Maturation of Human Tripeptidyl-peptidase I in Vitro*

Received for publication, January 21, 2004, and in revised form, May 12, 2004
Published, JBC Papers in Press, May 13, 2004, DOI 10.1074/jbc.M400700200

Adam A. Golabek‡§, Peter Wujek‡, Marius Walus‡, Sylvain Bieler¶, Claudio Soto¶,
Krystyna E. Wisniewski‡, and Elizabeth Kida‡,

From the ‡Department of Developmental Neurobiology, New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York 10314 and the ¶Department of Neurology, University of Texas Medical Branch, Galveston, Texas 77555

Tripeptidyl-peptidase I (TPP I, CLN2 protein) is a lysosomal aminopeptidase that cleaves off tripeptides from the free N termini of oligopeptides and also shows minor endopeptidase activity. TPP I is synthesized as a preproenzyme. Its proenzyme autoactivates under acidic conditions in vitro, resulting in a rapid conversion into the mature form. In this study, we examined the process of maturation in vitro of recombinant latent human TPP I purified to homogeneity from secretions of Chinese hamster ovary cells overexpressing TPP I cDNA. Autoprocessing of TPP I proenzyme was carried out at a wide pH range, from −2.0 to 6.0, albeit with different efficiencies depending on the pH and the type of buffer. However, the acquisition of enzymatic activity in the same buffer took place in a narrower pH “window,” usually in the range of 3.6–4.2. N-terminal sequencing revealed that mature, inactive enzyme generated during autoactivation at higher pH contained N-terminal extensions (starting at 6 and 14 amino acid residues upstream of the prosegment/mature enzyme junction), which could contribute to the lack of activity of TPP I generated in this manner. Autoprocessing was not associated with any major changes of the secondary structure of the proenzyme, as revealed by CD spectroscopy. Both the activation and proteolytic processing of the recombinant TPP I precursor were primarily concentration-independent. The addition of the mature enzyme did not accelerate the processing of the proenzyme. In addition, the maturation of the proenzyme was not affected by the presence of glycerol. Finally, the proenzyme with the active site mutated (S475L) was not processed in the presence of the wild-type enzyme. All of these findings indicate a primarily intramolecular (uni-molecular) mechanism of TPP I activation and autoprocessing and suggest that in vivo mature enzyme does not significantly participate in its own generation from the proenzyme.

Tripeptidyl-peptidase I (TPP I, CLN2 protein) (EC 3.4.14.9) is a lysosomal aminopeptidase that cleaves off tripeptides from

* This work was supported in part by the Batten Disease Support and Research Association, by Grant NS047355 from NINDS, National Institutes of Health, and by the New York State Office of Mental Retardation and Developmental Disabilities. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: New York State Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Rd., Staten Island, NY 10314. Tel.: 718-494-5208; Fax: 718-982-6346; E-mail: a.golabek@att.net.

¶ This paper is available on line at http://www.jbc.org/papers to guests on July 23, 2018

© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.
19-amino acid signal peptide, which is cleaved off cotranslationally when the protein is translocated into the endoplasmic reticulum lumen, and a 176-amino acid propeptide removed during the maturation process to yield a mature enzyme of 368 amino acid residues (5, 11, 15). The proteolytic removal of the inhibitory prosegment, leading to the release of the active enzyme, is also referred to as “activation” of the protease. The cleavage of the prosegment can be accomplished either by other enzymes (in an intramolecular event) or by the same enzyme, either in an intramolecular (in cis) or intermolecular (in trans) fashion. The most common factor triggering the autoactivation of the zymogens of the cysteine, aspartic, and metalloproteases classes is a drop in pH (16–18). Indeed, Lin et al. (11) demonstrated that TPP I proenzyme (pro-TPP I), similarly to other lysosomal proteases, is activated in vitro upon acidification.

Recently, we have shown that pro-TPP I is transported from the endoplasmic reticulum to the lysosomes mainly via the mannose 6-phosphate-dependent pathway. In lysosomes, pro-TPP I matures through a proteolytic cleavage facilitated by a serine-type protease sensitive to 4-(2-aminoethyl)benzenesufonyl fluoride (AEBSF) (19). Nevertheless, although our studies suggested that another serine-type protease assists in pro-TPP I processing in vitro, this does not preclude the possibility that under certain physiological and pathological conditions, autoactivation of pro-TPP I in vitro might play an important role. To date, no detailed description of the autoactivation mechanism of pro-TPP I in vitro has been presented.

Here, by using various methods, we studied the autoactivation and autoprocessing of human pro-TPP I purified from secretions of Chinese hamster ovary (CHO) cells overexpressing TPP I cDNA, as a function of pH, concentration, and temperature. We demonstrate that recombinant pro-TPP I is predominantly activated by a unimolecular autocatalytic mechanism and that the autocatalytic conversion of the proenzyme into the mature form is primarily the result of intramolecular processing.

**EXPERIMENTAL PROCEDURES**

**Purification of Human TPP I Proenzyme—** Human pro-TPP I was purified from serum-free secretions of CHO-DHFR− cells (ATCC CRL-9096) stably transfected with plasmid encoding the full-length human TPP I, either wild-type (wt) or S475L mutant. Vector preparation, cell overexpression—

**Purification of Pro-TPP I—** Twice-concentrated stock solutions of purified wt pro-TPP I in 5 mM Tris, pH 8.0, 0.2% Triton X-100, 200 mM NaCl at the final concentrations specified in the figure legends were mixed at zero time with an equal volume of 200 mM sodium acetate (activation buffer) and maintained at 37 °C. At the indicated time points, an aliquot of 10 µl was withdrawn and combined with either 10 µl of 0.5 M NaOH (for SDS-PAGE analysis) or 40 µl of 200 mM sodium acetate, pH 5.0 (for TPP I activity measurement). Experimental data points were analyzed by least square nonlinear curve fitting with the help of GraphPad Prism 3.0 software.

