The Human CLN2 Protein/Tripeptidyl-Peptidase I Is a Serine Protease That Autoactivates at Acidic pH*

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The CLN2 gene mutated in the fatal hereditary neurodegenerative disease late infantile neuronal ceroid lipofuscinosis encodes a lysosomal protease with tripeptidyl-peptidase I activity. To understand the enzymological properties of the protein, we purified and characterized C-terminal hexahistidine-tagged human CLN2p/tripeptidyl-peptidase I produced from insect cells transfected with a baculovirus vector. The N terminus of the secreted 66-kDa protein corresponds to residue 20 of the primary CLN2 gene translation product, indicating removal of a 19-residue signal peptide. The purified protein is enzymatically inactive; however, upon acidification, it is proteolytically processed and concomitantly acquires enzymatic activity. The N terminus of the final 46-kDa processed form (Leu196) corresponds to that of mature CLN2p/tripeptidyl-peptidase I purified from human brain. The activity of the mature enzyme is irreversibly inhibited by the serine esterase inhibitor diisopropyl fluorophosphate, which specifically and stoichiometrically reacts with CLN2p/tripeptidyl-peptidase I at Ser475, demonstrating that this residue represents the active site nucleophile. Expression of wild type and mutant proteins in CHO cells indicates that Ser475, Asp360, Asp517, but not His236 are essential for activity. These data indicate that the CLN2 gene product is synthesized as an inactive proenzyme that is autokatlytically converted to an active serine protease.

Late infantile neuronal ceroid lipofuscinosis (LINCL, OMIM 204500)† is a recessive fatal neurological disease characterized by lysosomal accumulation of proteinaceous autofluorescent storage material in neurons and other cell types (reviewed in Ref. 1). The CLN2 gene product was first identified as a normal abundance 46-kDa mannose 6-phosphorylated glycoprotein that was absent in brain autopsy specimens from LINCL patients, leading to the molecular characterization of the disease gene (2). The encoded protein has significant sequence similarities to two previously characterized bacterial endoproteases from Xanthomonas and Pseudomonas (3, 4). These proteolytic enzymes have been named bacterial pepstatin-insensitive carboxyl peptidases (BICPs) based on a series of studies that suggest that, like classic aspartyl proteases, their catalytic mechanism involves a pair of amino acids with carboxyl side chains that catalyze peptide bond hydrolysis at acidic pH but, unlike the classic aspartyl proteases, they are not inhibited by pepstatin. Based on these sequence relationships, we demonstrated that the CLN2 protein was a lysosomal protease that could degrade hemoglobin at acidic pH in the presence of aspartyl and cysteine protease inhibitors (2, 5). More recently, the CLN2 protein has been shown to have tripeptidyl exopeptidase activity. Peptide sequencing of tripeptidyl-peptidase I (TPP-I) purified from rat spleen demonstrated that it is the rhod ortholog of human CLN2p (6), and TPP-I activity is absent in LINCL specimens (7). Recently, Ezaki and colleagues (8) have shown that purified CLN2p has both TPP-I and endopeptidase activity.

Mechanistic studies on purified CLN2p/TPP-I by a number of groups indicate that enzymatic activity is not inhibited by standard serine, cysteine, metallo, or aspartyl protease inhibitors (6, 8, 9). Based on this, it has been proposed that the CLN2p/TPP-I is a pepstatin-insensitive carboxyl peptidase. However, Rawlings and Barrett (10) critically reviewed existing evidence for classification of the BICPs and concluded that the catalytic mechanism for these enzymes has not been rigorously established. These investigators alertly pointed out that a conserved Gly-Xaa-Ser sequence in CLN2p/TPP-I and the BICPs is characteristic of the active site motif of many serine peptidases. Interestingly, data base searches using the CLN2p/TPP-I sequence with different BLAST tools (CD-Search and PSI-BLAST) retrieve members of the S8 subtilisin family of serine peptidases (data not shown).

