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NΔ89 and CΔ274 Truncated Enzymes of Chondroitinase ABC I Regain More Imperturbable Microenvironments Around Structural Components in Comparison to their Wild Type

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Abstract

Immune response stimulation and inactivation of chondroitinase ABC I in physiological condition have been limited its use in various clinical conditions as a bacterial enzyme drug. In the present study, we have investigated some structural and functional features of NΔ89, CΔ274 and NΔ89CΔ274; three designed truncated cABC I, in order to clarify the unclear role of two terminal parts of cABC I i.e., the 1–89 and 747–1021 amino acids sequences of the full length enzyme through truncation. As a result, the numbers of potential epitopes, the susceptibility to trypsin digestion, ANS fluorescence spectra, and fluorescence quenching using KI and acrylamide were diminished for NΔ89 and CΔ274 in comparison to the wild type. Secondary and tertiary structure investigation for NΔ89 and CΔ274 revealed that the intrinsic fluorescence was increased and Far-UV CD spectra were changed accordingly. Relative to the wild type enzyme, 0.164, 0.195 remaining activity and lack of activity was shown with the zymographic assay for NΔ89, CΔ274 and NΔ89CΔ274 variants, respectively. The diminished enzyme activity and structural changes suggested a reorientation of microenvironments interactions including cation–π interactions around structural elements toward lowering regional mobility. Constructing applicable truncated cABC I with improved features could be regarded as a strategy to regain new possible functional advantages over the full length enzyme.

Keywords Truncated cABC I · Zymographic assay · Fluorescence quenching · Protein structural elements

Abbreviations

cABC I	Chondroitinase ABC I
cAC	Chondroitinase AC
PIC	Protein interaction calculator
LB	Luria–Bertani
IPTG	Isopropyl-ß-D-thiogalactopyranoside
PMSF	Phenylmethanesulfonyl fluoride
DAB	3,3′-Diaminobenzidine
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
ANS	8-Anilinonaphthalene-1-sulfonic acid

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1 Introduction

Potential promising effects of bacterial chondroitinase ABC I (cABC I; EC 4.2.2.4) in treatment of neurodegenerative disorders, cancers, amblyopia, cystic fibrosis disease, and a variety of other disorders have generally been linked with its ability in deceleration of overexpressed extracellular glycosaminoglycans following abnormal conditions [1–4]. However, degradation of injected cABC I as a drug into the targeted loci has stimulated the body immune response with secreting antidrug antibodies against its antigenic sites [5]. In addition, physiological inactivation of cABC I has motivated researchers to utilize several strategies including site direct mutagenesis with different approaches for improvement of cABC I catalytic efficiency [6–13].

Establishment of truncated enzymes could be considered as a protein engineering approach based on sustaining critical structure parts of the molecule to improve their activity and stability [14], minimizing the overall complexity of full length enzyme for specific target delivery, maximizing the effective concentration of truncated version over the full length [15], and removing potential T cell epitopes of full length enzyme as a protein-sequence modification approach [16, 17].

Closeness comparing, truncated versions of chondroitinase AC (cAC) have been reported to show different activities in comparison to its wild type as tested by zymographic assay. This might exhibit several advantages of the truncated enzyme in clinical usage over its full length variant as suggested, although no results have reported about the structure and stability of these variants [15, 18]. Chondroitinase ABC I with widely open substrate-binding domain is structurally comparable to cAC according to their sequence and structural topology similarity [19–21]. It has been shown that modification of loops located at N- and C-terminal domains of cABC I, results in facilitation of substrate accessibility to the enzyme catalytic machinery [8, 10]. Investigating the role of amino-acids using site direct mutagenesis through analysis of available crystal structure, molecular docking, superimposing and alignment of cABC I with its homologous domains have revealed the number of amino acids present in the active site of the enzyme and engagement of N- and C-terminal domains of cABC I in catalysis, although the exact function of the two terminal regions is still unclear and conceal behind their structural complexity [20–27]. In this study, three truncated variants of cABC I i.e., NΔ89 (1–89), CΔ274 (747–1021) and NΔ89CΔ274 (1–89 plus 747–1021) of cABC I with 1021 amino acids residue were designed in order to evaluate the deletion effect of these amino acids on the enzyme functionality.

2 Materials and Methods

2.1 Chemicals

IPTG was purchased from Bio Basic Inc. (Canada). Ni-NTA agarose was supplied by Qiagen (USA). Kanamycin, ANS and chondroitin 4-sulfate were obtained from Sigma-Aldrich (USA). All other chemicals were provided by Merck (Germany) and with analytical grade.