**TPP I Activity Measurement—** Unless otherwise stated, TPP I activity was measured in the presence of 100 µM substrate Ala-Ala-Phe-7-amino-4-methylcoumarin (AAF-AMC) (Bachem), 0.1% Triton X-100, and 0.1 M sodium acetate, pH 5.0, in a total volume of 100 µl. At this pH, TPP I demonstrates near-maximal activity, and no further activation of pro-TPP I takes place during TPP I activity measurements (see below). The reaction was carried out at 37 °C for 20 min and was terminated by the addition of 50 µl of 10% SDS. Released AMC was measured fluorometrically on a CytoFluor 2000 (Applied Biosystems) (excitation wavelength, 360 nm; emission wavelength, 460 nm; both slits set at 40 nm) after alkalizing the solution by adding 50 µl of 1 M Tris, pH 9.0.

**SDS-PAGE and Western Blotting—** Samples were solubilized in the reducing sample buffer (Bio-Rad), boiled for 5 min, and loaded onto 10% Tris/Tricine SDS-polyacrylamide gels. Electrophoretically separated proteins were stained with either Coomassie Brilliant Blue or silver nitrate or were electrotransferred onto nitrocellulose or Immobilon-P® polyvinylidene difluoride membranes (Millipore). Nitrocellulose membranes were subsequently blocked with 5% nonfat dry milk in phosphate-buffered saline with 0.05% Tween 20 (PBST), incubated overnight with monoclonal antibody 8C4, an anti-TPP I monoclonal antibody characterized previously (3), then washed extensively in PBST, incubated with peroxidase-conjugated secondary antibodies diluted 1:5,000, and developed by using the Enhanced Chemiluminescence Kit (ECL, Amersham Biosciences). Polyvinylidene difluoride membranes were stained with 0.1% Amido Black in 10% acetic acid and destained in 5% acetic acid. Appropriate bands were excised from the membranes and applied to automated Edman degradation on an Applied Biosystems 494/HT PROCISe sequencer, which was controlled by PROCISe software and a 610A data analysis system.

**Circular Dichroism Measurement—** Purified TPP I proenzyme was dissolved either in 50 mM phosphate buffer, pH 8.0, or in 50 mM sodium acetate, pH 3.5, 4.0, and 5.0, and 50 mM NaCl at a concentration of 60 µg/ml, incubated overnight with monoclonal antibody 8C4, an anti-TPP I monoclonal antibody characterized previously (3), then washed extensively in PBST, incubated with peroxidase-conjugated secondary antibodies diluted 1:5,000, and developed by using the Enhanced Chemiluminescence Kit (ECL, Amersham Biosciences). Polyvinylidene difluoride membranes were stained with 0.1% Amido Black in 10% acetic acid and destained in 5% acetic acid. Appropriate bands were excised from the membrane and applied to automated Edman degradation on an Applied Biosystems 494/HT PROCISe sequencer, which was controlled by PROCISe software and a 610A data analysis system.

**RESULTS**

**Purification of Pro-TPP I—** By using a combination of anion-exchange chromatography, concanavalin A affinity chromatography, and gel filtration chromatography, wt pro-TPP I was purified to an apparent homogeneity (Fig. 1). The overall yield was about 14 mg of purified protein/1 liter of conditioned medium with about 30% recovery. S475L pro-TPP I mutant was purified from conditioned medium of overexpressing CHO-DHFR− cells. Concentrated cell secretions of CHO-DHFR− cells overexpressing wt pro-TPP I (lane 1), Source 15Q eluate (lane 2), concanavalin A-Sepharose eluate (lane 3), and Superose 12 eluate (lane 4) were analyzed on 10% SDS-PAGE stained with Coomassie Brilliant Blue. 5 µg of protein was used in each lane.
SDS-PAGE analysis showed that similarly to autoactivation, autoprocessing of pro-TPP I is also pH-dependent (Fig. 2B). Pro-TPP I was fully processed to the mature form at pH 2.6–4.0 and was partially processed at pH 1.0–2.0 and pH 4.6–6.0. Of interest, mature TPP I polypeptide generated at higher pH (4.6 and above) displayed slightly lower, by −1–2 kDa, electrophoretic mobility than mature TPP I resulting from autoactivation at pH 2.6–3.6. Notably, in the same pH range (4.6–5.0) a significant pro-TPP I processing occurred without concomitant acquisition of TPP I activity (compare A and B of Fig. 2), suggesting that mature TPP I with small N-terminal extensions is enzymatically inactive.

**Time Course of Autoactivation**—The rate of autoactivation of pro-TPP I was measured at pH 3.5 and 4.0 as a function of time and proenzyme concentration. As illustrated in Fig. 3A, the rate of appearance of enzymatic activity *versus* time at pH 3.5 and 37 °C could be fit to a one-phase exponential association equation, indicating an apparent single-step process. The halftime of the acquisition of the maximal activity under these conditions is about 0.7 min. However, the rate of acquisition of enzymatic activity *versus* time at pH 3.5 at room temperature (Fig. 3C) could be fit to a two-phase exponential association equation, indicating an apparent two-step process. The slopes of the activation curves at both room temperature and 37 °C are largely independent of the starting pro-TPP I concentrations (Fig. 3, B and D), which suggests that the activation of the human pro-TPP I *in vitro* is principally an intramolecular (zero-order) process under the conditions tested.