A thorough understanding of the biochemical properties of the CLN2 gene product may provide valuable clues toward understanding and developing therapies for LINCL. In this study, we have characterized the enzymatic properties of CLN2p/TPP-I. We find that the protein is synthesized as an inactive zymogen that is autokatlytically converted to an active serine protease at acidic pH.

EXPERIMENTAL PROCEDURES

Hexahistidine-tagged CLN2p/TPP-I—Two different human CLN2 cDNAs encoding the His177 and Arg177 variants (GenBankTM accession number AF017456) were subcloned into pBAGus-1 (Novagen). The constructs encode full-length CLN2p/TPP-I (residues 1–563) with a C-terminal hexahistidine tag (residues 564–569). Both constructs were expressed at Kemp Biotechnologies (Fredrick, MD) using the Sf9 cell baculovirus system. Although the Arg177 variant has only been observed in one of several cDNAs sequenced to date, it was used for large scale production purposes because it gave significantly higher yield. Alexs of conditioned medium (1 liter) were stored at –80 °C until use. All purification steps were conducted at 4 °C, and TPP-I activity

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The supernatant was applied to a 53-cm³ butyl-Sepharose 4 Fast Flow column at 22–37 °C for at least 10 min. For incubations with inhibitors, the supernatant was applied to a 10-cm³ TALON immobilized cobalt column (CLONTECH Laboratories) in a 0.1% Tween 20, 20 mM sodium citrate, pH 5.1, 100 mM sodium citrate; pH 3.0–5.5, 100 mM sodium acetate) and ter-
minated by addition of reducing SDS-PAGE sample buffer. Samples (1 μg) were fractionated by SDS-PAGE using a 12% Tris-glycine gel (Novex) and visualized by sequential staining with Coomassie blue and silver (Novex). The lane labeled pH 8.0 represents starting material that was diluted directly into SDS-PAGE sample buffer.

Fractions containing TPP-I activity eluted at 0.5 M sodium chloride and were further purified by anion exchange chromatography on either a Mono Q HR 5/5 column (Amersham Pharmacia Biotech). The column was eluted with a 0–1M sodium chloride gradient in 20 mM Tris, pH 8.0. The purified protein eluted at ~170 mM sodium chloride and appeared homogeneous by SDS-PAGE and Coomassie Blue staining. Protein (1–2 mg/ml) was divided into aliquots and stored at ~8 °C until use. The total yield of purified protein was typically 6 mg/liter of culture medium.

Untagged CLN2p/TPP-I—Mature CLN2p/TPP-I was purified from frozen human brain autopsy specimens obtained from National Disease Research Interchange (Philadelphia, PA). Brain mannose 6-phosphorylated glycoproteins were isolated by chromatography on immobilized cation-independent mannose 6-phosphate receptor as described previously (11) except that the column was sequentially eluted with 5 mM mannose 6-phosphate and then with 100 mM glycine, pH 2.5. The glycine eluate contained the bulk of the mannose 6-phosphorylated CLN2p/TPP-I and was applied to a Mono S HR 5/5 column (Amersham Pharmacia Biotech). The column eluted using a 0–1 M sodium chloride gradient in 0.1% Tween 20, 20 mM sodium citrate, pH 5. Fractions containing TPP-I activity eluted at ~0.5 M sodium chloride and were further purified by gel filtration on a 1.0 × 30-cm Superose 12 (Amersham Pharmacia Biotech) column eluted using 0.1% Tween 20, 50 mM sodium chloride, 20 mM sodium formate, pH 4.0. The purified 46-kDa protein appeared homogeneous by SDS-PAGE and Coomassie Blue staining. Total yield of purified protein was typically 20 μg/100 g of human brain. Production of untagged human CLN2p/TPP-I precursor was diluted to a concentration of 0.1 mg/ml in saline/Triton 100 mM sodium acetate, pH 4.0, buffer and incubated at 23 °C. Aliquots were removed and either analyzed by SDS-PAGE as described in Fig. 1 or diluted 20-fold into saline/Triton 100 mM sodium acetate, pH 4.0, buffer containing 421 μM AAF-AMC and immediately assayed for TPP-I activity. In the upper panel, lane 1 represents proenzyme diluted directly into SDS-PAGE buffer, whereas lanes 2–14 represent reactions terminated after 0.5, 5, 10, 20, 30, 40, 50, 60, 90, 120, 15, 25, or 1440 min. The fraction mature was estimated by scanning the gels and calculating the background corrected intensities of the broad bands migrating in regions labeled proform and mature. Data were fit with a one-phase exponential association equation using Prism 3.0 (GraphPad).