2.2 Bioinformatics and Gene Synthesis

Three-dimensional structure of wild type and truncated variants (NΔ89, CΔ274, and NΔ89CΔ274) of cABC I were designed and analyzed by I-TASSER protein structure and function prediction server and Jmol viewer software using cABC I PDB entry code (1HN0). Interaction within proteins and their immunogenicity were analyzed by protein interaction calculator (PIC server) and prediction antigenic peptides servers (http://imed.med.ucm.es/Tools/antigenic.pl), respectively. The gene encoding wild type and truncated (NΔ89, CΔ274, and NΔ89CΔ274) cABC I from *Proteus vulgaris* were synthesized by GeneCust Company (Luxembourg).

2.3 Gene Expression, Optimization and Western Blot Analysis

The pET-28 harboring wild-type, NΔ89, CΔ274 and NΔ89CΔ274 genes were transformed into the *E.coli* BL21 (DE3) competent cells, grown in LB containing kanamycin (50 µg/ml). Different concentrations of IPTG (0.1 and 0.7 mM), temperatures (16 and 27 °C), and time (6 and 16 h) were applied for induction and incubation to assess the level of expressed proteins. Harvested bacterial pellets were suspended in buffer A (50 mM potassium phosphate, 300 mM NaCl, 5 mM imidazole, and 1 mM PMSF; pH 7). After sonication and centrifugation, soluble enzymes in supernatants were added onto a nickel-affinity chromatography column for purification. Enzyme refolding was performed using precipitated inclusion bodies of all proteins [28]. After two wash with buffer A, the inclusion bodies were solubilized in 5 ml of buffer B (20 mM Tris–HCl, 6 M guanidine hydrochloride; pH 6.8) and centrifuged at 13,000×*g* for 21 min. Denatured enzymes were loaded onto a Ni-CAM column that was equilibrated with buffer B. Purified unfolded enzymes were renatured with one step dialysis against Tris buffer pH 6.8 for 24 h with several buffer change. Western blot was carried out with anti-His-tag mouse monoclonal Ab conjugated to HRP (1:1000) and DAB as a substrate to verify enzymes according to their attached N terminal his-tag and molecular weights.

2.4 Activity Analysis

The enzymatic reaction was monitored spectrophotometrically based on increased double bond formation as the product which absorbs light at 232 nm. Kinetic parameters were calculated as previously described [8]. In order to screen the activity of enzymes, zymographic assay was also conducted. In this experiment, 100 µg of each enzyme was loaded on SDS-PAGE having 2 mg/ml chondroitin 4-sulfate as substrate in the resolving gel. Gels were then rinsed with 2.5% Triton X100 for 1.5 h at RT. The zymographic assay buffer for incubation was 20 mM Tris, 100 mM $CaCl_2$, pH 7 for 16 h. Alcian blue (2.5%) as a binding dye to C4S was added to zymogram gels for 12 h and destained bands were analyzed by Image J software using wild type enzyme as control [29, 30].

2.5 Circular Dichroism

Secondary structure contents of purified enzymes (0.2 mg/ml in 20 mM Tris buffer, pH 6.8 at 25 °C) were recorded using an Aviv spectropolarimeter (model 215 USA). FAR-UV CD spectra were expressed as molar ellipticity (deg cm^2/dmol) using the equation: $[\theta]\lambda = (\theta \times 100\ MRW)/(cl)$ where MRW is mean amino acids residue weight of wild-type and truncated NΔ89, CΔ274, NΔ89CΔ274 cABC I enzymes separately, θ is the observed degree of ellipticity, c is the protein concentration (mg/ml) and l is the light path length (cm). To analyze and quantify the information content of far UV circular dichroism spectra and calculate the percentage of alpha helix and beta sheet contents of cABC I and truncated version of cABC I, CDNN 2.1 software was used [31].

2.6 Fluorescence Spectroscopy

Intrinsic fluorescence was examined with 20 µg protein in 20 mM Tris buffer, pH 6.8, at 25 °C. Extrinsic fluorescence spectra were assessed with 100 µg protein in 20 mM Tris buffer, and ANS (30 µM) as a probe at 25 °C. Intrinsic and extrinsic fluorescence spectra were recorded with the slit width of 10 nm from 300 to 400 nm and 400–600 nm after excitation at 280 nm and 380 nm using a Perkin Elmer luminescence spectrophotometer (L55 Germany), respectively. Potassium iodide and acryl amid quenching experiments were carried out with 20 µg of protein concentration in 20 mM Tris buffer pH 6.8 at 25 °C. Ksv As simplest case of Collisional quenching occurs when a molecule can facilitate non- radiative transitions to the ground state. Ksv value; The Stern–Volmer constant, was calculated according to the ratio of fluorescence intensity in the absence and presence of the quencher; F0/F, using the equation $F0/F = 1 + ksv[Q]$, in which Q is the molar concentration of the quencher.