Slightly different activation curves were obtained for pro-TPP I proenzyme incubated at pH 4.0 at 37 °C. As depicted in Fig. 3E, an increase in activity *versus* time is characterized by a lag period, followed by a first-order process. The presence of the lag phase in autoactivation curves of pro-TPP I at pH 4.0 suggests that under these conditions, the activation is clearly a two-step reaction. However, as illustrated in Fig. 3F, the apparent rates of pro-TPP I activation are again independent of the initial proenzyme concentration, further pointing to an intramolecular mechanism of pro-TPP I autoactivation *in vitro*, with kinetics governed by the pH.

**Time Course of Autoprocessing**—To study the kinetics of TPP I autoprocessing, pro-TPP I at a concentration of 50 nM was activated at pH 3.5 and 4.0 at 37 °C, and aliquots were withdrawn at the time points indicated, neutralized, and analyzed by SDS-PAGE. Time course analysis of the reaction mixture, seen in Fig. 4, A and B, showed that pro-TPP I at time 0 consists of a single polypeptide with a molecular mass of ~70 kDa. During incubation at pH 3.5 and 4.0, the 70-kDa band diminishes in strength, while several new protein species with lower molecular masses appear. Split products of TPP I catalytic cleavage at pH 3.5 include a ~48-kDa band corresponding to the mature enzyme and 15–19-kDa polypeptide doublets corresponding to liberated fragments of TPP I prosegment. These proteolytic fragments appear early and concomitantly with the mature TPP I polypeptide as early as ~10 s after initiation of the activation (Fig. 4A, second lane). Autoprocessing progressed so rapidly that after 2 min only a weak band corresponding to pro-TPP I could be seen. An increase in intensity of the mature TPP I band correlated well with the intensity of the generated prosegment polypeptides. With time, however, the intensity of the doublet corresponding to prosegment fragments with a lower molecular mass increased at the expense of the fragments with higher molecular mass, suggesting that prosegment fragments are processed slowly by mature TPP I.

A similar pattern of pro-TPP I autoprocessing was observed at pH 4.0 (Fig. 4B). However, at this pH, the cleavage of...
pro-TPP I was significantly slower than at pH 3.5, requiring ~2 h for completion. In addition, at pH 4.0, the cleaved-off polypeptides appeared to be more stable than at pH 3.5, although they became detectable later. A band with an apparent molecular mass of ~60 kDa was also occasionally detected, especially on immunoblots, which suggests that the proteolytic removal of the prosegment occurred in at least two steps.

We also studied the processing of pro-TPP I as a function of its initial concentration. The kinetics of pro-TPP I autoprocessing was independent of initial pro-TPP I concentration in the range of 1.5 nM–1.5 µM (not shown). Autoprocessing of pro-TPP I was also temperature-dependent. Half-maximum cleavage of pro-TPP I at pH 3.5 occurred after 3 min at 25 °C and after 16 min at 0 °C (not shown). In addition, the full set of protease inhibitors in the activation solution (Complete, Roche Applied Science) affected neither the kinetics of autoprocessing nor the pattern of polypeptides generated (not shown), which suggests that in vitro maturation of pro-TPP I was not conferred by another, contaminating protease that might have co-purified in minute amounts along with TPP I. Edman degradation of the two prosegment fragments released during pro-TPP I autoprocessing at pH 3.5 (as in Fig. 4A) revealed that they both contain an intact N terminus of the prosegment, namely the sequence SYSPEP.

Time course analysis of the proteolytic generation of mature TPP I in relation to the acquisition of enzyme activity is presented in Fig. 5. Autocatalytic cleavage of pro-TPP I at pH 3.5 (Fig. 5A) was a rapid first-order process, correlating very well with the activation curve. However, at pH 4.0 (Fig. 5B), the acquisition of specific TPP I activity was delayed in comparison

FIG. 3. Time course analysis of pro-TPP I activation in vitro. Pro-TPP I at the concentrations indicated (in nM) was incubated at pH 3.5 and either 37 °C (A and B) or room temperature (C and D) or at pH 4.0 and 37 °C (E and F). At indicated periods, aliquots were analyzed for TPP I activity (A, C, and E). The values for each pro-TPP I concentration were then normalized as a percent of the maximum TPP I activity at this concentration (B, D, and E). 100 mM sodium acetate, 100 mM NaCl, and 0.1% Triton X-100 were used as a buffer. Data points (symbols) were fit to a one-phase exponential association equation (A and B), a two-phase exponential association equation (C and D), or a sigmoidal equation (E and F) (solid lines) by using GraphPad Prism software. RT, room temperature.
with generation of the mature polypeptide. The presence of the lag period in the activation curve versus proteolytic processing suggests that an additional step of processing, structural rearrangement, and/or dissociation is required before full activity of the newly generated enzyme could be realized. We hypothesized that this second step might involve a structural change in the vicinity of the catalytic cleft. To assess whether in vitro maturation of TPP I is associated with significant structural rearrangement in pro-TPP I, we used CD measurement of pro-TPP I before and after activation.

**CD Analysis**—The far-UV CD spectrum gives a measure of the secondary structure of the protein. Pro-TPP I was incubated for 2 h at pH 8.0, 5.0, 4.0, or 3.5, and its CD spectra were recorded. Apart from a small increase in mean residue ellipticity for pro-TPP I incubated at pH 5.0 and 4.0 in the 205–225 nm region of the spectrum, the CD spectra for TPP I incubated at various pH levels were similar (Fig. 6). These results indicate that there is no large scale conformational change in the secondary structure of pro-TPP I when the pH is lowered from neutral to acidic. If conformational changes had occurred, they would have been either localized and/or very small.

