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Unfolding of chondroitinase ABC I is dependent on thermodynamic driving force by kinetically rate constant-amplitude compensation: A stopped-flow fluorescence study

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ABSTRACT

We had previously investigated the role of a loop on the activity and conformational stability of chondroitinase ABC I (cABC I) by constructing some representative mutants in which a network interaction around Asp^{689} was manipulated. Here we extended our study by measuring the proteolytic resistance, long term and thermal stability as well as unfolding kinetics of these variants. Long term stability data at 4 and 25 °C for 3 weeks indicates that all mutants remain considerably active at 4 °C. Thermoinactivation rates for all variants shows that the wild type (WT) enzyme retained 50% of its activity after 2 min keeping at 40 °C, while L701T, H700N and H700N/L701T as conformationally stabilized variants, have slower inactivation rate. It was also found that compact and thermodynamically stabilized variants are more resistant to tryptolytic digestion. Also, kinetic curves of chemical unfolding of the enzyme variants from stopped-flow fluorescence measurements were best fitted into a three-exponential function with three rate constants and corresponding amplitudes. We found that the energy barrier of the fast unfolding phase is lower in stabilized variants; while the amplitude of this phase to the whole amplitude of the unfolding reaction is lower than that of destabilized variants, indicating more population of stabilized mutants unfold *via* slower unfolding phase. We concluded that the rate of local conformational change alone is not the same that is expected from global thermodynamic stability; however the corresponding amplitude can compensate the rate constant toward thermodynamic stability.

1. Introduction

In unfolding reaction of proteins, the structure of transition state is characterized by having different parts of its structure identical to the structural elements in the folded protein; however, some particular set of interactions differ from those of folded state according to the pathway of reaction [1,2]. In other words, native state switches to transitions state by only breaking and formation of the non-covalent interactions of some residues that are essential to lose the stabilizing contacts of the native structure. Using this assumption in statistical thermodynamics, if the whole population of protein molecules is exposed to different schemes of breaking and formation of non-covalent interactions it is said that this reaction have different pathways or different transition states [3,4]. Energetically; the process of formation the transition state is the rate limiting step in the unfolding reaction, i.e., it is the conformation that has the highest Gibbs free energy on the unfolding reaction of proteins [5]. One of the most important techniques in protein science for determining the role of residues in the structure of transition states during unfolding or refolding reaction includes using mutant proteins and measuring the changes in the rate constants and energy barrier of reaction between the WT and mutant proteins [6–9]. The site of mutation is used for probing the local conformational changes in the structure of transition state in the early stages of reaction. These studies are performed by rapid-mixing experiments which are able even to monitor two-stage collapsing

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kinetics of the coil-to-globule transition as well as microsecond refolding and unfolding kinetics in small biomolecules [10,11].

Chondroitinase ABC I (pdb code: 1HN0) is an endolytic enzyme responsible for metabolism of glycosaminoglycan *via* β-elimination of proteoglycans and release of O-linked glycosaminoglycans [12]. Chondroitin sulfate proteoglycans (CSPGs) are inhibitory extracellular matrix molecules that are upregulated after CNS injury. It was shown that degradation of CSPGs by chondroitinase ABC I can promote recovery of spinal cord injury [13–15]. Crystal structure of cABC I shows that it has three domains; among them the central or catalytic domain contains active site of enzyme [16].

We had previously investigated the stability of cABC I using different solvents and enzyme variants [17,18] and recently; we have made some mutants of cABC I in which the primary intension of making the mutants was to study the contribution of a network interaction around Asp^{689} on the conformational stability and activity of cABC I [19]. This residue is located in a long length loop at C-terminal domain of the enzyme. According to thermodynamic studies; we found that the H700N, L701T and H700N/L701T mutants are structurally stable relative to WT protein; however, their catalytic efficiency is lower than that of WT enzyme. We concluded that this loop in C-terminal domain is indirectly involved in substrate binding by affecting the open conformation of the active site and accessibility of substrate, so that increasing the flexibility of protein by weakening the aforementioned interactions results in improvement of catalytic efficiency of the enzyme [19]. The aim of this study is to determine the effect of this network interaction on the long term and thermal stability as well as proteolytic resistance. The correlation between thermodynamic parameters with those of unfolding kinetics of chondroitinase ABC I was also determined by stopped-flow fluorescence measurements of unfolding kinetics.

