at −80 °C until analyzed. The samples were further diluted with 20 μl of protein sample buffer, heated 3 min in a boiling water bath, and subjected to SDS-PAGE followed by protein staining with SYPRO-R (Fig. 3B). Calculations of the relative amounts of assemblin and An + Ac, at each time point showed that 36% of the assemblin

![FIGURE 3. Time course of I-site cleavage by HCMV H63G assemblin (PRn) treated with imidazole in bacteria and in vitro.](http://www.jbc.org/)

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had been cleaved to $A_n + A_c$ within the first hour, 77% after 24 h, and 86% after 3 days (Fig. 3D; day 2 and 3 data points not shown).

To confirm that the cleavage observed with in vitro chemical rescue was at the I site, the putative $A_n$ band was recovered and subjected to sequence analysis, as described above. The first six cycles of sequencing gave ATSLSG, as predicted for the amino end of authentic Ac.

Counterpart Mutations in Assemblin Precursor Show No Chemical Rescue—We next tested for chemical rescue of the same mutations in the context of the protease precursor (pPR), the primary translation product of the protease gene made in virus-infected cells, the additional carboxyl sequence of which contains the herpesvirus group conserved 14-cm gel to obtain better resolution of the M- and R-site cleavage products of pPR. Wild-type pPR was cleaved at the M site to yield PR, at the R site to yield $PR_n$, and at the I site to yield $A_n$ and Ac (Fig. 4). None of these proteins diagnostic of pPR M-, R-, and I-site cleavage was detected in the S118A- or H47A-pPR mutants (Fig. 4A). Dashes between lanes 3–4 and 5–6 indicate position of $PR_n$. A faint band was noted in the S118A-pPR sample (Fig. 4A, lane 4–5), which was not present in S118A-pPR preparations (Fig. 4A, lane 3–4). Although the H47A-pPR sample contained comparatively more nonimmunoreactive material than pPR, the amount was not increased by imidazole and is attributed to nonspecific proteolysis. A similar experiment was performed using the H47G-pPR sample and a 14-cm gel to obtain better resolution of the M- and R-site cleavage products. It can be seen more easily here that none of the products resulting from M- and R-site cleavage (i.e. PR and $PR_n$ and PR, respectively) was present in the H47G-pPR preparations (Fig. 4B, lanes 3 and 4). The bands indicated by dots above pPR, PR, and $PR_n$ are phosphorylated forms of each (Fig. 4B) (39, 40). The $A_n$ and Ac fragments are not retained in these gels.

Wild-type pPR was cleaved at the M site to yield PR, at the R site to yield assemblin ($PR_n$), and at the I site to yield $A_n$ and Ac (Fig. 4A, lanes 1–3). None of these proteins diagnostic of pPR M-, R-, and I-site cleavage was detected in the S118A- or H47A-pPR mutants (Fig. 4A, lanes 4–9). Although the H47A-pPR sample contained comparatively more nonimmunoreactive material smaller than pPR, the amount was not increased by imidazole and is attributed to nonspecific proteolysis.
We repeated the experiment using the HCMV assemblin precursor expressed in bacteria to reproduce the conditions that yielded rescue of I-site cleavage for HCMV assemblin. Western immunoblotting showed that the wild-type pPR or PR remained uncleaved and that most assemblin (PRn) was likewise cleaved to An and A1 (Fig. 5A, lanes 1and 2; A6, not detected by anti-N2). The patterns for the H63A- and H63G-pPR mutants showed none of the diagnostic fragments resulting from M-, R-, or I-site cleavage, most notably A6, from I-site cleavage, and were essentially indistinguishable from that of the catalytically dead S132A-pPR mutant, with or without imidazole (Fig. 5A, lanes 3–8).

Thus, under the same conditions that imidazole restored I-site cleavage activity to the His mutants of SCMV and HCMV assemblin, we found no evidence that it restored any cleavage activity to corresponding mutants of SCMV and HCMV pPR. We were unable to test for imidazole rescue of pPR in vitro, as was done for assemblin, because of “sticky” domains within the PRn sequence that interfere with eluting pPR from chromatography media under nondenaturing conditions (data not shown).

**DISCUSSION**

We have tested the feasibility of using chemical rescue to create a conditional-lethal mutant of the HCMV serine maturational protease, pUL80a, and unexpectedly found that the approach works for CMV assemblin but not its precursor. We initiated the study using assemblin, the 28-kDa structurally and enzymatically well characterized proteolytic portion of the larger precursor enzyme, and measured activity by monitoring cleavage at its internal (I) site. The critical His (SCMV, His-47; HCMV, His-63) of the active site triad was targeted for mutagenesis, and imidazole was used to restore activity. Inactivating substitutions of Ala or Gly were rescued for I-site cleavage in living cells and in vitro (Figs. 1 and 2). However, when the same mutations were tested for imidazole rescue in the precursor form of the enzyme, we detected no cleavage at any of its four sites (Figs. 4 and 5).