**Transactivation of Pro-TPP I in Vitro**—To determine whether the active enzyme could facilitate generation of mature TPP I from the proenzyme, pro-TPP I was activated at various pH levels in the presence or absence of mature TPP I and analyzed for both processing and activation. As depicted in Fig. 7, in the pH range of 2.8–6.2, the presence of the mature TPP I (so-called seed) at 1:100 molar ratio to pro-TPP I affected neither the pH-dependent processing (Fig. 7A) nor the activation profile (Fig. 7B) of pro-TPP I. These results confirm that in vitro maturation of pro-TPP I is an intramolecular event.

In addition, analysis of Western blots revealed an additional product of pro-TPP I cleavage, evident especially at higher pH (Fig. 7A, lanes 7 and 8, asterisk). This band had an apparent molecular mass of ~60 kDa and was often visible on immunoblots under various conditions used for autoprocessing studies, but it was hardly distinguishable on silver-stained gels. This polypeptide might represent a short lived, unstable intermediate of pro-TPP I autoprocessing.

**The Effect of Buffer Type on Maturation of Pro-TPP I in Vitro**—Because phosphate-citrate buffer, pH 4.6, afforded significant processing of pro-TPP I cleavage, evident especially at higher pH (Fig. 7A, lanes 7 and 8, asterisk). This band had an apparent molecular mass of ~60 kDa and was often visible on immunoblots under various conditions used for autoprocessing studies, but it was hardly distinguishable on silver-stained gels. This polypeptide might represent a short lived, unstable intermediate of pro-TPP I autoprocessing.

As illustrated in Fig. 8A, after 2-h incubation, autocatalytic cleavage of pro-TPP I in other types of buffers at pH 4.5. As illustrated in Fig. 8A, after 2-h incubation, autocatalytic cleavage of pro-TPP I in other types of buffers at pH 4.5. As illustrated in Fig. 8A, after 2-h incubation, autocatalytic cleavage of pro-TPP I in other types of buffers at pH 4.5. As illustrated in Fig. 8A, after 2-h incubation, autocatalytic cleavage of pro-TPP I in other types of buffers at pH 4.5. As illustrated in Fig. 8A, after 2-h incubation, autocatalytic cleavage of pro-TPP I in other types of buffers at pH 4.5. As illustrated in Fig. 8A, after 2-h incubation, autocatalytic cleavage of pro-TPP I in other types of buffers at pH 4.5. As illustrated in Fig. 8A, after 2-h incubation, autocatalytic cleavage of pro-TPP I in other types of buffers at pH 4.5. As illustrated in Fig. 8A, after 2-h incubation, autocatalytic cleavage of pro-TPP I in other types of buffers at pH 4.5.
ditions, the complete autoactivation was achieved only in phosphate buffer, while still significant but lower TPP I activity was also present in glycine and formate buffers (Fig. 8B), again independent of the presence of the seed. In contrast, only residual TPP I activity was present in pro-TPP I activated in acetate and citrate buffers at pH 4.5, despite distinct processing of pro-TPP I in these samples. Hence, these results suggested once again that at higher pH (≥4.0 and above), in vitro maturation of pro-TPP I involves at least two steps: initial cleavage of the prosegment region and dissociation and/or degradation of the released prosegment.

To evaluate whether inactive processed TPP I generated from pro-TPP I is able to resume full activity under the permissive pH, pro-TPP I was first incubated in acetate buffer at pH 4.5. Its activity was measured, and then the pH in the sample was reduced to 3.5 (Fig. 8C). After 3-h incubation at pH 4.5, a significant portion of pro-TPP I was processed to a mature 48-kDa polypeptide (not shown), although only residual TPP I activity was present in that sample (Fig. 8C, time 0). Nonetheless, upon buffer adjustment to pH 3.5 and further 30-min activation, the already processed TPP I became only partially enzymatically active, showing slower kinetics and lower maximal activity than the same amount of pro-TPP I directly activated at pH 3.5, suggesting that under suboptimal conditions, activation of pro-TPP I leads to generation of some dead-end products that cannot be reactivated. These data demonstrate that although pro-TPP I can be autoprocessed at a relatively wide pH range, the pH required for autoactivation is distinctly narrower.

In an attempt to elucidate lack of direct correlation between the processing and acquisition of enzymatic activity by pro-TPP I at higher pH, we performed the N-terminal sequencing of the...
mature TPP I polypeptides generated at various pH levels of activation buffer (Fig. 9). Mature TPP I generated after 3 h of autoactivation at pH 3.5 had an N terminus starting at Leu196, thus at the precise prosegment/mature enzyme junction. After autoactivation at higher pH (4.5 and 5.0), two major cleavage sites were identified, after Glu189 and Ser181, generating mature TPP I with N-terminal extensions containing 6 and 14 amino acid residues, respectively. At pH 5.0, the yields of both polypeptides were nearly equimolar, whereas at pH 4.5, the polypeptide starting at Val190 predominated. In addition, a minor polypeptide with molecular mass of approximately 60 kDa, thus of intermediate size between pro-TPP I and the mature enzyme that was generated at pH 4.5 and 5.0 (Fig. 9, arrowhead), had an N-terminal sequence starting at Thr98. These data suggest that the lack of tripeptidyl peptidase activity of mature TPP I generated during pro-TPP I processing in vitro may be caused by the presence of small N-terminal extensions on enzymes generated in this manner.

