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Total Chemical Synthesis of Dengue 2 Virus Capsid Protein via Native Chemical Ligation: Role of the Conserved Salt-bridge¹

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Abstract

The dengue capsid protein C is a highly basic alpha-helical protein of ~100 amino acid residues that forms an amphipathic homodimer to encapsidate the viral genome and to interact with viral membranes. The solution structure of dengue 2 capsid protein C (DEN2C) has been determined by NMR spectroscopy, revealing a large dimer interface formed almost exclusively by hydrophobic residues. The only acidic residue (Glu87) conserved in the capsid proteins of all four serotypes of dengue virus forms a salt bridge with the side chains of Lys45 and Arg55'. To understand the structural and functional significance of this conserved salt bridge, we chemically synthesized an N-terminally truncated form of DEN2C (WT-DEN2C) and its salt bridge-void analog ^{E87A}DEN2C using the native chemical ligation technique developed by Kent and colleagues. Comparative biochemical and biophysical studies of these two synthetic proteins using circular dichroism spectroscopy, fluorescence polarization, protein thermal denaturation, and proteolytic susceptibility assay demonstrated that the conserved salt bridge contributed to DEN2C dimerization and stability as well as its resistance to proteolytic degradation. Our work provided insight into the role of a fully conserved structural element of the dengue capsid protein C and paved the way for additional functional studies of this important viral protein.

Keywords

Dengue capsid protein; Native chemical ligation; Solid phase peptide synthesis; Salt bridge; DEN2C

1. Introduction

Dengue virus (DENV) belongs to the flavivirus genus and is a member of the Flaviviridae family. Other well-known members of the same genus include, but are not limited to, yellow fever virus, West Nile virus, Japanese encephalitis virus, and tick-borne encephalitis virus.¹ As the most prevalent mosquito-borne pathogen, dengue virus is responsible for the highest

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rates of disease and mortality among members of the flavivirus genus, affecting 50–100 million people yearly according to the World Health Organization (WHO). Sequential infections by multiple serotypes of dengue virus (DENV 1–4) can lead to dengue hemorrhagic fever, of which there are an estimated ~500,000 annual cases worldwide, resulting in 22,000 deaths (<http://www.who.int/csr/disease/dengue/impact/en/>). No vaccines or effective antivirals are currently available to prevent or treat dengue infection.

Dengue virions comprise a single, positive-strand RNA genome packaged by capsid proteins (C) in a host cell-derived lipid bilayer and an outer glycoprotein shell consisting of 180 copies each of a membrane protein (prM/M) and envelope protein (E).^{2–4} After clathrin-mediated entry and uncoating of the nucleocapsid, the viral genome is released into the cytoplasm and the positive-sense RNA is translated into a single polyprotein that is further processed by cellular and virus-derived proteases into the three structural proteins C, prM/M, and E as well as seven non-structural (NS) proteins involved in viral RNA replication. Virus maturation occurs while in transit through the secretory pathway, a process characterized by low pH-triggered conformational changes predominantly in the E protein and by proteolytic excision of the “pr” peptide from prM.⁵ In the immature virion, the E and prM proteins heterodimerize, and the resultant E/prM heterodimers further trimerize, forming 60 trimeric “spikes” projecting away from the viral surface.⁶ In the mature virion, however, the E protein homo-dimerizes to form a “smooth” protein shell, covering M proteins embedded in the viral membrane.

The highly basic capsid protein C dimerizes in solution and encapsidates the viral genome during virus assembly. The solution structure of DENV-2 capsid protein of 100 amino acid residues, or DEN2C, was solved by NMR spectroscopy, revealing a symmetric dimer structure of the alpha-fold class that comprises residues 21–100.⁷ (The N-terminal 20 amino acid residues are disordered in the solution structure, thus “invisible”.) Each DEN2C monomer contains four alpha helices, of which the first three ($\alpha 1$ – $\alpha 3$) forms a right-handed helix bundle, and the longest helix $\alpha 4$ (residues 74–96) extends out from the 3-helix core. DEN2C dimerization is mediated primarily by extensive inter-molecular hydrophobic interactions involving two pairs of antiparallel helices, $\alpha 2$ – $\alpha 2'$ and $\alpha 4$ – $\alpha 4'$. The solvent-exposed edge of $\alpha 4$ – $\alpha 4'$ is densely charged with basic residues, presumably engaging viral RNA, whereas the $\alpha 2$ – $\alpha 2'$ interface presents a deep apolar cleft for interactions with viral membrane.⁷