Comparable results were obtained with mammalian, insect (data not shown), and bacterial cells, indicating the applicability of imidazole-mediated rescue to a broad range of living systems, an approach also exploited recently in studies of the protein tyrosine kinase, Csk (41), and the influenza M protein ion channel (42). Although the observed rescue in living cells could have alternate explanations (e.g., because of imidazole metabolite), its reproducibility in vitro using affinity-selected mutant assemblin supports the conclusion that it is due to a direct influence of imidazole on the enzyme. We also considered that the observed imidazole-mediated cleavage might be a general property of all herpesvirus assemblin homologs or that failure to rescue the precursor might be peculiar to the CMV homolog. We found, however, that the counterpart mutants of the herpes simplex virus assemblin and precursor homologs did not respond to chemical rescue (data not shown from expression of H43A and H43G HSV assemblin and pPR in HEK cells and in bacteria), consistent with a requirement for factors specific to CMV assemblin (e.g., I site, I-site loop, or both).

Of the four amino acid side chains known to be involved directly in catalysis by assemblin (e.g., HCMV Ser-132, His-63, His-157, Arg-166), only that of the critical His-63 was compensated functionally by imidazole. Failure of imidazole to rescue mutations of the serine nucleophile and His-157 was not surprising, given the structural dissimilarity of imidazole to serine and the nonessential nature of His-157. More noteworthy was its inability to restore activity to the oxyanion hole Arg (data from transfection experiments performed with the SCMV counterpart mutants, R151A and R151G assemblin, see supplemental Fig. S1), considering that this residue is essential for assemblin activity (43) and that there is precedent for mutations of catalytic arginines in other enzymes responding to chemical rescue by imidazole (44–46).

The comparatively better rescue of Gly substitutions than Ala for the critical His in both SCMV and HCMV assemblin is attributed to the smaller size and greater structural flexibility of Gly, allowing imidazole a better functional fit at the mutant His position (37). This interpretation is consistent with (i) the inability of imidazole to enhance activity of a mutant containing the larger Gin substitution (Fig. 1A, lanes 9 and 10) and (ii) results showing no rescue by larger compounds structurally related to imidazole (data not shown from in vitro assays done with the four compounds listed under “Experimental Procedures”). The capacity of H47Q to enable limited I-site cleavage by assemblin and production of additional cleavage products (Fig. 1A) indicates that Gin can partially substitute for the catalytic His and suggests that its substitution alters cleavage specificity.

The inability of imidazole to restore activity to mutants in the protease precursor was unexpected, considering how effectively it rescues the same mutants of assemblin under the same conditions. The difference was not due to a low concentration (e.g., insufficient for activation by dimerization) or inactivity of pPR (e.g., protein insolubility), as determined by comparison with other pPR mutants that were expressed in comparably high amounts in mammalian, insect, and bacterial cells and were active (Figs. 4 and 5; data not shown from studies in progress to purify active pPR). It is also unlikely that the difference was due to a reduced accessibility of imidazole to the pPR active site, given that much larger molecules (e.g., its 38-kDa substrate, pUL80.5) gain access. Instead, we interpret this finding to reveal a structural difference between the two forms of the enzyme, with implications for the design and screening of protease inhibitors.

It has been suggested that dimerization of assemblin results in a small positional shift of the oxyanion hole Arg that results in activation (47–49). It has also been suggested that without R-site cleavage, conformation of the pPR sequence destined to form the carboxyl terminus of assemblin would be different (14) and possibly unable to participate in the interactions and changes, which accompany dimerization and activation. Thus, one explanation of our finding is that imidazole rescue of I-site cleavage works only when the active site residues are in the relative orientation they assume in the activated assemblin dimer, which may be different in the precursor. Sensitivity of the imidazole rescue effect to such small changes is compatible with the >10-fold improved I-site cleavage observed when Gly was substituted for the critical His rather than Ala (e.g., Figs. 1 and 2).

It is also possible that the I-site substrate of pPR has comparatively reduced access to the active site. The crystal structure of assemblin places the I site within an unstructured loop in the vicinity of the active site (11–14). Extension of the loop toward the interior of the protease would situate the I site in proximity to the catalytic residues. This structural feature could give the I site privileged access to the catalytic residues, perhaps bypassing the normal mode of substrate binding. Constraints on movement of the loop in pPR could restrict its access to the active site, eliminating the proximity that may be required for chemical rescue.

The inability of imidazole to restore either M- or R-site cleavage to the H47G SCMV or the H63G HCMV pPR mutants presents a major challenge to developing the imidazole-dependent mutant virus that we had envisioned because cleavage at these two sites is likely to be essential for the replication of all herpesviruses (50). On the other hand, the possibility that the protein fold of the precursor may differ sufficiently within the nascent capsid to enable rescue of the H63G mutant during virus replication is not formally ruled out.

Our finding that imidazole can restore I-site cleavage to mutants in...
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assembling but not in its precursor distinguishes the two forms of the CMV protease and reinforces other evidence for enzymatic differences between them (51, 52). We suggest that the difference reflects a conformational change in the active site environment and that the imidazole rescue effect may provide a useful tool for evaluating whether and how the conformation and mechanism of the enzyme are influenced by changes that accompany its processive maturational cleaving and folding.

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