The Effect of Glycerol and Tripeptidyl Substrate on Pro-TPP I Maturation in Vitro—To further test the possibility that pro-TPP I maturation in vitro is a unimolecular process, we analyzed autoactivation and autoprocessing of the proenzyme in the presence of glycerol. Glycerol increases solution viscosity, and any reaction involving an intermolecular event would be associated with the diffusion process and thus should be slower in the presence of glycerol (22). However, as illustrated in Fig. 10A, addition of 10% glycerol did not affect the kinetics of pro-TPP I autoprocessing in the entire pH range studied (3.4–5.0). MOCAc (7-methoxy coumarin-4-acetyl)-Gly-Lys-Pro-Ile-Pro-Phe-Phe-Arg-Leu-Lys-(DNP)-NH2, a substrate of endoproteolytic activity of TPP I (2), also did not affect the kinetics of pro-TPP I autoprocessing at pH 3.5, thus, the value near the pH optimum of endopeptidase activity of TPP I (not shown). These results confirm the unimolecular mechanism of pro-TPP I autocatalytic activation and processing in vitro.

Transactivation of wt Pro-TPP I and Pro-TPP I with Active Site Mutated—As a final approach to characterize the mechanism of pro-TPP I activation in vitro, we analyzed the kinetics of activation of pro-TPP I in the presence of active TPP I. As illustrated in Fig. 10C, addition of 10% (mol/mol) of preactivated TPP I to a 50 nM solution of pro-TPP I did not significantly affect the kinetics of pro-TPP I activation. In addition, when the activity of the seed was subtracted from the mixture, the TPP I activation curve was identical to that of pro-TPP I activated alone. Similar results were obtained with a 1 and 0.1% (mol/mol) ratio of the seed to pro-TPP I (not shown).

Our earlier studies showed that TPP I proenzyme with the active site nucleophile mutated (S475L-pro-TPP I) is processed in vitro into a mature 48-kDa polypeptide with the assistance of AEBSF-sensitive protease (19). Thus, we hypothesized that if autoactivation of TPP I in vitro was an intermolecular event, enzymatically active wt TPP I could also process S475L-pro-TPP I. To test this possibility, we studied the effect of the seed on the processing of human S475L-pro-TPP I. As expected, purified mutant protein was unable to autoprocess at pH 3.5.

Fig. 9. Cleavage sites observed during the activation of pro-TPP I at various pH levels. Shown are Amido Black-stained polyvinylidene difluoride membrane (top) and partial sequences of TPP I prosegment and prosegment/mature enzyme junction (bottom) with cleavage sites identified at pH 3.5 and at pH 4.5 and 5.0 by N-terminal sequencing of bands corresponding to the mature enzyme (bold arrow on the blot and sequence) and corresponding to an intermediate activation product (arrowhead on the blot and sequence).
Maturation of TPP I in Vitro

31065

In summary, our studies suggest that TPP I activity is not involved in the major proteolytic step leading to generation of the mature enzyme, indicating that autoactivation of human pro-TPP I in vitro occurs by an intramolecular mechanism.

DISCUSSION

In accord with the results of Lin et al. (11), we demonstrate that maturation of human pro-TPP I in vitro is a pH-driven process. Autoprocessing of purified wt pro-TPP I incubated under acidic conditions was associated with generation of the mature polypeptide and a number of small species corresponding to fragments of TPP I prosegment. An intermediate product with molecular mass of ~60 kDa also was detectable, but in variable and usually very small amounts in activation buffers with pH ≥ 4.0.

The efficiency of pro-TPP I autoprocessing and the pattern of proteolytic processing of pro-TPP I, either four distinct bands (in phosphate-citrate buffer) or two doublets (in acetate buffer) of prosegment fragments were present. Interestingly, however, incomplete autoprocessing of pro-TPP I that occurred at pH 2.0 and 4.5–5.6 in the same buffers was associated with the formation of fewer proteolytic fragments, with higher molecular masses than those generated at more permissive pH. This incomplete conversion to mature species was coupled with a lack of tripolyphosphatidylphosphatase activity, which suggests that at low pH, liberated mature polypeptide and a number of small species corresponded to generation of the mature enzyme, indicating that autoactivation of human pro-TPP I occurs by an intramolecular mechanism.

Time course analysis of pro-TPP I activation revealed, similarly to autoprocessing, a rapid and efficient progression at pH 3.5, with half-time of ~0.7 min at 37 °C and ~3 min at room temperature. At this pH, the pro-TPP I activation at 37 °C could best fit to a one-phase exponential association curve, whereas data obtained for pro-TPP I activated at room temperature could best fit to a two-phase exponential association curve. However, a logistic curve was obtained for pro-TPP I activated at pH 4.0 at 37 °C, and the comparison of autocatalytic and autoprocessing curves of pro-TPP I at this pH demonstrated that acquisition of enzymatic activity was slightly delayed relative to autoprocessing.

The pH- and temperature-dependent transition of the activation curves from the logistic curve to the two-phase and finally the one-phase exponential association curve indicates that TPP I maturation in vitro is performed in two stages. The first step, relatively fast and less pH-dependent, includes proteolytic scission, followed by a limiting, strongly pH-dependent step involving the liberation of the prosegment, or fragment thereof, from its cognate protease, either by proteolytic cleavages or simply by dissociation. Alternatively, the second step might involve the displacement of the newly formed N terminus of the mature enzyme from the catalytic cleft or other structural rearrangements within the substrate-binding site.

Because (i) specific activity of TPP I is higher at pH 4.0 than 3.5 (2, 23), (ii) the activation of pro-TPP I is faster at lower pH, and (iii) exopeptidase and endopeptidase substrates do not affect the rate of pro-TPP I autoprocessing, we propose that the endopeptidase and exopeptidase activity of the newly formed enzyme can be excluded as a limiting factor in pro-TPP I activation. The strong dependence on pH (these studies) and ionic strength of the second step of pro-TPP I activation suggests that this step involves disruption of existing salt bridges.