Recently, the immunogenicity and protective capacity of a recombinant DEN2C protein has been reported,⁸ where mice were inoculated with crude recombinant DEN2C and subsequently challenged intracranially with homologous lethal virus. Statistically significant protection of the animals without the induction of antiviral antibodies was observed, making the capsid protein an attractive vaccine candidate against dengue by inducing a cellular immune response.⁹ Other studies began to unravel the complex molecular mechanism by which dengue virus capsid protein usurps ER-derived lipid droplets via conserved hydrophobic residues (located in the apolar cleft formed by $\alpha 2$ – $\alpha 2'$) for RNA encapsidation, viral particle formation, and replication.^{10,11}

These interesting recent findings motivated us to carry out additional biochemical studies of DEN2C to better understand how the viral protein works at the molecular level. DEN2C contains 26 cationic residues and only three anionic residues, Asp3, Glu19, and Glu87. As the only acidic residue conserved in the capsid proteins of all four serotypes of dengue virus, Glu87 forms salt-bridges with the side chains of Lys45 and Arg55' as illustrated by the NMR structure,⁷ representing the only charge interaction that stabilizes the dimer interface. To elucidate the structural and functional role of the conserved salt bridge in DEN2C, we chemically synthesized, using the native chemical ligation (NCL) technique pioneered by

Kent and coworkers,^{12,13} and biochemically and biophysically characterized an N-terminally truncated DEN2C (residues 21–100), or ^{WT}DEN2C, as well as its salt bridge-void analog ^{E87A}DEN2C.

2. Materials and methods

2.1. Materials

Boc-(L)-amino acids were obtained from Peptides International (Louisville, KY); p-Methyl-BHA resin and Boc-Leu-OCH₂-PAM resin were purchased from Applied Biosystems (Foster City, CA); dichloromethane, N,N-dimethylformamide, and HPLC grade acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA), and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU) was acquired from Oakwood Products Inc. (West Columbia, SC). Trifluoroacetic acid (TFA) was purchased from Halocarbon (River Edge, NJ) and hydrogen fluoride (HF) was from Matheson Tri-gas (Montgomeryville, PA). N,N-diisopropylethylamine (DIEA), 4-mercaptophenylacetic acid (MPAA) and *p*-cresol were from Fluka (Switzerland), and ultrapure guanidine hydrochloride was obtained from ICN Biochemicals (Irvine, CA). Fluorescein-5-Maleimide (FAM-5-Mal) and tris(2-carboxyethyl)phosphine (TCEP) were from Thermo Scientific (Rockford, IL).

2.2. Solid phase peptide synthesis (SPPS) of (21–57)DEN2CαCOSR (R = CH₂CH₂CO-Arg₆-NH₂) and (58–100)DEN2C

The three peptide fragments, i.e., (21–57)DEN2CαCOSR, (58–100)DEN2C and E87A-(58–100)DEN2C were individually synthesized on appropriate resins on an automated peptide synthesizer ABI 433A using the optimized HBTU activation/DIEA in situ neutralization protocol developed by Kent and colleagues for Boc-chemistry SPPS.¹⁴ To increase the solubility of the N-terminal fragment, a six-Arg residue solubilizing tag was introduced to the thioester moiety as described.^{15,16} The three crude peptides, after HF cleavage, were precipitated with cold ether and purified by preparative C18 reversed-phase (RP) HPLC, and their molecular masses were ascertained by electrospray ionization mass spectrometry (ESI-MS).

2.3. Native chemical ligation, protein folding and labeling, and circular dichroism (CD) spectroscopy

Native chemical ligation was carried out in 0.2 M phosphate buffer containing 6 M guanidine hydrochloride, 30 mM MPAA, and 20 mM TCEP, pH 7.2 (Supplemental Figure 1). The ligated products were purified to homogeneity by preparative C18 RP-HPLC.

