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Structural and functional characterization of a recombinant leucine aminopeptidase

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ABSTRACT

The function of proteins, such as the catalytic enzyme activity, depends on the interaction of their active sites with their specific substrates and the environment conditions that affect the stability of those sites. This study presents a structure-to-function characterization of the folding process of a recombinant 6×-His tag leucine aminopeptidase (*r*LAP) based on a platform of analytical techniques. The results demonstrated an increase up to 31 U/mg in the activity of the enzyme after folding as revealed by circular dichroism, intrinsic fluorescence, differential scanning calorimetry, and free thiol analysis. Collectively, these techniques revealed a larger number of covalent and non-covalent bonds within the protein seen as an increase in the chemical and thermal stability, while exhibited a lower level of non-bonded cysteines after the protein was folded. Mass spectrometry analysis showed the maintenance of the distribution of the enzyme isoforms related to N-terminal histidine residues after folding, which confirmed that the enzymatic activity of *r*LAP depends on its three-dimensional structure rather than N-terminal self-processing activity. In summary, the studied attributes allow a better understanding of the structure-to-function relationship of *r*LAP, that permit a more proficient manufacturing of the enzyme that would improve the bioprocesses in which is employed.

1. Introduction

DNA technology has been widely used for the production of recombinant proteins such as therapeutic proteins and enzymes. Currently, the production of recombinant proteins in prokaryotic hostess is an advantageous process because allows yielding higher amounts of product [1,2]; nevertheless, the expression of recombinant proteins in *Escherichia coli*, and the purification of insoluble inclusion bodies represent major challenges in determining the correct structure of the recombinant proteins that ensure their biological activity [3–6].

The study of the physical attributes of proteins, and their effects on their biological function, is an important asset in the establishment of the processes and conditions to obtain the appropriate protein structure [7,8]; in addition, is also important to determine the optimal conditions in which these proteins will be preserved and used under specific environments [9–11]. Additionally, identifying the principles of protein folding allows understanding the forces that drive and/or preclude this phenomenon [12,13].

The gaining of knowledge about the relationship between structure, function, and the environment conditions; depends on the study of variables that affect folding, such as: protein concentration, pH, temperature, and ionic strength [14–16]. So, adjustments on the conditions of the protein environment can be planned toward the improvement of its manufacturing bioprocesses. On this regard, enzymes are ideal model proteins, because they act on specific substrates that can be employed to measure their biological activity, and thus investigate the impact of environmental variables on their functionality.

Recombinant leucine aminopeptidase (*r*LAP) is an enzyme commonly used by the biopharmaceutical industry for the remotion of N-terminal methionine [17–21]. The mature 6×-His tag *r*LAP under study is a non-glycosilated monomeric protein of 304 amino acids, with an apparent averaged mass of 32.8 kDa (by reducing SDS-PAGE) [17]. This enzyme exhibits a characteristic secondary structure, as reported by circular dichroism [22]; furthermore, as

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in the wild type leucine aminopeptidase (LAP_{wt}) [19,23], *r*LAP has only one disulfide bond in its mature form which makes easier to study its folding.

In a previous study, we reported an increase in the enzymatic activity of *r*LAP after an incubation period that was conceived as the best for folding [22]. Herein, we focus on the structural changes of *r*LAP and the repercussion on its functional attributes headed to the improvement of bioprocesses.

To achieve this objective, a set of sensitive techniques capable to detect conformational changes in proteins was employed, such as: circular dichroism (CD), intrinsic fluorescence (IF), differential scanning calorimetry (DSC), and mass spectrometry (MS), along with colorimetric free thiol analysis, and enzymatic activity assays that helped to track the changes of *r*LAP subjected to folding and/or induced denaturation. Collectively, these techniques provided a quick and reliable platform to assess structural and functional changes in the protein associated to predetermined variations on its environment.

2. Materials and methods

2.1. Materials

Wild type leucine aminopeptidase from *Vibrio proteolyticus*, Leucine-*p*-nitronilide (L-*p*NA), zinc chloride ($ZnCl_2$), DL-dithiotreitol, tricine, acetonitrile (LC–MS grade), formic acid (LC–MS grade), and water (LC–MS grade) were obtained from Sigma–Aldrich (St. Louis, MO); citric acid, sodium citrate, mono-dibasic sodium phosphate, sodium carbonate, and sodium bicarbonate from J. T. Baker (Center Valley, PA); Urea from Millipore Corp. (Billerica, MA); DyLight 488 Maleimide and Slyde-A-Lyzer 10 kDa MWCO Cassettes from Thermo Fisher Scientific Inc. (Waltham, MA); and Recombinant leucine aminopeptidase (rLAP) in-house produced.

