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Kinetics and conformational stability studies of recombinant leucine aminopeptidase

Ana V. Hernández-Moreno[a], Francisco Villaseñor[b], Emilio Medina-Rivero[c], Néstor O. Pérez[c], Luis F. Flores-Ortiz[c], Gloria Saab-Rincón[d,**], Gabriel Luna-Bárcenas[a,*]

[a] Polymer and Biopolymer Research Group, Cinvestav Querétaro, QRO, Mexico
[b] Department of Biochemical Engineering, Instituto Tecnológico de Celaya, GTO, Mexico
[c] Research & Development Group, PROBIOMED S.A. de C.V., Edo de México, Mexico
[d] Departamento de Ingeniería Celular y Biocatálisis, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, MOR, Mexico

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ABSTRACT

Leucine aminopeptidase from *Vibrio proteolyticus* is a broad specificity N-terminal aminopeptidase that is widely used in pharmaceutical processes where the removal of N-terminal residues in recombinant proteins is required. We previously reported the expression of a heterologous construction of the mature protein fused to a 6-histidine tag that presents a reasonable refolding rate for its use at industrial level. Here, we investigate this recombinant leucine aminopeptidase (*r*LAP) to explain the gain of activity observed when incubated at 37 °C after its production. Unfolding transitions of *r*LAP as a function of urea concentration were monitored by circular dichroism (CD) and fluorescence (FL) spectroscopy exhibiting single transitions by both techniques. Free energy change for unfolding measured by CD and FL spectroscopy are 2.8 ± 0.4 and 3.7 ± 0.4 kcal mol^{-1}, respectively. Thermal stability conformation of *r*LAP is 2.6 ± 0.1 and 6.1 kcal mol^{-1} for CD and Nano-Differential Scanning Calorimetry (Nano-DSC), respectively. Enzyme activity was assessed with L-leucine-*p*-nitroanilide (L-*p*NA) as substrate. The catalytic efficiency was 3.87 ± 0.10 min^{-1} μM^{-1} at 37 °C and pH 8.0. Kinetic and conformation studies show differences between the enzyme native and *r*LAP; however *r*LAP is selective and specific to remove N-terminal groups from amino acids.

1. Introduction

Aminopeptidases have important roles in living systems and applications in biopharmaceutical industrial processes [1]. These peptidases are widely distributed in bacteria, yeast, plant, and animal tissues [2]. Their functions include protein maturation, protein degradation, hormone level regulation, and cell-cycle control. These proteins cleave amino acid residues from the N-terminal of polypeptides [3] resulting in a posttranslational modification that is involved in directed degradation and cellular targeting processes [4].

Leucine aminopeptidase belongs to a group of exopeptidases, hydrolases and metalloenzymes [2]. This enzyme prefers substrates with hydrophobic residues in their N-terminal [5]. The protein is synthesized as a precursor peptide that after a maturation process yields a monomeric enzyme of 29.5 kDa [6], containing two Zn(II) ions per mole [7]. This protein has an active dinuclear site, usually formed by zinc, but can also be activated by cobalt, nickel, and copper [8]; it also presents heteronuclear co-catalytic sites [9,10]. The thermal stability of the mature enzyme has been estimated at 70 °C for a minimum of 5 h [4,6]. This enzyme is inhibited by hydroxamates of amino acids [11], L-leucinethiol [12], peptide derived thiols [13], 1-phenyl-2 thiourea, urea [14], and aliphatic as well as aromatic alcohols [15].

Recombinant leucine aminopeptidase (*r*LAP) is used industrially for N-terminal methionine excision of recombinant human proteins like interferon alpha-2b, growth hormone and granulocyte macrophage-colony stimulating factor. The *r*LAP production process is devoted to reduce production costs yet keeping similar functions as the enzyme, leucine aminopeptidase from *Vibrio proteolyticus* (AVP), formerly classified as *Aeromonas proteolytica* [16].