Table I

| Inhibitor | Recombinant enzyme | Human brain |
|-----------|--------------------|-------------|
|           | Insect cell | CHO cell | Purified | Homogenate |
| None      | 100         | 100       | 100      | 100       |
| 1, 10 phenanthroline (1 mM) | 62 | 60 | 61 | 63 |
| EDTA (5 mM) | 101 | 107 | 97 | 102 |
| E-64 (250 μM) | 97 | 100 | 92 | 100 |
| Pepstatin (10 mM) | 98 | | | |
| PMSF (1 mM)⁷ | | | | |
| AAF-CMK (1 μM) | | | | |
| DFP (5 mM) | | | | |

⁷Activated enzyme was preincubated with 2.5 times the indicated concentration of inhibitor for 1 h at 22 °C, diluted 2.5-fold into substrate solution to give the indicated concentration of inhibitor, and then assayed for TPP-I activity. Preincubation and assay buffers were saline/Triton with 100 mM sodium citrate, pH 4.25.

AMC substrate concentration was 200 μM.

Protein Chemistry—For peptide mapping, the activation and DFP reaction conditions were as described above except that volatile buffers (150 mM ammonium acetate, pH 4.0) were used to facilitate downstream processing. Samples (typically 100 μg) were evaporated to dryness in a vacuum centrifuge, resuspended in 100 μl of 6 M guanidine hydrochloride, 2 mM EDTA, 375 mM Tris, pH 8.1, and incubated for 1 h at 50 °C. Samples were reduced by adding 4 μl of 1 M diithiothreitol (incubation, 1.5 h at 50 °C) and alkylated by adding 16 μl of 0.5 M iodoacetamide (incubation in the dark for 30 min at 25 °C). The mixtures were desalted on a Superdex Peptide column (Amersham Pharmacia Biotech) equilibrated with 20% acetonitrile, 0.1% trifluoroacetic acid. The peak fractions were pooled, evaporated to dryness in a vac-

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The hexahistidine-tagged CLN2p/TPP-I was activated and then preincubated with the indicated concentrations of inhibitors for 70 min at 25 °C in saline/Triton, 100 mM sodium acetate, pH 4.5. The samples were diluted 10-fold into buffer containing 200 μM AAF-AMC substrate supplemented with or without inhibitor to give the final indicated concentration and assayed immediately for TPP-I activity.

**Table II: Reversibility of inhibitors**

| Treatment | During preincubation | Inhibitor concentration | During TPP-I assay | Relative TPP-I activity |
|-----------|----------------------|-------------------------|-------------------|------------------------|
|           | AAF-CMK | DCI | DFP | AAF-CMK | DCI | DFP |       |
| 1         | nm     | nm  | nm  | 100    |      |
| 2         | 30     | 30  | 30  | 6       |      |
| 3         | 30     | 3   | 30  | 43      |      |
| 4         | 3      | 3   | 30  | 39      |      |
| 5         | 300    | 300 | 300 | 37      |      |
| 6         | 300    | 30  | 30  | 49      |      |
| 7         | 30     | 30  | 30  | 77      |      |
| 8         | 2      | 3   | 30  | 1       |      |
| 9         | 2      | 0.2 | 0.2 | 2       |      |
| 10        | 0.2    | 0.2 | 0.2 | 58      |      |
| 11        | 30     | 2   | 0.2 | 38      |      |