2.7 Trypsinolysis Study

Trypsin digestion of proteins was performed with 0.2 mg/ml protein concentration in 20 mM Tris buffer containing 10 mM $CaCl_2$, pH 7.5 at 25 °C. After incubation of proteins with 0.2 µg/ml trypsin for 0, 25 and 45 min, PMSF; 1 mM final concentration, was added to the reaction mixture to stop the lysis enzyme action. Digestion pattern of proteins were visualized by SDS-PAGE.

3 Results

3.1 Bioinformatics

According to the available crystal structure, molecular docking and PIC server data, the area in cABC I that hold non-potential immunogenic amino acids (Fig. 1) are located in N-terminal (1–89) and C-terminal (747–1021) parts of the enzyme which were selected for truncation. Moreover, antigenic prediction results using "The Antigenic Index Server" and regarding antigenic index above 1.0, confirmed the above mentioned theoretical results (Fig. 3). Based on several available bioinformatics studies about crystal structure of cABC I, none of amino acids in the two selected terminal regions of enzyme in this study for truncation are still reported as a critical amino acids, therefore the reported functional elements of the cABC I are not removed upon truncation (Figs. 1, 2).

3.2 Enzymes Expression

Soluble forms of wild type and NΔ89 enzymes were obtained incubating 6 h induction of the cells with IPTG 0.7 mM at 27 °C and 16 h with 0.1 mM IPTG at 16 °C as inducing reagents, respectively. The CΔ274 and NΔ89CΔ274 variants were solubilized by refolding methods. In order to compare structural differences with their

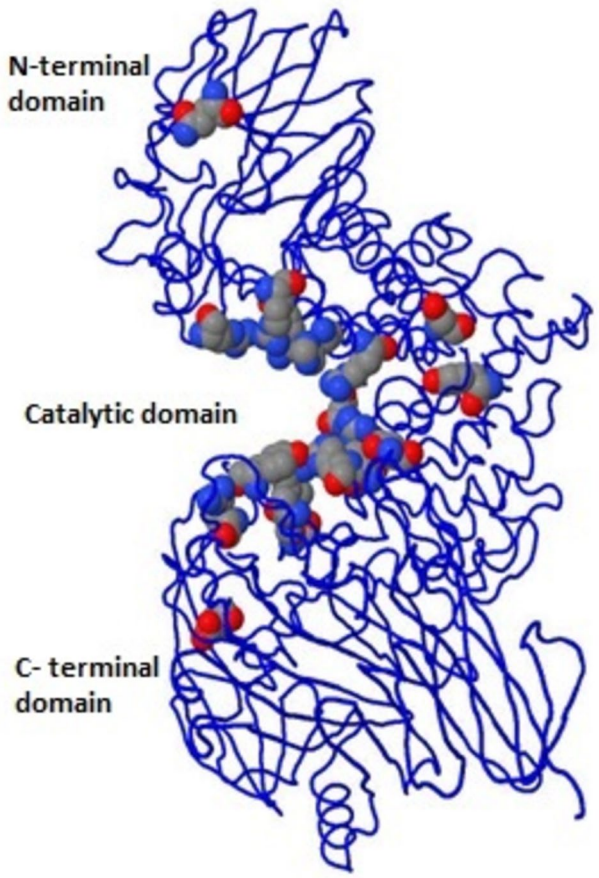

Fig. 1 View of critical amino acids in terms of catalysis and substrate binding site of cABC I e.g., Arg^{105} from N-terminal and His^{712} from C-terminal with CPK sapacefill style

