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Analysis of catalytic properties of tripeptidyl peptidase I (TTP-I), a serine carboxyl lysosomal protease, and its detection in tissue extracts using selective FRET peptide substrate

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ABSTRACT

Tripeptidyl peptidase I (TPP-I), also named ceroid lipofuscinosis 2 protease (CLN2p), is a serine carboxyl lysosomal protease involved in neurodegenerative diseases, and has both tripeptidyl amino- and endopeptidase activities under different pH conditions. We developed fluorescence resonance energy transfer (FRET) peptides using tryptophan (W) as the fluorophore to study TPP-I hydrolytic properties based on previous detailed substrate specificity study (Tian Y. et al., J. Biol. Chem. 2006, 281:6559–72). Tripeptidyl amino peptidase activity is enhanced by the presence of amino acids in the prime side and the peptide NH_2-RWFFIQ-EDDnp is so far the best substrate described for TPP-I. The hydrolytic parameters of this peptide and its analogues indicated that the S_4 subsite of TPP-I is occluded and there is an electrostatic interaction of the positively charged substrate N-terminus amino group and a negative locus in the region of the enzyme active site. KCl activated TPP-I in contrast to the inhibition by Ca^{2+} and NaCl. Solvent kinetic isotope effects (SKIEs) show the importance of the free N-terminus amino group of the substrates, whose absence results in a more complex solvent-dependent enzyme: substrate interaction and catalytic process. Like pure TPP-I, rat spleen and kidney homogenates cleaved NH_2-RWFFIQ-EDDnp only at F—F bond and is not inhibited by pepstatin, E-64, EDTA or PMSF. The selectivity of NH_2-RWFFIQ-EDDnp to TPP-I was also demonstrated by the 400 times higher k_{cat}/K_M compared to generally used substrate, NH_2-AAF-MCA and by its resistance to hydrolysis by cathepsin D that is present in high levels in kidneys.

1. Introduction

Tripeptidyl peptidase I (TPP-I), also named ceroid lipofuscinosis 2 protease (CLN2p), is a lysosomal acidic and pepstatin insensitive peptidase, and the only member of the sedolisin (serine-carboxyl peptidases) family (MEROPS S53 clan SB) so far found in mammals. Sedolisins resemble subtilisin but they are larger, and the characteristic features of these peptidases are the catalytic triad Ser-Glu-Asp (with Glu replacing the canonical His in subtilisin), and with an aspartic acid residue present in the oxyanion hole [1,2]. Based on the crystal structure and analogy to subtilisin the catalytic mechanism of TPP-I was proposed where S^{475} acts as the nucleophile, attacking the polarized carbonyl group of the scissile peptide bond to form a tetrahedral intermediate, E^{272} is the proton acceptor and D^{360} side chain helps to create the oxyanion hole, stabilizing the tetrahedral intermediate of the reaction. The presence of aspartic acid (D^{276}) replacing asparagine of subtilisins (N^{155}) creates the oxyanion hole, leading to activity at acidic pH [3,4]. TPP-I is a globular protein with the bacterial subtilisin-like fold and an octahedrally coordinated Ca^{2+} binding site [5]. The enzyme has several glycosylation sites, with *N*-glycosylation of N^{286} required for activity and maturation through the mannose 6-phosphate receptors pathway to the lysosome [6,7]. Like most subtilases and subtilase-like proteases, mature TPP-I (consisting of 368 amino acids) is obtained from an autocatalytic intramolecular removal of a prosegment of 176 amino acid residues from the zymogen, [8,9]. The TPP-I prosegment is a slow binding inhibitor of its parent enzyme with a strong dependence of pH, thus acting either as an inhibitor or as a molecular chaperon of the mature enzyme, helping deliver

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the required peptidase activity into the lysosome [10]. Although the probable common precursor of sedolisins and classical serine peptidases, sedolisins seem also to make use of some catalytic features of aspartic peptidases [11,12] that were confirmed by mechanical/molecular mechanical (QM/MM) molecular dynamics and free energy simulations [12–15].

TPP-I is widespread in the human body, with high expression levels in brain [16,17], spleen [18] and kidney [19]; however, the naturally occurring substrate(s) and biological function of TPP-I are still elusive. Mutations described for the *TPP-I* gene result in deficiency of the peptidase leading to accumulation of autofluorescent lipopigments constituted by the subunit c of mitochondrial ATP synthase complex (F_0F_1-ATPase) in the lysosome. This results in the Late-Infantile Neuronal Ceroid Lipofuscinoses (LINCL), a fatal childhood inherited neurodegenerative disease [20–23]. On the other hand, overexpression of TPP-I, like others lysosomal peptidases, was reported in breast, esophageal and colorectal cancers, suggesting a role in metastasis [24,25]. Besides the putative role of TPP-I in degradation of subunit c of mitochondrial ATP synthase, it also exhibits *in vitro* activity on other physiological substrates such as angiotensin-II, cholecystokinin, neuropeptide neuromedin B and Bid during apoptosis [26–31].