The normal production of leucine aminopeptidase involves the heterologous expression of the propeptide and ulterior treatment with proteases to yield the mature active enzyme with the consequent increase in process cost and the potential contamination with

* Corresponding author at: Libramiento Norponiente No. 2000, Fracc. Real de Juriquilla, Queretaro, QRO 76230, Mexico. Tel.: +52 442 211 9908; fax: +52 442 211 9938.
** Corresponding author at: Av. Universidad #2001, Col. Chamilpa, Cuernavaca, MOR 62210, Mexico. Tel.: +52 777 329 1640; fax: +52 777 317 2388.
E-mail addresses: gsaab@ibt.unam.mx (G. Saab-Rincón), gluna@qro.cinvestav.mx, glunascf@yahoo.com (G. Luna-Bárcenas).

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other proteases [17]. Previous attempts to express heterologously the mature protein have yielded protein in inclusion bodies that could not be solubilized [17]. In a previous report by Pérez-Sánchez et al. [18], the gene coding for the mature protein is fused to a 6-histidine tag for its purification. Albeit most of the protein precipitates in inclusion bodies, it can be solubilized and refolded to a low activity conformation whose activity increases upon incubation at 37 °C.

The aim of this work is to understand the relationships between the structure and activity of the previously reported heterologously expressed mature-active leucine aminopeptidase from *V. proteolyticus* fused to a 6-histidine tag at its N-terminus [18], which we will call *r*LAP from now on. The protein is obtained heterologously in *Escherichia coli* as inclusion bodies and refolded from there. Here, we investigated the thermodynamic stability of *r*LAP by circular dichroism (CD) and fluorescence (FL) using urea as denaturant, thermal unfolding by CD, Nano-Differential Scanning Calorimetry (Nano-DSC) and intrinsic fluorescence. The folding process of *r*LAP was monitored by intrinsic fluorescence to understand structural changes associated with the enzyme function when it is incubated to 37 °C for 72 h at pH 8.0. Additionally, it was determined the kinetic behavior of recombinant protein using leucine-*p*-nitroanilide (L-*p*NA) as substrate. Enzyme stability at 37 °C and pH 8.0 was determined, since these are the conditions at which the enzyme is used for interferon alpha-2b processing, albeit these are not its optimal conditions.

2. Materials and methods

2.1. Expression and purification of rLAP

The recombinant leucine aminopeptidase was expressed and purified as described previously [18]. Briefly, the coding region for the mature 32 kDa leucine aminopeptidase from *V. proteolyticus* was synthesized optimizing the codon usage for *E. coli* and adding the codons for a 6-histidine tag at the 5′ end. The resulting gene was cloned in *E. coli* and expressed as inclusion bodies after thermoinduction. Inclusion bodies were extracted and solubilized with an 8 M urea solution and purified by immobilized metal ion affinity chromatography (IMAC). The purified protein was dialyzed against 10 mM tricine buffer and 1 mM of $ZnCl_2$, pH 8.0, and subsequently lyophilized [18].

2.2. CD spectra of rLAP

The circular dichroism spectra of *r*LAP and AVP were recorded at 25 °C from 260 to 190 nm using an 8 s averaging time and 0.1 nm step size, and an average of eight scans was recorded on a Jasco J-715 spectropolarimeter™ equipped with a Peltier temperature control using a 0.1 cm path length cell. Sample for spectropolarimetry was equilibrated with 10 mM phosphate buffer, pH 8.0 and 0.5 mM of $ZnCl_2$, by dialysis. The concentrations of enzymes were of 17 and 18 μM to *r*LAP and native, respectively.