**RESULTS**

**Autocatalytic Processing of CLN2p/TPP-I**—We purified a C-terminal hexahistidine-tagged version of the CLN2p/TPP-I secreted by an insect cell production system as described under “Experimental Procedures.” When maintained at nearly neutral pH, the protein had an apparent size of 66 kDa by SDS-PAGE (Fig. 1). Edman degradation revealed that the N terminus of the 66-kDa species (SYSPE . . . ) corresponds to residue 20 of the predicted CLN2p precursor. Differences in the predicted and actual molecular mass of the His-tagged CLN2p/TPP-I (residue 20–569; 60,118 Da) are probably due to N-linked glycosylation (2, 9). In acidic conditions (pH <4.5), the protein is converted to lower molecular mass forms (Fig. 1). Note that in addition to the major species of ~46 kDa, faster migrating bands also appear (~20 kDa), suggesting that the processing occurs through an endoproteolytic mechanism.

More detailed analysis of the conversion at pH 4.0 indicates that the 66-kDa species is rapidly (t1/2 = 7 min) converted to a 46-kDa species that is stable upon prolonged (24 h) incubation (Fig. 2). Note the transient appearance of lower molecular mass species near the bottom of the gel that appear to decrease in size and eventually disappear at long time points (Fig. 2, lane 14), consistent with endoproteolytic liberation and eventual degradation of the propeptide. Also, at early time points there appears to be a ~49-kDa species that transiently appears (Fig. 2, lanes 2 and 3), suggesting that the initial endoproteolytic cleavage may be upstream of the terminally processed 46-kDa mature form. Edman degradation revealed that the N terminus of the 46-kDa species (LHLOV) corresponds to residue 196 of the predicted CLN2p precursor, identical to that of human brain CLN2p (2). Enzyme activity measurements indicate that the 66-kDa form has very low TPP-I activity and that the time course for acquisition of activity (t1/2 = 6 min) is nearly identical to that of proteolytic processing (Fig. 2). Similar findings were obtained using untagged proenzyme purified from CHO cells (data not shown). Taken together, these data indicate that the CLN2 protein is synthesized as an inactive proenzyme that upon acidification undergoes autocatalytic conversion to an enzymatically active species.

**Inhibition of CLN2p/TPP-I Activity**—To investigate the catalytic properties of the CLN2 protein, we incubated the processed protein at pH 4.25 in the presence of different group-specific protease inhibitors and then measured TPP-I activity. Essentially identical results were obtained using purified His-tagged CLN2p/TPP-I produced in insect cells, untagged CLN2p/TPP-I purified from a CHO cell expression system, mature CLN2p/TPP-I purified from human brain, and crude human brain homogenates (Table I). Consistent with previous studies, there was essentially no effect on activity by the metalloproteinase inhibitor EDTA, by the cysteine protease inhibitor E-64, by the aspartyl protease inhibitor pepstatin, or by the serine protease inhibitor phenylmethylsulfonyl fluoride. A relatively minor effect was seen with the metalloproteinase inhibitor 1,10-phenanthroline. Also, confirming the results of others (6, 15–17), a tripeptide substrate-based chloromethylketone (AAF-CMK) inhibited activity (Table I) in a competitive manner (data not shown). Unexpectedly, TPP-I activity was inhib-
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Fig. 3. Time and concentration dependence of DFP inhibition. Top panel, concentration dependence. Samples were incubated with the indicated concentration of DFP for 10 min (filled squares), 40 min (filled triangles), 2 h (filled diamonds), 6 h (open squares), or 23 h (open triangles). For clarity of presentation, the 80- and 250-min time points are not depicted. The initial rate of TPP-I activity in the presence of inhibitor was normalized to that of samples that did not contain inhibitor (v0/vo). Curves were fit for the second order rate constant k2 using the equation v = vo - k2[D(FP)]. The depicted theoretical curves were obtained from fits performed independently for each concentration series at a fixed time point and yielded k2 values of 81, 54, 39, 39, 31, 27, and 22 M⁻¹min⁻¹ for the 10-, 20-, 40-, 80-, 120-, 250-, 360-, and 1380-min time points, respectively. The variation in the calculated rate constants may in part be due to hydrolysis of DFP during the incubation period. Middle panel, time dependence. Log transformed relative TPP-I activity from samples incubated with a fixed concentration of DFP (1 mM (filled circles), 0.0316 mM (open circles), 0.01 mM (filled squares), and 0.00316 mM (open squares)) were fit by linear regression with the negative slope of the lines yielding the apparent first order rate constant. Bottom panel, double reciprocal plots of the apparent first order rate constant (see above) versus inhibitor concentration. Inset, plot of the calculated second order rate constants versus inhibitor concentration. After preactivation, hexahistidine-tagged CLN2p/TPP-I was incubated in saline/Triton 100 mM sodium citrate, pH 4.25, containing the indicated concentration of DFP. At the indicated times aliquots were removed and diluted into the same buffer/inhibitor solution containing AAF-AMC substrate and immediately assayed for TPP-I activity.