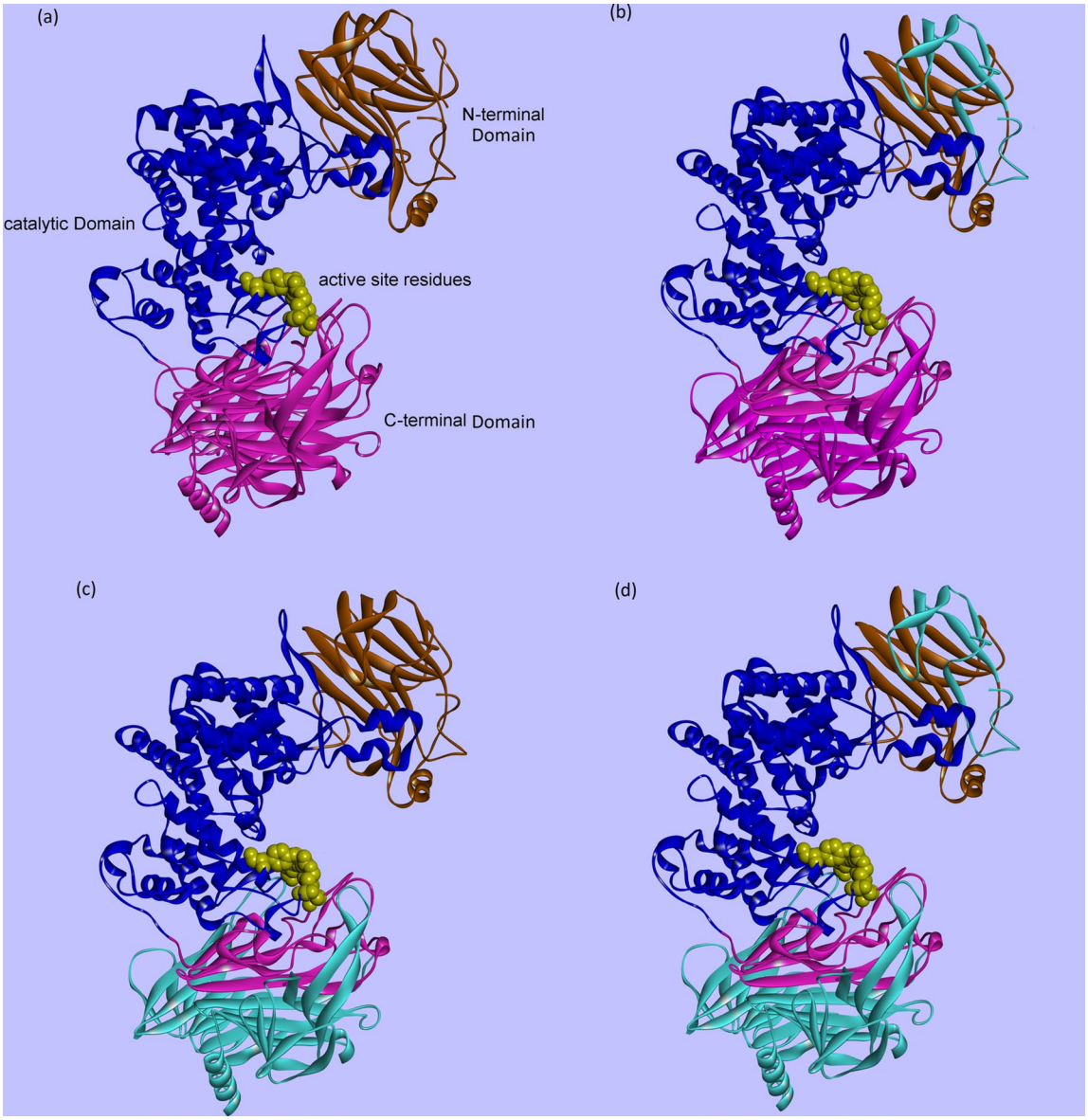

Fig. 2 3D view of **a** wild type cABC I containing three domains: N-terminal (1–234), catalytic domain (235–617) including active site, C-terminal (618–1021), **b** residue 1–89 selected amino acids of N-terminal with cyan color for NΔ89 truncated form, **c** residue 747–1021 selected amino acids of C-terminal with cyan color for CΔ274 truncated form and **d** residue 1–89 selected amino acids of N-terminal plus residue 747–1021 selected amino acids of C-terminal with cyan color for NΔ89CΔ274 form. (Color figure online)

native soluble forms, wild type and NΔ89 enzymes were also prepared in their refolded structures. Integrity and molecular mass of all proteins were confirmed by Western blot or SDS-PAGE (data not shown).

3.3 Activity Analysis

Vmax (μM/min), Km (μM), kcat (min^{-1}), kcat/Km (μM^{-1}/min) as kinetic parameters of wild type cABC I were 0.012 ± 0.0025, 0.52 ± 0.08, 2223 ± 205 and 4275, respectively (mean ± SD for at least three measurements). No catalytic activity could be detected for truncated forms spectrophotometrically. Zymographic assay was carried out to analyze the probable activity of truncated enzymes and the wild type cABC I as a control (Fig. 4). NΔ89, CΔ274 showed 0.164, 0.195 as compared to the wild-type enzyme, respectively and NΔ89CΔ274 was inactive (Fig. 5).