FIG. 10. The effect of glycerol, tripeptidyl substrate, and mature TPP I seeds on in vitro maturation of wt pro-TPP I and the effect of wt TPP I on autoprocessing of the S475L mutant of pro-TPP I. A, pro-TPP I, at 12.5 μM, was activated in 100 mM sodium acetate, pH 3.5, 100 mM NaCl, 0.1% Triton X-100 at room temperature in the absence or presence of glycerol at indicated concentrations and was analyzed for TPP I activity at the indicated time points. B, pro-TPP I, at 100 μM, was activated for 20 min at 37 °C in the absence (top) or presence (bottom) of tripeptidyl substrate at 1 mM in phosphate-citrate buffer at the indicated pH values and was analyzed by Western blotting. C, pro-TPP I, at 12.5 μM, was activated in 100 mM sodium acetate, pH 3.5, 100 mM NaCl, 0.1% Triton X-100 at room temperature in the absence or presence of 1.25 mM mature TPP I and was analyzed for TPP I activity at the indicated time points. D, S475L mutant pro-TPP I was not activated (lane 1), activated alone (lane 2), or activated with wt pro-TPP I (lane 5) and was analyzed by Western blotting. Lane 3, wt pro-TPP I not activated; lane 4, wt pro-TPP I activated. Both pro-TPP I and S475L mutant pro-TPP I were used at 100 μM, and activation was performed in 100 mM sodium acetate, pH 3.5, 100 mM NaCl, 0.1% Triton X-100 at 37 °C for 2 h.

(A, A. Golabek, unpublished results.)
and protonation of either a single or multiple carboxylic side chain(s) with pKₐ between 3.5 and 4.0, thus pointing to dissociation of the prosegment fragment(s). This dissociation would be followed or proceeded by some structural rearrangements as the mechanism responsible for the second step of pro-TPP I activation.

Because we used highly purified, homogeneous pro-TPP I preparation for these studies and because the in vitro maturation was not affected by the presence of a mixture of protease inhibitors, all of the proteolytic activities leading to pro-TPP I activation can be attributed solely to pro-TPP I. Pro-TPP I could be activated at pH as low as 2.0, and in fact, the activation at pH 2.0–3.0 was the quickest. However, mature polypeptide generated at this pH was either rapidly (pH 2.0) or slowly (pH 2.6–3.0) inactivated. As it was already reported, mature TPP I is less stable in this pH range (2, 24). Interestingly, although proteolytic processing of pro-TPP I was carried out in a wide pH range from ~2.0 to 6.0, albeit with different efficiencies, the acquisition of enzymatic activity in the same buffer took place in a narrow pH window of ~3.5–4.2. Furthermore, no significant tripeptidyl peptidase activity was present in pro-TPP incubated at pH ≥ 4.6.

Several factors could contribute to the lack of pro-TPP I activation despite its processing that we observed at higher pH. Under conditions in which pro-TPP I was processed without gaining activity, the mature enzyme contained N-terminal protrusions (6 and 14 amino acid residues), which could inhibit the acquisition of enzymatic activity by TPP I similarly to what was observed for cathepsin L (25) and asparagusinyl endopeptidase (26). TPP I polypeptide generated at higher pH also might not acquire “active” structure; thus, its processing might be “non-productive,” as was described for prohormone convertase 2 (27). In addition, at pH ≥ 4.6, cleaved-off TPP I prosegment, or fragments thereof, might still be tightly bound to the active site of the mature enzyme, inhibiting its activity. For a number of peptidyl hydrolases, including serine proteases, the prosegment has been shown to be a potent inhibitor for its cognate protease (28–31). The presence of the cleaved-off prosegment still complexed to mature TPP I could explain not only the lack of activity of TPP I processed at pH 4.6 and higher but also the delay in appearance of activity in newly processed TPP I at pH 4.0 (Fig. 5B). According to this scenario, this delay could be caused by the time necessary for the dissociation and/or by the time needed for cleavage of the prosegment (or fragment thereof) from the prosegment-mature protein complex. Our preliminary unpublished results from gel filtration experiments suggest that indeed prosegment remains bound to mature TPP I activated at pH 4.5 but not at pH 3.5. It remains unclear and must be elaborated by further studies whether a polypeptide with molecular mass of ~60 kDa starting at Thr cannot be a normal intermediate of pro-TPP I autoprocessing that is extremely unstable at lower pH or represents a “dead-end” product generated exclusively at higher pH.

Unlike other families of proteases, a high resolution three-dimensional structure of the proenzyme is not available for any of the sedolisins. Hence, the exact mechanism of inhibition and activation of zymogens of the sedolisins is not known. In other protease families, i.e. cysteine or serine proteinases, the common property of zymogens is that the catalytic machinery (bond-cleavage apparatus, substrate binding cleft) that is required for productive cleavage of peptide bond is preserved (32). By using CD spectroscopy, we have demonstrated that human pro-TPP I does not undergo a major structural change during its in vitro maturation. However, this does not preclude a localized structural rearrangement or some tertiary structure modifications because the conversion process of zymogens often involves significant conformational changes in regions that are adjacent to the active site or within the prosegment that is subsequently removed (32). Detailed analysis of the catalytic mechanism of TPP I will be possible after resolving its high resolution three-dimensional structure.