2.3. Equilibrium unfolding of rLAP by urea

The unfolding of *r*LAP was induced by adding urea as denaturing agent. Previously to the experiments, the protein was dialyzed against 4 changes of 10 mM phosphate buffer, 0.5 mM $ZnCl_2$ at pH 8.0 and its concentration was measured by UV-absorbance at 278 nm in a Cary-14 spectrophotometer™ using an extinction coefficient of 38 700 $M^{-1}\,cm^{-1}$. Samples were prepared to a final protein concentration of 18 μM by diluting 50 μL of a stock protein solution (305 μM) into 950 μL of buffer containing various concentrations of urea (0–8 M). Unfolding experiments were carried out at a temperature of 25 °C in a 10 mM sodium phosphate buffer at pH 8.0. The samples were equilibrated at 25 °C for 1 h prior to collecting data to assure that equilibrium was reached. Ultrapure, biological-grade urea solutions were, prepared fresh for each test, from Fluka-Biochemika (99.5% of purity). CD spectra were recorded from 260 to 190 nm with the same spectropolarimeter described in Section 2.2 for each concentration of urea-enzyme. To assure the reversibility of the reaction protein was equilibrated in an 8 M urea, 10 mM sodium phosphate solution at pH 8.0 for 1 h. Aliquots of the unfolded protein solution were diluted to different urea concentrations, maintaining the final protein concentration at 15 μM. After equilibrating for 1 h under the different refolding conditions, the CD spectra were recorded as described above.

To probe unfolding by fluorescence (FL), *r*LAP was dialyzed for 24 h against 4 changes of 10 mM phosphate buffer, 0.5 mM $ZnCl_2$ at pH 8.0, in dialysis cassettes of 10 MWCO. Samples with varying urea concentrations of *r*LAP were prepared in a similar fashion as for the CD experiments. Total intensity fluorescence spectra were recorded on a Fluoromax-3 spectrofluorometer equipped with Czerny-Turner monochromators for excitation and emission using 1 cm path length cuvettes. Excitation was carried out at 280 nm and the emission from 300 to 500 nm was recorded. The temperature was maintained at 25 °C using a circulator water bath (Thermo Scientific NESLAB RTE 740™). Initial protein concentration was measured at 278 nm (absorbance) on a GE 65000 spectrophotometer™.

The CD and FL data were fitted using Savuka version 5.12 [19] and an in-house nonlinear least-squares program [20]. Wavelength of maximal emission ($\lambda_{max\text{-}emission}$) and ellipticity of unfolding curves were fit to two-state model F ↔ U, where F and U represent the folded and unfolded states, respectively.

2.4. Thermal unfolding of rLAP by CD

The thermal unfolding and refolding of *r*LAP were followed by CD at 190–290 nm from 5 to 90 °C and then reversibility was induced by cooling from 90 to 5 °C immediately after the heating phase. Unfolding and refolding were performed at a scan rate of 1 °C min^{-1}. Protein concentration was set to 46 μM. The test was measured using 0.1 nm step size on a Jasco J-815 spectropolarimeter™ equipped with a Peltier temperature control in a 0.1 mm path length cell, the bandwidth was 1 nm and 2 scans were accumulated. For setting and determination of thermodynamic parameters it was considered the change of the mean residue ellipticity measured at 220 nm as a function of temperature.

2.5. Nano-Differential Scanning Calorimeter (Nano-DSC)

Calorimetric analysis was performed in a Nano-Differential Scanning Calorimeter TA model 6300™. Prior to analysis, the samples were degassed under vacuum for 15 min while stirring at 15 °C. The concentration of *r*LAP was 45.7 μM. Samples were heated at a scan rate of 1 °C min^{-1} from 10 to 100 °C under a 3 atm pressure. 300 μL of each degassed *r*LAP sample plus 10 mM phosphate buffer (reference) at pH 8.0 were loaded into the capillary cells. The enthalpies of the transitions (ΔH_{cal}) were estimated by calculating the area under the thermal transition after subtracting the blank and fitting a baseline using Nano Analyzer™ Software.

2.6. Thermal unfolding of rLAP by intrinsic fluorescence

The heat-induced unfolding of *r*LAP was observed by the intrinsic fluorescence of 5–90 °C at a scan rate of 1 °C min^{-1}. The lyophilized enzyme was resuspended at a concentration of 6 μM and equilibrated in 10 mM phosphate buffer solution, pH 8.0 and 0.5 mM $ZnCl_2$. 200 μL of protein solution was placed in a quartz cells of 3 mm. Fluorescence spectra were monitored with a