Identification of the Active Site Serine—To determine the site treated by the serine protease inhibitor DFP (Table I). Similar results were obtained when activity was assayed using fluorescein isothiocyanate-hemoglobin substrate (data not shown). In addition, the serine protease inhibitor 3,4-dichloroisocoumarin (DCI) partially inhibited activity (Table I). Note that at the acidic pH used for these experiments, DCI formed an insoluble precipitate at millimolar or higher concentrations, thus compounding analysis of the concentration dependence of this inhibitor.

We conducted a series of dilution experiments to ascertain the stability of the enzyme-inhibitor complex. Activated enzyme was preincubated with inhibitor for 1 h, and then the initial TPP-I activity was measured immediately after diluting the preactivation reaction into a substrate solution adjusted to contain the same or a 10-fold lower final concentration of inhibitor (Table II). This was compared with the activity of a parallel sample where low concentration of inhibitor was present in both the preincubation and assay mixture. These experiments revealed that the inhibition by AAF-CMK was readily reversible, indicating that the enzyme-inhibitor complex was in rapid equilibrium (Table II, compare treatments 2 and 4). The inhibition of activity by high concentrations of DCI was partially reversed following rapid dilution (Table II, compare treatments 5 and 7), and the reversal was nearly complete after 3 h (data not shown). In contrast, the inhibition by DFP was essentially irreversible (Table II, compare treatments 8 and 10), even when measured 24 h after dilution (data not shown). The DFP inhibition could be prevented by preincubating the enzyme with the competitive inhibitor AAF-CMK (Table II, compare treatment 9 with treatments 11 and 4) or by the AAF-AMC substrate (data not shown). Taken together, these data indicate that a serine in the active site of CLN2p/TPP-I plays a key role in catalysis.

We investigated different parameters that affected inactivation of CLN2p/TPP-I by DFP. Inactivation was dependent on time and DFP concentration (Fig. 3, top panel). Plots of the log of the residual activity versus time yielded a series of straight lines for different DFP concentrations, indicating that the inactivation reaction followed pseudo-first order kinetics (Fig. 3, middle panel). A double reciprocal plot of the inverse apparent first order rate constant versus inverse DFP concentration yielded a straight line passing near the origin, suggesting that DFP does not form a Michaelis complex with CLN2p/TPP-I (Fig. 3, bottom panel), and the second order rate constant (k_agg/[DFP]) was ~30 m⁻¹min⁻¹ at 25 °C and pH 4.25 (Fig. 3, bottom panel, inset). Note that this analysis ignores hydrolysis of DFP during the reaction period and is likely to slightly underestimate the true second order rate constant (see Fig. 3 legend).