2. Material and methods

2.1. Solutions and samples

All buffers used in this study were prepared with two-distilled water. Isopropil-beta-D-thiogalactopyranoside (IPTG) and urea were purchased from Sigma-Aldrich. Stock solution of urea (10 M) was prepared according to the protocol adapted from the method described by Pace [20].

2.2. Site-directed mutagenesis studies

Quik-Change method was used for site-directed mutagenesis to produce mutants of cABC I as previously described [19].

2.3. Expression and purification of cABC I and mutants

Recombinant cABC I and mutants were expressed in *E. coli* BL21 and purified as previously described [18]. The purity of proteins was confirmed by observing a single band on SDS-PAGE and protein concentration was determined by the Bradford method [21].

2.4. Enzyme activity measurements

The activity of cABC I and its mutants was assessed in 50 mM potassium phosphate buffer, pH 6.8 at 25 °C using Chondroitin 4-sulfate (C4S) with chain length of ~50 kDa as substrate. The activity of enzyme was measured by recording the increase in absorbance at 232 nm. This is because that it catalyzes the cleavage of C4S and leads to the formation of double bond-containing products which act as chromophore at 232 nm with absorption coefficient (ε) of 3800 M^{-1} cm^{-1} [19].

2.5. Unfolding kinetics

A Biologic μ-SFM-20 fluorescence detected stopped-flow, equipped with a 0.8 cm cuvette (FC-08) was used for kinetic measurements (excitation 290 nm, emission 320 nm; both excitation and emission slits were set to 5 nm). The fluorescence emission was measured at a 90° angle relative to the excitation light and the typical dead time of the instrument was 2.6 ms. In unfolding studies, one volume of folded protein in 50 mM phosphate buffer and pH 6.8 was mixed with 3 vol of concentrated urea (10 M) in the same buffer. This result in final concentration of urea equals to 7.5 M. Data collected from at least 4 scans and analyzed using Bio-kine analysis software (Bio-kine 32 V4.49-1).

Kinetic traces were analyzed by fitting into an exponential function as Eq. (1):

$$Y = at + b\sum_{i}^{N} A_i \exp(-k_i t) \quad (1)$$

In which, a is the slope and b is the offset of kinetic curve corresponding to the baseline, A_i and k_i are the amplitude and rate constant, respectively. According to the biological concept and the accuracy of fitting, kinetic traces were fitted into a three-exponential function by setting N = 3 in Eq. (1).

The free energies of transition states were calculated from the transition-state theory using Eq. (2):

$$k = (k_B T/h)\exp(-\Delta G^{\ddagger}/RT) \quad (2)$$

Where k is the unfolding rate constant, k_B the Boltzmann constant, h the Planck constant and $\Delta G^{\ddagger}$ is the free energy of activation or the difference in energy between the transition and ground states [22].

2.6. Determination of long term and thermal stability

To determine the long term stability of cABC I and its mutants, remaining activity of the enzymes was evaluated at 4 and 25 °C for 3 weeks. Thermal inactivation rate (k_i) of these variants was measured at 25, 30, 37, 40 and 42 °C at a period of 1–30 min, if possible. In all experiments of both long term and thermal stability, aliquots of purified enzymes in 50 mM phosphate buffer, pH 6.8 were first incubated at specified temperature. Samples then were snap-cooled on ice and their residual activity was measured after 30 min [23]. The thermal stability of enzyme was quantified by measuring the half-life ($t_{1/2}$) of the enzyme activity. The final concentration of the enzyme in all measurements was 1 μM. Each experiment was carried out at least three times and data were reported as mean ± standard deviation for triplicate samples.

2.7. Calculation of inactivation thermodynamic parameters

The rate constant of inactivation (k_i) is used to calculate the activation energy according to the Arrhenius equation:

$$k_i = A\exp(-E_a/RT) \quad (3)$$

Where k (s^{-1}) is the inactivation rate constant at temperature T (K), A as a pre-exponential factor related to steric effects and the molecular collision frequency, R the gas constant (8.314 J mol^{-1} K^{-1}) and E_a the activation energy of the reaction. Hence, a plot of ln k versus 1/T gives a straight line with the slope equals to $-E_a/R$. The thermodynamic parameters of the inactivation measurements were determined as follows:

$$\Delta G^{\#} = (RT\ln k_B T/h) - (RT\ln k) \quad (4)$$

$$\Delta H^{\#} = E_a - RT \quad (5)$$

$$\Delta S^{\#} = (\Delta H^{\#} - \Delta G^{\#})/T \quad (6)$$