DEN2C polypeptides were dissolved in water at a concentration of 20 mg/ml, and subjected to size-exclusion gel filtration chromatography on a Superdex-75 column (prep grade) running phosphate buffer containing 1.0 M sodium chloride, pH 7.4, at a flow rate of 0.5 ml/min. Dimeric proteins were dialyzed extensively against PBS (pH 7.4) in the presence of 0.5 mM TCEP and concentrated. Protein solutions were quantified by UV absorbance measurements at 280 nm using a molar extinction coefficient of 5500 calculated according to a published algorithm.¹⁷

DEN2C proteins were fluorescently labeled with FAM-5-mal. Specifically, ^{WT}DEN2C or ^{E87A}DEN2C was dissolved in PBS (pH 7.0) at a concentration of 2 mg/ml, to which a 10-fold molar excess of FAM-5-mal was added. The reaction proceeded for 1 h with stirring and the product was purified by preparative RP-HPLC. FAM-^{WT}DEN2C and FAM-^{E87A}DEN2C were lyophilized and stored in dark.

CD spectra of DEN2C proteins at 20 μ M in 10 mM phosphate buffer (pH 7.2) were obtained at room temperature on a J-810 spectropolarimeter (Jasco, Easton, MD) using a 1-mm cuvette.

2.4. Fluorescence polarization measurements

Fluorescence polarization measurements were performed on a Tecan Infinite M1000 multimode plate reader. Two-fold serial dilutions of FAM-WT^{DEN2C} or FAM-E87A^{DEN2C} in PBS (pH 7.4), ranging from 4.0 μ M to 7.8 nM, were incubated at room temperature in a 96-well plate (100 μ l per well) for 2 hours before polarization was measured at $\lambda_{\text{ex}} = 470$ nm and $\lambda_{\text{em}} = 530$ nm.

2.5. Thermal denaturation

Thermal denaturation of WT^{DEN2C} or E87A^{DEN2C} was conducted at a concentration of 10 μ M in PBS containing 0.5 mM TCEP, pH 7.4, on a Perkin-Elmer LS55 fluorescence spectrometer equipped with a PTP1 peltier temperature controller. Data collection was performed from 25 to 90 °C in 1 °C increments with a 30 sec incubation and 10 sec integration. Fluorescence intensity of the only Trp residue, Trp69, was measured using excitation/emission wavelengths of 280/350 nm, and the data were subjected to a 6-parameter non-linear regression analysis using the following equation:¹⁸

$$F = \{(a_1 + b_1 \times T) + (a_2 + b_2 \times T) * e^{\left[-\Delta H \times (1 - T/T_m) / (R \times T)\right]}\} / \{1 + e^{\left[-\Delta H \times (1 - T/T_m) / (R \times T)\right]}\}$$

where F is measured Trp fluorescence, T is absolute temperature, T_m is protein melting temperature, ΔH is van't Hoff enthalpy.

2.6. Proteolytic stability

WT^{DEN2C} or E87A^{DEN2C} at 0.5 mg/ml each was incubated at room temperature with 2.5 μ g/ml trypsin in 50 mM Tris/HCl buffer containing 20 mM CaCl_2 and 0.001% Triton X-100, pH 8.3. Aliquots were withdrawn at different time intervals (0, 15, 30 min, 1, 2, 4, 8, 24 and 54 h) and acidified by acetic acid before quantitative analysis on a Waters XBridge C18 column (3.5 μ m, 4.6 $\times$ 150 mm) running a linear gradient of 25–85% acetonitrile containing 0.1% TFA. The percentage of residual DEN2C protein was calculated based on the ratio of integrated peak areas of the intact molecule (starting material) at a given time *versus* at time 0.

3. Results and Discussion

3.1. Protein synthesis via native chemical ligation

The strategy for the synthesis of N-terminally truncated (21–100)DEN2C proteins of 80 amino acid residues is illustrated in Figure 1. We mutated Thr58 to Cys, which is distal to the dimer interface and expected to have no impact on DEN2C folding and dimerization, in order to produce a C-terminal peptide fragment permissible for native chemical ligation. This mutation also made it possible to site-specifically label the proteins using thiol chemistry under non-denaturing conditions. It should be pointed out that DEN2C proteins can be synthesized via native chemical ligation without resorting to the T58C mutation. An alternative and certainly more elegant strategy involves the use of an auxiliary Ala-to-Cys mutation at an appropriate site for ligation followed by desulfurization to revert Cys to Ala after ligation.^{19,20}