The rate of TPP-I/CLN2p inactivation by DFP was most rapid at pH ~4.5 (Fig. 4, top panel). This pH dependence was similar to that of enzymatic activity assayed using the AAF-AMC TPP-I substrate (Fig. 4, bottom panel). Experiments using nonactivated precursor and preactivated enzyme indicated that the proenzyme reacted very slowly if at all with DFP (data not shown). Given that the DFP reaction occurs by nucleophilic attack of an activated serine (18), these data indicate that both proteolytic processing and acidic pH are required for activation and/or accessibility of the catalytic serine to external substrates.
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DISCUSSION

In this study, we find that CLN2p/TPP-I is synthesized as a catalytically inactive protein that upon acidification is autocatalytically processed to an active protease. More detailed kinetic studies indicate that activation can occur both by intermolecular and intramolecular events, with the latter route predominating at low proenzyme concentrations. Based on the sequence of the precursor and mature protein, residues 20–195 (M = 19,523) are removed following acidification.Processing is likely to entail an endoproteolytic cleavage of the CLN2p/TPP-I as shown by the appearance of small (~20 kDa) fragments that transiently appear after acidification. Consistent with this, CLN2p/TPP-I has recently been shown to have intrinsic endoproteolytic activity as well as tripeptidyl exopeptidase activity (8). The appearance of a transient 49-kDa species suggests that the initial endoproteolytic cleavage may occur upstream of the Leu196 N terminus of the mature form. However, identification of processing intermediates is hindered by the appearance of TPP-I activity that can further trim various species including endogenous CHO cell CLN2p/TPP-I, are capable of cleaving the catalytically inactive mutant. We also analyzed two constructs that had alanine substitutions at Asp196 and Asp197, which align to residues important for function of the bacterial pepstatin-insensitive proteases (19, 20). The two aspartate mutants were similar to the S475A mutation in terms of lack of enzyme activity and processing (Fig. 7). Finally, in a preliminary attempt to find other catalytically important residues, we also analyzed a H236A mutant and found that it resembled the wild type construct in regard to processing and activity (Fig. 7).

Fig. 4. pH dependence of DFP inactivation and TPP-I activity. Top panel, DFP inactivation. Preactivated CLN2p/TPP-I was incubated at 25 °C in the absence and presence of 0.5 mM DFP in 100 mM formate-buffered saline/Triton of the indicated pH. At various times (0.17, 1, 2, 3, 4, and 5 h) aliquots were diluted into 250 mM formate-buffered Triton/saline, pH 4.0, containing AAF-AMC and assayed for TPP-I activity at 30 °C. Second order rate constants (k_{app}/[DFP]) were obtained as described in the legend to Fig. 3. Bottom panel, TPP-I activity. Preactivated CLN2p/TPP-I was diluted into 100 mM formate-buffered saline/Triton of the indicated pH containing AAF-AMC and assayed for TPP-I activity at 30 °C.

Edman degradation of the purified DFP-modified tryptic peptide was complicated by poor retention of the peptide on the polyvinylidenefluoride membrane during sequencing (data not shown). However, in analysis of three different preparations, for cycles 1–16 we could clearly assign all residues to that predicted from the cDNA sequence except for cycle 10, which was predicted to be Ser475. Inspection of the repetitive yield of serine showed that serine clearly was present at cycle 7, 12, and 21 but absent at cycle 10. In contrast, serine was clearly present at cycle 10 in sequencing of the unmodified tryptic peptide. Finally, Ser475 represents the only conserved serine among CLN2p/TPP-I and the BPICPs in the tryptic peptide. Taken together, these data indicate that Ser475 represents the active site nucleophile of CLN2p/TPP-I and, by extension, the BPICPs.