3.4 Fluorescence Studies

Evaluation of local stability of proteins around their aromatic residues by intrinsic fluorescence indicated the higher

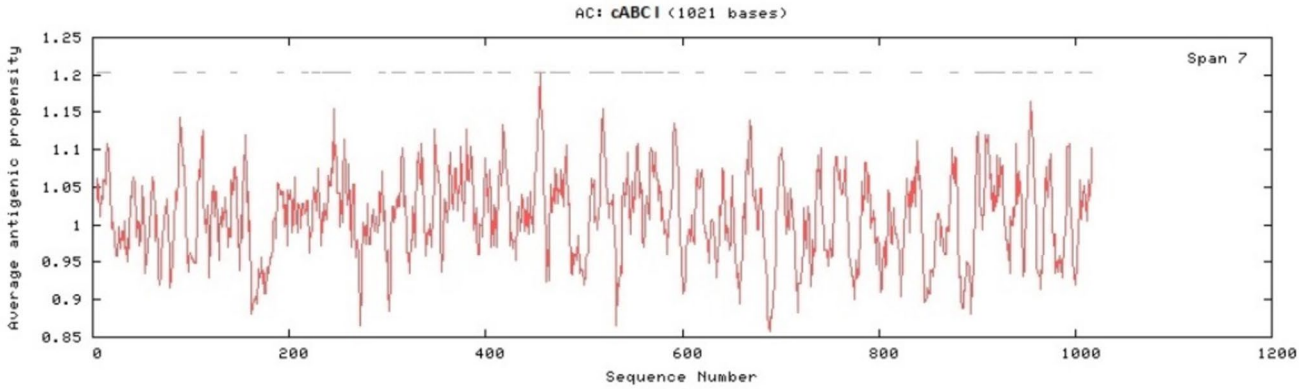

Fig. 3 Average antigenic propensity plot result for wild type cABC I sequence. Residues having propensity index above 1.0 are potentially antigenic (the reported accuracy of this method is about 75%)

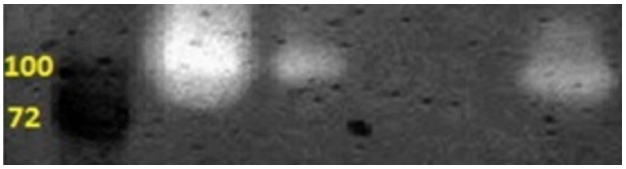

Fig. 4 Analyses of cleavage activity of wild type cABC I and its truncated forms using zymographic assay in the presence of C4S as a substrate. Lane 1: wild type cABC I, lane 2: NΔ89, lane 3: NΔ89CΔ274 and lane 4: CΔ274

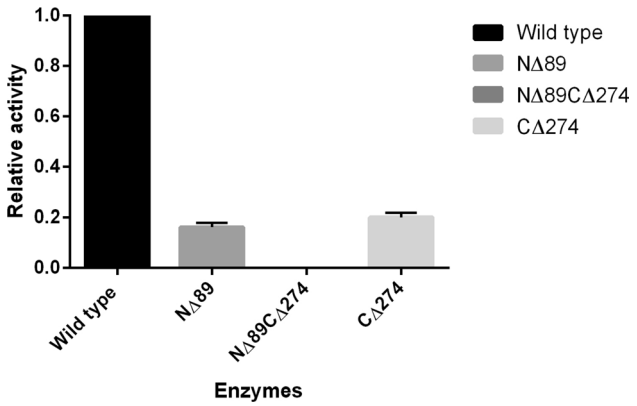

Fig. 5 Relative activities of truncated forms of cABC I in comparison to the wild type enzyme as control

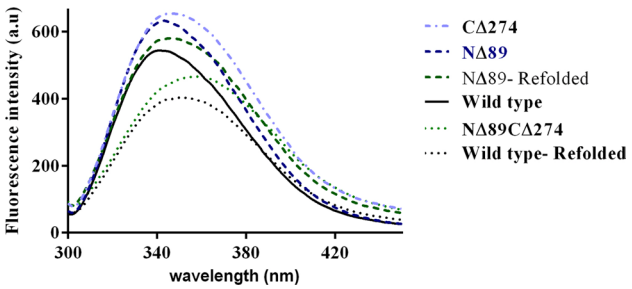

Fig. 6 Intrinsic fluorescence spectra of wild type and truncated cABC I (20 µg/ml protein concentration) in 50 mM phosphate buffer, pH 6.8