The N-terminal thioester peptide is extremely hydrophobic and presented an initial challenge to peptide solubilization and purification – a well-recognized technical hurdle in chemical

protein synthesis.¹⁶ Poly-Arg tags have been commonly used to enhance the solubility of hydrophobic peptide fragments used for native chemical ligation.^{15,16} Organic solvents such as DMF and DMSO^{21,22} and lipid bilayer systems²³ have also been employed to improve peptide solubility. In the present work we introduced an Arg₆ tag to the thioester moiety to improve synthesis and purification as well as ligation efficiency. Both (21–57)DEN2CaCOSR and (58–100)DEN2C peptides were obtained, after purification to homogeneity by RP-HPLC, at a yield of 20–25% on a synthetic scale of 0.25 mmol.

The ligation reaction was performed at room temperature at a concentration of 10–15 mg/ml for the thioester peptide in the presence of a 2-fold molar excess of (58–100)DEN2C. MPAA was used as a catalyst instead of the more traditional thiophenol as the former is known to accelerate ligation reaction with improved yields.²⁴ However, we found that the ligation of (58–100)DEN2C to (21–57)DEN2CaCOSR was exceedingly slow despite the fact that *in situ* transthioesterification between the thioester peptide and MPAA was almost complete in 30 min. After an overnight reaction and subsequent HPLC purification, the ligation product was obtained at a typical overall yield of only 5%. The low yield likely stemmed from (1) an inefficient nucleophilic displacement of the thioester intermediate by (58–100)DEN2C, (2) hydrolysis of the thioester intermediate during an extended period of the ligation reaction, and (3) reactants and product heterogeneity arising from the ligation reaction. Since both the N- and C-terminal segments are heavily cationic, we speculate that a strong electrostatic repulsion force may have prevented efficient collision of two peptide molecules in solution, resulting in a slow ligation reaction rate. Shown in Figure 2 are purified DEN2C and DEN2C characterized by analytical RP-HPLC, ESI-MS and size-exclusion gel filtration chromatography. The determined molecular masses of 9251.5 Da (WTDEN2C) and 9192.8 Da (E87ADEN2C) were within experimental error of the theoretical values of 9252.3 Da and 9194.3 Da calculated on the basis of the average isotopic compositions of the proteins. Both proteins eluted on Superdex-75 predominantly as dimers under high-salt conditions²⁵ as calibrated by molecular weight standards (data not shown).

As expected, both proteins adopted a largely alpha-helical conformation characterized by the double negative peaks at 208 and 222 nm and the single positive peak at 195 nm (Figure 3), consistent with the known structural features of DEN2C. Importantly, both proteins showed nearly identical CD spectra, suggesting that deletion of the conserved salt bridge did not impact the global structure of the protein.

3.2. Fluorescence polarization as a tool to study DEN2C dimerization

Fluorescence polarization (FP) is a powerful tool to study protein dimerization in solution. FP measurements are based on the principle that a protein monomer tumbles faster than its dimer, and upon excitation by polarized light, it “scrambles” the polarization of emitted light more efficiently.^{26,27} Thus, protein dimerization necessarily causes decreased rotational rates and increased polarization of emitted light. A titration curve is generated from measurements of polarized fluorescence, and dimerization can be quantitatively evaluated using known mathematical models.

Thus, we expected that DEN2C would exist as a dimer of a high polarization value at high concentrations and, as the protein concentration is lowered, start to dissociate to monomers of low polarization. Surprisingly, however, the opposite was observed in our experiments. As FAM-WT-DEN2C was serially diluted in PBS from 4 μ M to 7.8 nM, FP increased from ~150 mP to 330 mP in a dose-dependent manner (Figure 4). Similar results were obtained for FAM-E87A-DEN2C, where the mutant protein in fact displayed an identical dose-dependency to FAM-WT-DEN2C within the concentration range of 500–50 nM. While these findings are completely counter-intuitive, they probably can be explained by the existence in monomeric DEN2C of an extremely large hydrophobic surface as revealed by NMR

spectroscopy. The structural studies have shown that DEN2C dimerization is driven predominantly by the burial of solvent-exposed non-polar residues at the dimer interface, generating a contact surface area of 1,650 Å².⁷ It is therefore plausible that monomeric DEN2C is energetically unstable in solution and may have a strong tendency to form soluble aggregates to shield from aqueous solution its large apolar surface. The hydrophobic nature of fluorescein may have also contributed to this process. This (aggregation) event at low concentrations should effectively lead to an increase in fluorescence polarization as DEN2C dissociates.