Expression of Wild Type and Mutant CLN2p/TPP-I—To directly investigate the function of Ser475 we expressed wild type CLN2p/TPP-I and a S475A mutant in CHO cells. The cells expressing wild type human CLN2p/TPP-I had elevated TPP-I activity and Western blotting revealed elevated levels of both precursor and processed protein (Fig. 7). The cells expressing the S475A mutant had activity similar to the neo-transfected control, indicating that the mutant was inactive compared with the wild type CLN2p/TPP-I protein. Western blotting indicated that the majority of the protein was in the precursor form, suggesting that processing was impaired (Fig. 7). However, the increased amount of ~46-kDa protein compare with the neo-transfected control indicates that other proteases, possibly including endogenous CHO cell CLN2p/TPP-I, are capable of cleaving the catalytically inactive mutant. We also analyzed two constructs that had alanine substitutions at Asp196 and Asp197, which align to residues important for function of the bacterial pepstatin-insensitive proteases (19, 20). The two aspartate mutants were similar to the S475A mutation in terms of lack of enzyme activity and processing (Fig. 7). Finally, in a preliminary attempt to find other catalytically important residues, we also analyzed a H236A mutant and found that it resembled the wild type construct in regard to processing and activity (Fig. 7).
replacement therapy for LINCL patients, where the recombinant proenzyme could be administered as an inactive prodrug that is converted to active enzyme after proper delivery to its site of action.

Proteases are typically classified by catalytic type based on their susceptibility to different types of inhibitors, with DCI and DFP being diagnostic for serine proteases (21). We find that DCI inhibits CLN2p/TPP-I in a reversible manner. Although DCI is generally thought to be an irreversible inhibitor, some bona fide serine proteases do regain activity following DCI inactivation (22). Our clear finding that DFP inactivates CLN2p/TPP-I is somewhat unexpected given the reported insensitivity of TPP-I (6, 8, 17) and a bacterial pepstatin-insensitive protease (23) to this inhibitor. However, other studies have reported partial to complete inhibition of TPP-I activity with high concentrations of DFP (15, 16). These discrepancies may reflect the kinetics and pH dependence of the DFP reaction. Even at its optimal pH, DFP inhibition of CLN2p/TPP-I is slow compared with that of other serine esterases (24), so it is possible that the experimental conditions were not optimized to detect the reaction.

Serine proteases typically contain a catalytic triad of aspartate, histidine, and serine residues, with the serine hydroxyl group being activated by the histidine, and the aspartate stabilizing the histidine imidazole ring as it gains and loses a proton (25). Although most serine proteases are inactive at acidic pH, this is not true for carboxypeptidase II and cathepsin A. In both of these enzymes, the environment of the active site includes a cluster of acid groups that may lower the histidine $pK_a$ (26, 27) and thus allow it to participate in both general acid and base catalysis at acidic pH (25). There are also examples of serine proteases that lack the classic catalytic triad such as members of the SF and SG clan, which have a catalytic dyad consisting of a lysine and serine (28).

The results presented in this study, as well as our previous detection of a S475L mutation in a LINCL patient (29), demonstrate that Ser$^{475}$ represents the active site nucleophile of CLN2p/TPP-I. Previous studies reported that diethylpyrocarbonate inhibited TPP-I activity (6, 16), suggesting that a histidine may be involved in catalysis. Although His$^{236}$ is excluded...
from the catalytic triad based on our mutagenesis data, it is possible that some other histidine activates the serine. If so, the alignments among CLN2p/TPP-I, the BPICPs, and other apparently related proteins (29) do not reveal an obvious candidate histidine.

The lack of activity of the Asp^{360} and Asp^{517} mutants are consistent with a role for these residues in catalysis. One possibility is that one aspartate participates in a classical catalytic triad and the other helps decrease the $pK_a$ of the histidine. Alternatively, if a histidine is not important for catalysis, one or both of the aspartates may assume a novel role in directly activating the catalytic serine. Protein engineering combined with structural approaches will be required for detailed insights into the catalytic process.

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