Our studies, however, clearly demonstrate that both autoactivation and autoprocessing of pro-TPP I in vitro are primarily intramolecular processes. This conclusion is based on the following findings. (i) Both autoactivation and autoprocessing were independent of the initial concentration of the proenzyme. (ii) Glycerol, which should slow a bimolecular process, did not significantly affect in vitro maturation of the proenzyme. (iii) Preactivated TPP I had no effect on the activation or processing rate of pro-TPP I, and (iv) S475L-pro-TPP I was not processed by the active wt TPP.

If the chemical reaction has the option to proceed in either a unimolecular or bimolecular manner, the unimolecular pathway will usually predominate. We have demonstrated that indeed both autoactivation and autoprocessing of pro-TPP I in vitro are largely, if not exclusively, intramolecular events. However, our earlier studies showed the existence of the in trans activation of human pro-TPP I in vivo, which was accomplished not by mature TPP I but by another, not yet identified AEBSP-sensitive serine protease (19).

Given that the results of our study showed that effective autoactivation of purified pro-TPP I is possible in a narrow pH range, it appears that TPP I autoactivation could not encounter favorable conditions in the lysosomal milieu in vivo. For many years, it was believed that lysosomes have the most acidic lumen among all intracellular organelles and relatively stable pH values under steady-state conditions. However, the exact values of lysosomal pH reported in the literature differ significantly, depending on the method used for the measurements, the type of cell analyzed, or whether the study was done in living cells or in isolated lysosomal fraction. Thus, even if the values of lysosomal pH around 4.5–5.5 have been reported most often (33–38), values as high as 7.0 in individual lysosomes of CHO cells (39), as low as 3.55 for pancreatic adenocarcinoma cells from a patient with cystic fibrosis (40), and below 4.0 for heterophagolysosomes in breast cancer cells (41) have been found. Although the pH within the lysosomes, as well as other intracellular vesicular compartments, is controlled by a family of ATP-dependent proton pumps known as the vacuolar (H⁺)-ATPases (42), our knowledge of the precise mechanisms involved in regulation of lysosomal pH in vivo is still far from being complete. Butor and co-workers (43) formulated the idea that lysosomal pH may be more dynamic than previously thought and that it fluctuates over a wide pH range, with a preference for a more acidic state. Furthermore, it appears that cells can enhance acidification of the endosomal/lysosomal system in response to specific biological requirements, as was recently shown for monocytes differentiated into dendritic cells (44). The results of our study showed that efficient processing and activation of pro-TPP I were possible in one of the activation buffers used (monosodium/disodium phosphate) even at pH 4.5. Furthermore, our unpublished data indicate that polyanions, compounds also present in lysosomes of mammalian cells, shift the range of activity of TPP I and allow pro-TPP I to generate active enzyme in less acidic pH. Thus, it appears that autoactivation and generation of an enzymatically active mature form of human TPP I can also proceed in vivo.

TPP I is the only tripeptidyl peptidase identified to date in lysosomes of mammalian cells. Mutations in TPP I lead to the classic late-infantile neuronal ceroid lipofuscinosis, one of the most devastating neurodegenerative disorders of childhood as-
associated with generalized lysosomal storage and severe neuronal death in the brain. TPP I is up-regulated in various pathological conditions including tumors, neurodegenerative disorders, or some lysosomal storage disorders (3, 45). In this regard, utilization of two different pathways of pro-TPP I processing, intramolecular autoactivation and proteolytic processing with the assistance of another protease, could secure the availability of the active enzyme under conditions unfavorable for the alternative pathway or when high amounts of the active enzyme are needed by the cell. It was demonstrated for the bacterial serine protease subtilisin that although in vivo its zymogen is clearly capable of intramolecular processing, in vivo both cis and trans activation mechanisms may function, with the trans activation route occurring during periods of maximum synthesis (46, 47). Thus, which pathway of pro-TPP I activation predominates in vivo under both physiological conditions and in pathology must be elucidated by further studies.

Understanding of the factors and conditions that determine efficient processing and activation of TPP I will help in development in the future of a rational treatment strategy for individuals with the CLN2 disease.

Acknowledgment—We thank Maureen Stoddard Marlow for co-editing the manuscript.