Interestingly, the loss of the conserved salt bridge in DEN2C did not significantly affect protein dimerization until in the low concentration range (< 50 nM). WT DEN2C plateaued at 330 mP, whereas E87A DEN2C progressively increased to 350 mP. These differences in FP suggest that the dimer of E87A DEN2C is indeed less stable than that of WT DEN2C.

3.3. Loss of the conserved salt bridge destabilizes DEN2C

We compared the two synthetic proteins WT DEN2C and E87A DEN2C in a thermal denaturation study where Trp fluorescence was measured as a function of temperature. The intrinsic fluorescence of Trp is known to decrease as temperature increases.²⁸ Burial of a Trp residue in the hydrophobic environment of a protein typically enhances its fluorescence intensity and blue-shifts its emission maximum as compared with an solvent-accessible Trp residue in aqueous solution.^{29,30} Trp69 is fully conserved in all flavivirus C proteins and plays an important energetic role in DEN2C dimerization by stabilizing the dimer interface.⁷ In fact, Trp69 lies at the dimer interface and its indole ring packs against several C-terminal hydrophobic residues of helix α4' from the second molecule. Thus a decrease in Trp fluorescence resulting from heat-induced denaturation of DEN2C is expected to accelerate as the protein “melts” during the transition from its folded state to an unfolded state. The characteristic “melting temperature” or T_m at which 50% of the protein unfolds is indicative of DEN2C thermal stability. As shown in Figure 5, both WT DEN2C and E87A DEN2C displayed, above 55 °C, a typical cooperative, two-state unfolding transition. The T_m values were determined for WT DEN2C and E87A DEN2C as 71.5 and 64.3 °C, respectively. The wild type protein is more stable than the mutant by 7 °C, confirming that the conserved salt bridge is indeed stabilizing, as expected.

3.4. Loss of the conserved salt bridge increases susceptibility to proteolysis

Despite the fact that WT DEN2C is more stable than E87A DEN2C, both proteins are fully folded at physiological temperature, i.e., 37 °C. To better understand the physiological role of the conserved salt bridge in DEN2C, we subjected WT DEN2C and E87A DEN2C to a proteolytic stability test where the proteins were treated with trypsin and their time-dependent degradation was quantitatively evaluated by analytical HPLC and ESI-MS.³¹ As shown in Figures 6 and 7, a predominant initial cleavage occurred between Arg98–Arg99, effectively excising the C-terminal dibasic residues Arg99Arg100 from both proteins within 60 min of incubation with trypsin. This finding is consistent with the structural features of DEN2C where the very end of the C-terminus is disordered,⁷ thus susceptible to proteolysis. The C-terminally truncated intermediate (E87A DEN2C-Δ2 or WT DEN2C-Δ2) populated to a maximum concentration at 2 h before being gradually degraded by the protease to smaller peptide fragments. The time for a 50% decay after reaching the maximum concentration was estimated to be ~18 h for E87A DEN2C-Δ2 and ~36 h for WT DEN2C-Δ2. At the last time point of 54 h, 28% of WT DEN2C-Δ2 remained intact, while only 11% of E87A DEN2C-Δ2 was uncleaved. It is important to note that the E87A mutation effectively freed up two basic residues (K45 and R55') that were previously engaged in electrostatic interactions with E87, presumably making the mutant protein more susceptible to trypsin digestion. However, we do not have any experimental evidence to support that those two sites (K45 and R55')

directly contributed to trypsin susceptibility of the protein. Our findings strongly suggest that the conserved salt bridge is important for in vivo stability of DEN2C.