emission intensities for NΔ89 and CΔ274 as compared to the wild type enzyme (Fig. 6). In these two variants, 4 and 14 of 64 total residues of Trp and Tyr were decreased upon truncation, respectively. The ksv values obtained for NΔ89 and CΔ274 variants by quenching experiments using KI and acrylamide, were lower in comparison with the wild type, indicating compactness of the structures (Fig. 7; Table 1). 8-Anilino-1-naphthalenesulfonic acid (ANS) is believed to strongly bind cationic groups of proteins and polyamino acids through ion pair formation. A paucity of data exists on the fluorescent properties of ANS in these interactions. ANS binding to arginine and lysine derivatives was studied by fluorescence and circular dichroism spectroscopies to augment published information attained by isothermal titration calorimetry (ITC). Extrinsic fluorescence were monitored using ANS as a probe for hydrophobic patches as binding sites or positively charged amino acids at the surface of the enzymes. ANS is mainly non-fluorescent in aqueous solution, but fluorescence enhancement results from the ion pairing between charged group of Arg (or Lys) and the sulfonate group of ANS that reduce the intermolecular charge transfer (CT) rate constant. The intramolecular CT process affects upon a positive charge near the –NH group of ANS producing a blue shift of fluorescence [32]. Based on ANS binding analysis, the slightly red shifts as well as decrease in ANS fluorescence intensity were shown for NΔ89 and CΔ274 proteins relative to the wild type enzyme. Thus, NΔ89 and CΔ274 proteins showed less exposed hydrophobic patches or positively charged amino acids on their surfaces upon deletions of 18 of 64 total hydrophobic residues and 38 of 105 total residues of positive charge amino acids (Fig. 8).

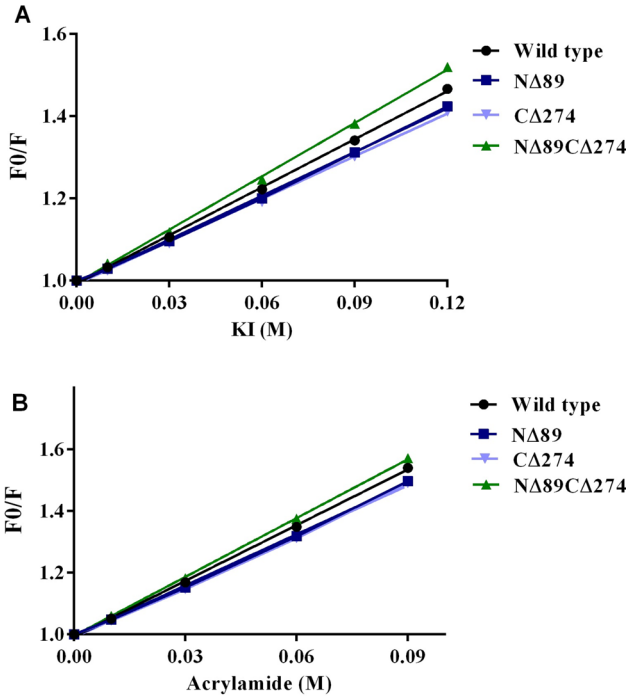

Fig. 7 Stern–Volmer plot of fluorescence quenching by KI (**a**) and acrylamide (**b**) for wild type cABC I and the truncated forms

3.5 Trypsinolysis Patterns

In order to assess the sensitivity of the enzymes to proteolysis, trypsin digestion experiment was performed for the variants. NΔ89 and CΔ274 digestion patterns revealed a more resistance against trypsinolysis in comparison to the wild type as shown in Fig. 9. In these two variants, 8 and 30 of 105 total residues of Arg and Lys were decreased upon truncation, respectively.

3.6 Secondary Structure Determination

Secondary structure content of the proteins was calculated by CDN software following analysis of Far-UV CD spectra of proteins. Increased β-sheets and decreased turn contents were reported for NΔ89 and CΔ274 variants in comparison to the wild type (Fig. 10).

4 Discussion

Utilizing cABC I as a therapeutic enzyme have been encountered different precautions related to the obstacles against approach to an appropriate applicable version [2, 23, 33].

In the case of protein truncation strategy, a number of truncated enzymes have showed increased activity or thermal stability of new shorter length enzymes in comparison to their wild types ;although there have been enzymes in which activity and stability were drastically diminished as a consequence of amino acid deletions, indicating the importance or hindrance effect of the deleted segments [14, 34–40].

In this study, we investigated the 1–89 and 747–1021 amino acid deletions effect on the whole structure and function of cABC I through generation of new truncated proteins. The exact role of these two parts in the enzyme activity and/or stability is not studied but in comparison to the wild type enzyme, antigenic prediction results indicated a decrease in the number of potential epitopes at the surface of NΔ89, CΔ274 and, NΔ89CΔ274 variants as a result of truncation.