REFERENCES
1. Tomkinson, B. (1999) Trends Biochem. Sci. 24, 355–359
2. Ezaki, J., Takeda-Ezaki, M., Oda, K., and Kominami, E. (2000) Biochem. Biophys. Res. Commun. 288, 904–908
3. Kida, E., Golabek, A. A., Walus, M., Wujek, P., Kaczmarski, W., and Wisniewski, K. (2001) J. Neuropathol. Exp. Neurol. 60, 280–292
4. Kurachi, Y., Oka, A., Itoh, M., Misuguchi, M., Hayashi, M., and Takashima, S. (2001) Acta Neuropathol. 102, 20–26
5. Sleat, D. E., Donnelly, R. J., Lacklack, H., Liu, C.-G., Sohar, I., Pullarkat, R. K., and Lobel, P. (1997) Science 277, 1802–1805
6. Sleat, D. E., Gin, R. M., Sohar, I., Wisniewski, K. E., Sklower Brooks, S., Pullarkat, R., Palmer, D. N., Lerner, T. J., Boustatany, R. M., Uldall, P., Siakotos, A. N., Donnelly, R. J., and Lobel, P. (1999) Am. J. Hum. Genet. 64, 1511–1523
7. Kida, E., Wisniewski, K. E., and Golabek, A. A. (2001) Adv. Genet. 45, 35–68
8. Wisniewski, K. E., Kida, E., Golabek, A. A., Kaczmarski, W., Connell, F., and Zhong, N. (2001) Adv. Genet. 45, 1–34
9. Wisniewski, K. E., Kida, E., Connell, F., Kaczmarski, W., Kaczmarski, A., Michalewski, M. P., and Zhong, N. (1999) Mol. Genet. Metab. 66, 248–252
10. Wisniewski, K. E., Kida, E., Walus, M., Wujek, P., Kaczarmarski, W., and Golabek, A. A. (2001) Eur. J. Cell Biol. 5, Suppl. A, 73–79
11. Lin, L., Sohar, I., Lacklack, H., and Lobel, P. (2001) J. Biol. Chem. 276, 2249–2255
12. Wlodawer, A., Li, M., Gustchina, A., Oyama, H., Dunn, B. M., and Oda, K. (2003) Acta Biochim. Pol. 50, 81–102
13. Wlodawer, A., Li, M., Dauter, Z., Gustchina, A., Uchida, K., Oyama, H., Dunn, B. M., and Oda, K. (2001) Nat. Struct. Biol. 8, 442–446
14. Comellas-Bigler, M., Fuentes-Prior, P., Maskos, K., Huber, R., Oyama, H., Uchida, K., Dunn, B. M., Oda, K., and Bode, W. (2002) Structure 10, 865–876
15. Liu, C.-G., Sleat, D. E., Donnelly, R. J., and Lobel, P. (1998) Genomics 50, 206–212
16. Erickson, A. H. (1989) J. Cell. Biol. 40, 31–41
17. Takeda-Ezaki, M., and Yamamoto, K. (1993) Arch. Biochem. Biophys. 304, 352–358
18. Mach, L., Mort, J. S., and Glossl, J. (1994) J. Biol. Chem. 269, 13030–13035
19. Golabek, A. A., Kida, E., Walus, M., Wujek, P., Mehta, P., and Wisniewski, K. E. (2003) J. Biol. Chem. 278, 7135–7145
20. Ho, S. N., Hunt, H. D., Horton, R. M., Pulken, J. K., and Pease, L. R. (1989) Gene (Annu.) 77, 51–59
21. McIvaine, T. C. (1921) J. Biol. Chem. 49, 183–186
22. Rozman, J., Stojan, J., Kuhelj, R., Turk, V., and Turk, B. (1999) FEBS Lett. 458, 358–362
23. Junaid, M. A., Wu, G., and Pullarkat, R. K. (2000) J. Neurochem. 74, 287–294
24. Vines, D., and Warburton, M. J. (1998) Biochem. Biophys. Acta 1384, 233–242
25. Ishidoh, K., and Kominami, E. (1994) FEBS Lett. 352, 281–284
26. Li, D. N., Matthews, S. P., Antoniou, A. N., Mazzeo, D., and Watts, C. (2003) J. Biol. Chem. 278, 38380–38390
27. Lamango, N. S., Apletalina, E., Liu, J., and Lindberg, I. (1999) Arch. Biochem. Biophys. 362, 275–282
28. Creemers, J. W., Yew, M., Schafer, W., Ayoubi, T. A., Roebroek, A. J., Klink, H. D., Garten, W., and Van de Ven, W. J. (1995) J. Biol. Chem. 270, 2695–2702
29. Zhou, A., Paquet, L., and Mains, R. E. (1995) J. Biol. Chem. 270, 21509–21516
30. Anderson, E. D., VanSlyke, J. K., Thulin, C. D., Jean, F., and Thomas, G. (1997) EMBO J. 16, 1568–1518
31. Boudreault, A., Gauthier, D., and Lazure, C. (1998) J. Biol. Chem. 273, 31574–31580
32. Rob, A. R., and James, M. N. G. (1998) Protein Sci. 7, 815–836
33. Okuma, S., and Poole, B. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 3327–3331
34. Mellman, I., Fuchs, R., and Helenius, A. (1986) Annu. Rev. Biochem. 55, 663–700
35. Vergne, I., Constant, P., and Laneelle, G. (1998) Anal. Biochem. 255, 127–132
36. Bach, G., Chen, C. S., and Pagano, R. E. (1999) Clin. Chim. Acta 280, 173–179
37. Golabek, A. A., Kida, E., Walus, M., Kaczarski, W., Michalewski, M., and Wisniewski, K. E. (2000) Mol. Genet. Metab. 70, 203–213
38. Chen, C.-S. (2002) BMC Cell Biol. 3, 21
39. Yamashiro, D. J., and Maxfield, F. R. (1987) J. Cell Biol. 105, 2723–2733
40. Van Dyke, R., Root, K. V., Schreiber, J. H., and Wilson, J. M. (1992) Biochem. Biophys. Res. Commun. 184, 300–305
41. Montourier, P., Mangeat, P. H., Valeiob, C., Salazar, G., Sabaquet, A., Dupray, C., and Rochefort, H. (1994) J. Cell Biol. 127, 2361–2391
42. Nishi, T., and Forgac, M. (2002) Science 299, 1409–1403
43. Junaid, M. A., Clark, G. M., and Pullarkat, R. K. (2000) Int. J. Biol. Markers 15, 129–134
44. Li, Y., and Inouye, M. (1996) J. Mol. Biol. 262, 591–594
45. Hu, Z., Haghijo, K., and Jordan, P. (1996) J. Biol. Chem. 271, 3375–3384
Maturation of Human Tripeptidyl-peptidase I in Vitro
Adam A. Golabek, Peter Wujek, Marius Walus, Sylvain Bieler, Claudio Soto, Krystyna E. Wisniewski and Elizabeth Kida

J. Biol. Chem. 2004, 279:31058-31067. doi: 10.1074/jbc.M400700200 originally published online May 13, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M400700200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 47 references, 16 of which can be accessed free at http://www.jbc.org/content/279/30/31058.full.html#ref-list-1