TPP-I has predominantly tripeptidyl exopeptidase activity, with an optimum pH activity around 5.0, catalyzing the release of sequential tripeptides from the N-termini of substrates as well as a weak endopeptidase activity with optimum pH around 3.0 [30,31]. The preference for hydrolysis of tripeptides *en bloc*, a unique characteristic among S53 family members, is related to occluding interactions of the putative S_4 subsite as suggested by the crystal structure [8,10]. TPP-I specificity was elucidated using synthetic peptide libraries, which indicated preference for cleavage between hydrophobic residues at P_1 and P_1' and for positively charged residues at P_3 (Schechter and Berger nomenclature [32]) [33,34].

Efficient and convenient substrates for TPP-I are still required for its detection, quantitation and analysis of hydrolytic properties. In the present paper, based on previous detailed substrate specificity of TPP-I [34], we report the synthesis of fluorescence resonance energy transfer (FRET) peptides with a free N-terminus amino group and containing tryptophan (W) in the P_2 position as the fluorescence donor and Q-EDDnp (glutaminyl-[*N*-(2,4-dinitrophenyl)-ethylenediamine]) as fluorescence quencher. We found the most efficiently hydrolyzed peptide so far obtained for TPP-I, NH_2-RWFFI-Q-EDDnp, with a free N-terminus amino group. This substrate was used for a pH-profile activity analysis in H_2O and D_2O, solvent kinetic isotope effects (SKIEs) with proton inventory and detection of TTP-I in crude extracts of rat spleen and kidney. For comparison, similar experiments were done using as the standard commercial peptide AAF-MCA substrate. The weak endopeptidase properties for TPP-I were also studied and this activity was better detected with FRET peptide substrate MCA-KLFFSKQ-EDDnp, which was used to evaluate pH rate profiles and SKIEs with proton inventory activities of TPP-I.

2. Materials and methods

2.1. Materials

Anhydrous dimethyl sulfoxide (DMSO), heavy water with 99.9% deuterium content were from Sigma. All buffer salts were reagent-grade and purchased from Fisher Scientific (Pittsburgh, PA) or Sigma.

2.2. Peptides

FRET peptides containing tryptophan (W) or MCA were synthesized by solid-phase synthesis [35,36]. An automated bench-top simultaneous multiple solid-phase peptide synthesizer (PSSM 8 system, Shimadzu, Japan) was used to synthesize peptides using the Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) procedure. The molecular weight and purity (94% or higher) of synthesized peptides were confirmed by amino acid analysis and MALDI-TOF mass spectrometry using a Microflex—LT mass spectrometer (Bruker e Daltonics, Billerica, MA, USA). Stock solutions of peptides were prepared in DMSO and the concentration was measured spectrophotometrically using a molar extinction coefficient of 17.300 $M^{-1}\,cm^{-1}$ at 365 nm from EDDnp.

2.3. Solutions

Buffer solutions for the proton inventory studies were prepared gravimetrically by mixing appropriate quantities of buffers made in H_2O or D_2O at the required pH or pD. The pD of deuterium oxide solution was obtained from pH meter readings according to the relationship pD = pH (meter reading) + 0.4 [37]. The fraction of deuterium in each buffer (n) was calculated accounting for density, mass of the enzyme stock solution, and presence of buffer salts.

2.4. Enzyme

Recombinant TPP-I was obtained as previously described [33]. Cathepsin D from bovine spleen was purchased from Sigma (C3138).

2.5. Determination of the substrate cleavage sites

The scissile bonds of hydrolyzed FRET peptides were identified by isolation of the fragments using analytical HPLC and the molecular mass of each product was determined by MALDI-TOF and by LC/MS using an LCMS-2020 system equipped with the ESI-probe (Shimadzu, Japan). Analytical HPLC conditions utilized an Ultrasphere C18 column (5 μM, 4.6 × 250 mm) eluted with the solvent system A (water/TFA, 1:1000) and B (ACN/water/TFA 900:100:1) at a flow rate of 1 mL/min and a 0–80% gradient for 20 min monitored by absorbance at 220 nm.

2.6. Edman degradation peptide library analysis

To determine the primed side cleavage specificity, a 100 μM solution of MCA-GXXFXXQ-EDDnp was incubated with TPP-I as previously described [38] and the N-terminal amino acid sequence was determined by Edman degradation using a PPSQ-23 Model Protein Sequencer (Shimadzu, Tokyo, Japan). Amino acid preference in a given cycle was calculated by dividing the amount of a particular residue by the amount of prevalent amino acid residue in that cycle. The data were then corrected for bias present in the library by dividing each value by the relative amount of that particular amino acid in the starting mixture.

2.7. Kinetic parameter determination

Pseudo first-order rate constants were measured at [S]«K_M and calculated by nonlinear regression data analysis using the GraFit software version 5.0 (Erithacus Software, Horley, Surrey, United Kingdom). The specificity rate constants (k_{cat}/K_M) were obtained by dividing the first-order rate constant by the enzyme concentration present in the reaction mixture. The kinetic parameters, k_{cat} and K_M, were determined from the initial rate measurements at 8–10 substrate concentrations between 0.15 and 15 K_M. The enzyme