Resistance of NΔ89 and CΔ274 variants against digestion by trypsin suggested that truncation caused deletion of exposing Arg and Lys residues or flexible parts of the proteins in their new tertiary structures. However, in accordance to digestion patterns, the protease digestion and ANS fluorescence results revealed tertiary structure changes of proteins in different manners [32, 41–43]. ANS fluorescence intensities of NΔ89 and CΔ274 were decreased with a red

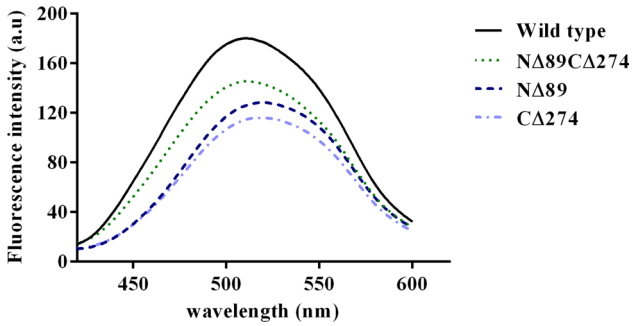

Fig. 8 Extrinsic fluorescence spectra of wild type and truncated cABC I proteins

Table 1 Structural parameters of wild type cABC I and its truncated variants

Variants	% α-helix	% β-sheet	% Turn	% Random coil	K_{sv} (M^{-1}) for acrylamide	K_{sv} (M^{-1}) for KI
Wild type	27	11.5	27.5	34.4	4.67 ± 0.4	3.14 ± 0.28
NΔ89	27.86	21.16	17.18	33.78	4.27 ± 0.3	2.85 ± 0.13
CΔ274	30.74	19.79	17.31	32.13	4.18 ± 0.43	2.74 ± 0.1
NΔ89CΔ274	14.97	31.77	16.88	36.27	5 ± 0.18	3.51 ± 0.08

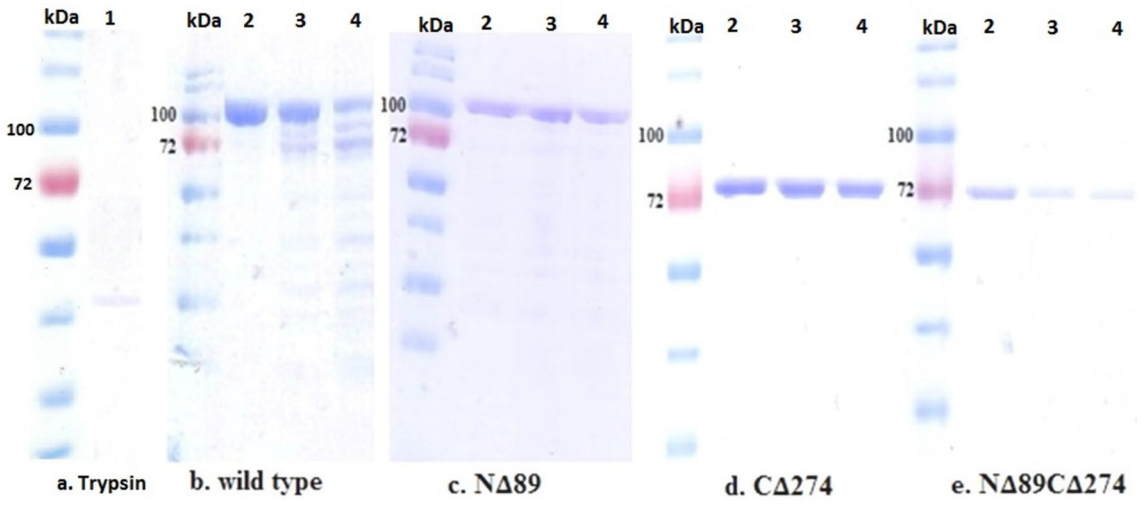

Fig. 9 SDS-PAGE analysis of limited proteolysis of wild type and truncated forms of cABC I by trypsin with 1/200 ratio to each enzyme at 25 °C. **a** 1—Trypsin, **b** 2—non-treated wild type, 3—treated wild type in 25 min, 4—treated wild type in 50 min, **c** 2—non-treated NΔ89, 3—treated NΔ89 in 25 min, 4—treated NΔ89 in 50 min, **d** 2—non-treated CΔ274, 3—treated CΔ274 in 25 min, 4—treated CΔ274 in 50 min, **e** 2—non-treated NΔ89CΔ274, 3—treated NΔ89CΔ274 in 25 min, 4—treated NΔ89CΔ274 in 50 min

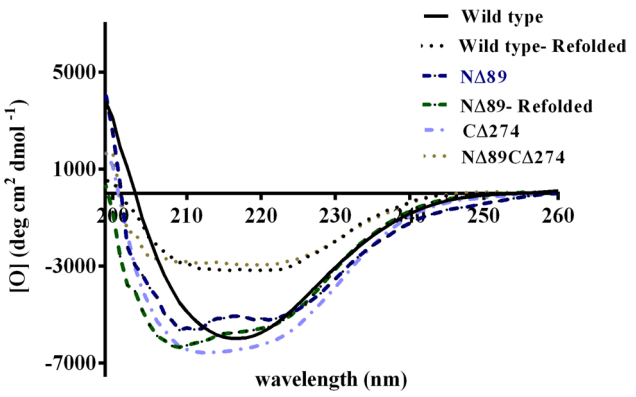

Fig. 10 Far-UV CD spectra of wild type and truncated cABC I

shift indicating either the low binding affinity of ANS to Lys and Arg as positively charged amino acids at the surface of proteins or having lesser binding sites for ANS at the surface of NΔ89 and CΔ274 truncated forms [32].