2.2. Activity assay

Enzymatic activity was determined by hydrolysis of L-*p*NA. The assays were conducted by triplicate for each sample of *r*LAP in a final volume of 480 μL; 0.5 mM L-*p*NA and 3×10^{-8} M of purified enzyme were incubated in 10 mM tricine, 1 mM $ZnCl_2$ buffer at pH 8.0. A negative control without enzyme was used to account for non-enzymatic hydrolysis of the substrate. The reactions were incubated at 25 °C for 5 min, and then 20 μL of 20 mM DL-dithiotreitol were added to quench the reaction. Absorbance from *p*-nitroaniline at 405 nm (ε = 10,800 M^{-1} cm^{-1}) [24], and from *r*LAP (ε = 38,700 M^{-1} cm^{-1}) was measured in a Beckman Coulter DU 640 spectrophotometer (Beckman Coulter Inc.; Brea, CA). One enzyme unit (U) was defined as the amount of enzyme that released 1 μmol of *p*-nitroaniline at 25 °C in 1 min.

2.3. Folding conditions

3 μM *r*LAP (in the folding state in which was purified from the inclusion bodies, referred as unfolded) was incubated at 37 °C in 10 mM phosphate, 10 μM $ZnCl_2$ buffer at pH 6.0 for 72 h (main folding conditions). The samples were collected every 12 h to track the enzyme activity. Three different combinations of *r*LAP and $ZnCl_2$ concentrations were also evaluated to observe their effect on the folding process: (1) 30 μM and 20 μM, (2) 152 μM and 50 μM, and (3) 305 μM and 100 μM respectively. After 72 h of folding, the protein was dialyzed in Slyde-A-Lyzer 10 kDa MWCO cassettes, in 10 mM tricine, 1 mM $ZnCl_2$ buffer pH 8.0, at 4 °C, for 24 h; the protein obtained from this step was referred as $rLAP_{fd}$ (folded *r*LAP). Enzyme no subjected to folding was dialyzed under the same conditions prior to be analyzed and was referred as $rLAP_{uf}$ (unfolded *r*LAP). For the mass spectroscopy analysis the enzyme was incubated at 15 °C under the main folding conditions, and was analyzed every 24 h.

2.4. Circular dichroism (CD)

The analysis was conducted at 15 μM of enzyme in the far UV CD region (190–270 nm) at 25 °C using a 0.1 cm path length cell in a Jasco J-815 spectropolarimeter (Jasco Corporation Ltd.; Tokyo, Japan). Three scans per test were accumulated at 1 nm bandwidth.

2.5. Intrinsic fluorescence (IF)

The analysis was conducted at 3 μM of enzyme in 3 mm quartz cells. Fluorescence spectrum was monitored from 290 to 400 nm at 25 °C, using an excitation wavelength of 283 nm in a Fluorolog-3 spectrofluorometer (Horiba Ltd.; Tokyo, Japan). The wavelength of maximal emission (λ_{max}) was defined as the point of maximal intensity from the fluorescence spectra.

2.6. Thermal analysis by IF

IF was monitored in a range from 5 to 90 °C, at a scanning rate of 1 °C min^{-1} using the same instrument and settings mentioned above.

2.7. Fluorescence lifetime using time correlated single photon counting (TCSPC)

Fluorescence lifetime analysis by the Time-Correlated Single Photon Counting method (TCSPC) was performed at 25 °C using 332 nm as emission wavelength, 2 nm band-pass, and a 280 nm ± 10 Nanoled as excitation source. Data analysis was carried out with the DAS6 software (Horiba Ltd.; Tokyo, Japan) using two exponential decay components. The intensity decays were calculated as the sum of exponentials using the Eq. (1) [25].

$$I(t) = \sum_i \alpha_i \exp\left(\frac{-t}{\tau_i}\right) \quad (1)$$

where $\Sigma\alpha_i$ is normalized to the unit, α_i is the pre-exponential factor, and τ_i is the lifetime. The lifetime average (τ) was calculated using the Eq. (2) [26].

$$\langle\tau\rangle = \frac{\sum_{i=1}\alpha_i\tau_i^2}{\sum_{i=1}\alpha_i\tau_i} \quad (2)$$

2.8. Chemical denaturation observed by IF and TCSPC

Solutions of 6 μM *r*LAP were prepared at different concentrations of urea (0–8 M) in 10 mM tricine, 1 mM $ZnCl_2$ buffer at pH 8.0. Urea-induced unfolding was analyzed by IF and TCSPC at 25 °C as described in the methodology of those techniques.

2.9. Chemical denaturation evaluated by enzymatic activity

The urea-induced unfolding of 6 μM *r*LAP was measured by its specific enzymatic activity at different urea concentrations (from 0 to 8 M) in 10 mM tricine, 1 mM $ZnCl_2$ buffer at pH 8.0 at 25 °C. Reversibility of the chemical denaturation was evaluated by subjecting the enzyme to decreasing urea dilutions from 8 M to 0.4 M.

2.10. Differential scanning calorimetry (DSC)

Transition temperatures (T_m) and enthalpies (ΔH_{cal}) were measured at 46 μM of *r*LAP using a nano-DSC system from TA