4. Conclusion

We have demonstrated the feasibility of chemically synthesizing the capsid protein C of dengue-2 virus and its salt bridge-void analog using the native chemical ligation strategy. The synthetic proteins are correctly folded as verified by several biophysical techniques. Our data support dimer as a stable and functional form of DEN2C and shed light on the importance of the conserved salt bridge between Glu87 and the side chains of Lys45 and Arg55' for DEN2C dimerization and stability. Our findings also provide insight into the possible physiological role of the conserved salt bridge in enhancing proteolytic stability of DEN2C in vivo. Since DEN2C plays critical roles in the life cycle of dengue virus,^{32–34} facile synthetic access to DEN2C should facilitate future studies of this important viral protein to better understand how it functions at the molecular level.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Strategy for chemical protein synthesis of (21–100)DEN2C using native chemical ligation. Thr58 was mutated to Cys to introduce the ligation site Leu57-Cys58. As shown in the NMR solution structure of DEN2C (PDB code: 1R6R), Thr58 is located between the $\alpha 2$ and $\alpha 3$ helices and distal to the dimer interface. The salt bridge between Glu87 and the side chains of Lys45 and Arg55' are shown in sticks. Colored in red are helices $\alpha 1$ and $\alpha 2$ (or $\alpha 1'$ and $\alpha 2'$), and helices $\alpha 3$ and $\alpha 4$ (or $\alpha 3'$ and $\alpha 4'$) are colored in blue. DEN2C dimerization is mediated primarily by extensive inter-molecular hydrophobic interactions involving two pairs of antiparallel helices, $\alpha 2$ - $\alpha 2'$ and $\alpha 4$ - $\alpha 4'$.

Figure 2.

^{WT}DEN2C (panels A and C) and ^{E87A}DEN2C (panels B and D) characterized by HPLC, ESI-MS and size-exclusion chromatography. The HPLC chromatograms were obtained at 40 °C on a Waters XBridge C18 column (4.6 × 150 mm, 3.5 μm) running a gradient of 5% to 65% acetonitrile in water, containing 0.1% TFA, at a flow rate of 1 mL/min. The determined molecular masses of 9251.5 Da (^{WT}DEN2C) and 9192.8 Da (^{E87A}DEN2C) are respectively within experimental error of the theoretical values of 9252.3 Da and 9194.3 Da calculated on the basis of the average isotopic compositions of ^{WT}DEN2C and ^{E87A}DEN2C. Size-exclusion chromatographic analyses of ^{WT}DEN2C and ^{E87A}DEN2C were performed on a Superdex-75 prep-grade column running a phosphate buffer containing 1.0 M sodium chloride, pH 7.4, at a flow rate of 0.5 ml/min. The proteins were prepared in PBS at 20mg/mL each, from which 100 μl was injected. *M_w* standards were used for calibration.

Figure 3. CD spectra of ^{WT}DEN2C (dot) and ^{E87A}DEN2C (circle) at 20 μ M in 10 mM phosphate buffer, pH 7.2, obtained at room temperature.

Figure 4. Concentration-dependent dimerization or monomerization of FAM-^{WT}DEN2C (dot) and FAM-^{E87A}DEN2C (circle) as monitored by fluorescence polarization in PBS at $\lambda_{\text{ex}} = 470$ nm and $\lambda_{\text{em}} = 530$ nm.

Figure 5.

Heat-induced two-state denaturation of WT DEN2C (dot) and E87A DEN2C (circle), each at 10 μ M in PBS containing 0.5 mM TCEP, pH 7.4, monitored by Trp fluorescence at $\lambda_{ex} = 280$ nm and $\lambda_{em} = 350$ nm. Nonlinear regression analyses yielded the melting temperature (T_m) of 344.7 K for WT DEN2C and of 337.5 K for E87A DEN2C (A). Fluorescence-based denaturation data were also normalized to fractional denaturation (B).

Figure 6. Hydrolysis kinetics monitored over 60 min for ^{WT}DEN2C and ^{E87A}DEN2C and over 54 h for their C-terminally cleaved products. The data were obtained from two independent experiments with error bars representing average deviations of the measurements.

Figure 7. Digestion of WT DEN2C (A) and E87A DEN2C (B) by trypsin at 15 min, 2 h and 24 h. The HPLC conditions were essentially the same as described in Figure 2 legend except that a 25% to 85% elution gradient was used here. The two Arginine residues (⁹⁹R¹⁰⁰R) at the C-termini of both proteins were completely cleaved in 2 hours.