Structural flexibility changes in truncated cABC I were supported by fluorescence results and Far-UV CD data. Intrinsic fluorescence emission was increased without blue shift for NΔ89 and CΔ274 as compared to the wild type. Intramolecular quenching effect of amino acid residues such as Arg and Lys on the photon emission of the wild type enzyme could be lowered as a consequence of truncation. Partially exposed tryptophan residues could be flanked with positively charged amino acid side chains in the wild type enzyme. This could be regarded as an explanation for more quenching the of wild type enzyme than NΔ89 and CΔ274 variants by KI as an ionic quencher [44, 45]. On the other hand, the Stern–Volmer value of acrylamide quenching for NΔ89 and CΔ274 was lowered; suggesting that channels were affected by interior dynamic re-arrangement.

Tertiary structure experiments suggested that cationic-aromatic contacts might have been undergoing changes in truncated cABC I in comparison to its wild type (Figs. 6, 7, 8). For example the PIC server analysis showed that 27 cation–π interactions within 6 Å distance; e.g., between Tyr^{508} and Arg^{560} amino acids in the catalytic site of wild-type cABC I, were diminished in truncated variants as the amino acids were deleted. It is evident that the negatively charged electron clouds of any aromatic amino acids with the side chains of positively charged residues play a significant role in protein structure, substrate binding and catalysis of enzymes [46, 47].

Far-UV CD spectra indicated an increased local stability of ß-sheet content and decreased irregular turn structures of NΔ89 and CΔ274 as compared to the wild type. Turns are well-suited to participate in substrate binding since they are mostly surface-exposed parts of the proteins [48].

The overall look at the CD and fluorescence spectra (Figs. 6, 10) have drawn our attention to a dramatic difference between native wild type enzyme and its refolded soluble conformations. Also, there seems some similarity in the secondary and tertiary structures of native NΔ89 with its refolded variant. These findings revealed a probable role of 89 amino acids sequence from N-terminal of cABC I in its exact folding and re-folding pathways, although further information is needed. In normal expression condition for CΔ274 and NΔ89CΔ274 proteins, they could not be extracted in the soluble fraction; this might be attributed to the role of the 274 amino acids sequence deletion from the C-terminal domain of

these variants in soluble expression process. A matter which should be more studied.

Although digestion of C4S as substrate was observed by zymographic assay for NΔ89, CΔ274 and the wild type enzyme (Fig. 5), the activity of NΔ89, CΔ274 and NΔ89CΔ274 variants could not be determined spectrophotometrically, so not any kinetic parameters could be calculated. It seems that enough amounts of unsaturated disaccharide products are necessary to be detected spectrophotometrically. Catalytic machinery of cABC I have revealed the formation of substrate recognition site, catalytic site and product release area in the enzyme structure. Among the amino acids engaged, Arg105, Gln140, Arg221, Lys312, His388, His501, Tyr392, Arg395, Arg500, Tyr508, Arg560, His561, Asn564, Asn587, Glu653, His712 are recognized. These amino acids are involved in catalysis for protonation and deprotonatation of glycoside bond, substrate binding site, stabilizing inolate intermediate, maintaining the integrity of catalytic site and neutralizing GAGs molecules. The right orientation of amino acids in the enzyme catalysis might have been drastically altered upon amino acids re-arrangements through cation–π interactions, the turn content of the secondary structures and imbalance in stability-flexibility of the truncated forms [49]. These variations could led to an uncompleted ß-elimination mechanism of substrate subsite by truncated cABC I representing the critical effect of deleted parts for catalysis [15, 19, 20, 27, 29, 46, 50]. However, apart from type of products, the digestion of substrate could be visualized by zymographic assay.

In summary, the protease digestion, ANS fluorescence, intrinsic fluorescence, fluorescence quenching by KI and acrylamide, and Far-UV CD studies on cABC I revealed that internal structural microenvironment components were dynamically more stable for NΔ89 and CΔ274 variants than the wild type enzyme. Activity analysis showed that the deleted parts of the enzyme perform a significant role in cABC I catalysis.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

Research Involving Human and Animal Participants This article does not contain any studies with human participants or animals performed by any of the authors.

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