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Thermal stability of the hemagglutinin-neuraminidase from Sendai virus evidences two folding domains

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Abstract The domain structure of hemagglutinin-neuraminidase from Sendai virus (cHN) was investigated by studying the thermal stability in the 20–100°C range. Differential scanning calorimetry evidences two conformational transitions. The first transition is apparently a reversible two-state process, with T_m 48.3°C, and is shifted to 50.1°C in the presence of the substrate analogue 2,3-dehydro-2-deoxy-*N*-acetyl neuraminic acid, meaning that the substrate binding domain is involved in the transition. The second transition, with apparent T_m 53.2°C, is accompanied by irreversible loss of enzymatic activity of the protein, and the presence of the substrate analogue does not affect the T_m. The data indicate that cHN is composed of two independent folding domains, and that only one domain is involved in the binding of the substrate. Our results suggest that the paramyxovirus neuraminidases have the folding properties of a two-domain protein.

Key words: β-Propeller; Differential scanning calorimetry; Paramyxovirus; Thermal denaturation; Neuraminidase; Protein folding

1. Introduction

Paramyxoviridae are a family of enveloped viruses which cause a large number of pathologies in both humans and animals. The envelope of paramyxovirus contains two glycoproteins, the fusion protein (F) and the hemagglutinin-neuraminidase (HN). The three-dimensional structure of a paramyxovirus neuraminidase has recently been reported [1], showing that the HN has the same folding topology as influenza neuraminidase. The experimentally determined structure confirms the prediction obtained by comparing conserved residues of viral and bacterial neuraminidases, whose three-dimensional structure is known, with paramyxovirus HN [2,3]. However, there are some functional differences between the neuraminidases of paramyxoviruses and the other neuraminidases [4,5] because HN is involved in the fusion process, being necessary for the activity of the F protein [6–11].

The presence of multiple functions is a unique feature of paramyxovirus HN, with no counterpart on influenza virus or bacterial neuraminidases. Thus it is interesting to know how the three-dimensional structure can account for all the activities of HN.

In the presence of detergents that disrupt the viral envelope, HN is cleaved selectively by trypsin at arginine 131 [12], and the C-terminal part is released as a soluble protein (cHN). cHN is a disulfide-linked homodimer, which fully retains the enzymatic activity of the intact protein. In the present report we have investigated the thermal stability of the cHN and we show that the structure is formed by at least two different folding domains.

2. Materials and methods

Unless otherwise stated, all experiments were performed in 10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS).

Sendai virus was grown in the allantoic cavity of 10-day embryonated eggs, and purified as previously described [13]. The water-soluble cHN was prepared with a new procedure. Briefly, HN was solubilized as previously described [14], and was treated with trypsin (5% by weight of the viral proteins) for 20 min at room temperature. The solution was passed through a column of Amberlite XA2 (9×1.7 cm) to remove the detergent, and the flow-through was poured into a phosphocellulose column (9×1.7 cm) equilibrated in 10 mM MES, 150 mM NaCl, pH 6.0. The unabsorbed material, containing cHN, was concentrated by centrifugation on Centricon CF25 (Amicon), and appeared as a single band on SDS–PAGE. Despite the apparent purity, cHN was still contaminated by a very small amount of an unknown proteolytic activity, which was removed by gel filtration on a column (1.2×100 cm) of Sephacryl S-200 (Pharmacia), equilibrated in PBS. The cHN concentration was determined by the absorbance at 280 nm, with $E1\% = 10.83$ [15].

Enzymatic activity was assayed at 37°C as previously described [16]. Proteolytic activity was assayed using azocasein (Sigma) as substrate.

Fluorescence measurements were performed in a Perkin-Elmer spectrofluorimeter with thermostated cell holder, and the temperature of the sample was read in the cuvette with a PT100 thermoresistance. The heating rate was maintained at 1.0 ± 0.2°C/min. The fraction of denatured protein at temperature T, fd(T), was calculated from:

$$\mathrm{fd}(T) = \frac{F(T) - F_{\min}(T)}{F_{\max}(T) - F(T)}$$

where $F(T)$ is the fluorescence at temperature T, $F_{\min}(T)$ and $F_{\max}(T)$ are the extrapolated values from the linear regression of the ln (fluorescence) versus $1/T$ before and after the transition respectively. The fluorescence emission was read at 330 and 350 nm with excitation at 280 nm.

Differential scanning calorimetry (DSC) measurements were performed with a Microcal VP microcalorimeter, and the data were processed with the software provided by the manufacturer. Baseline was constructed by fitting a third order polynomial to the data points before (26–36°C) and after (65–80°C) transition. Curve fitting was used to estimate the T_m and the enthalpy change associated with

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the transition of the component peaks when the thermogram was the summation of overlapping peaks. The deconvolution was based on the assumption that the thermograms can be described in terms of a linear combination of more than one independent transition, each approximating a two-state transition.

Selective proteolysis was performed using the partially purified cHN (before the final gel filtration step). The protein was extensively dialyzed against PBS, and, after incubation at the desired temperature, peptides were separated by SDS–PAGE and blotted onto PVDF membranes (Millipore). Bands, stained with Coomassie blue, were cut and subjected to amino-terminal sequencing.

3. Results

3.1. Calorimetric studies of cHN denaturation

Thermal denaturation of cHN, studied by DSC, shows a two-component endothermic transition. The experimental curve and the theoretical curves that best fit the experimental data are reported in Fig. 1 (lower trace), and show the presence of partially overlapping peaks, the first with T_m 48.3°C, and the second with T_m 53.2°C. In the presence of the substrate analogue, 2,3-dehydro-2-deoxy-*N*-acetyl neuraminic acid (DNANA), the best fit of the heat capacity vs. temperature plot was again obtained by two overlapping peaks. The temperature transition of the first peak, previously centered at 48.3°C, increased to 50.5°C while the temperature of the second peak remained constant (53.1°C) (Fig. 1, upper trace).

These data suggest that cHN is composed of two different folding domains, but only one involved in the binding of the substrate. The overall transitions observed by DSC appear to be irreversible, since no significant transitions were detected during cooling or a second heating cycle.

3.2. Fluorescence study of cHN denaturation

Temperature-induced unfolding of cHN was investigated determining the solvent accessibility of the tryptophan residues, by measuring the ratio of the fluorescence emission at 330 and 350 nm in the 25–75°C range. When the temperature was raised to 75°C, the 330/350 ratio slowly decreased from 1.20 to 1.15, without any evidence of a cooperative change (Fig. 2). This means that the solvent exposure of the tryptophan residues does not change in the temperature range studied, and therefore that cHN does not unfold at temperatures where DSC evidences the thermal transitions.

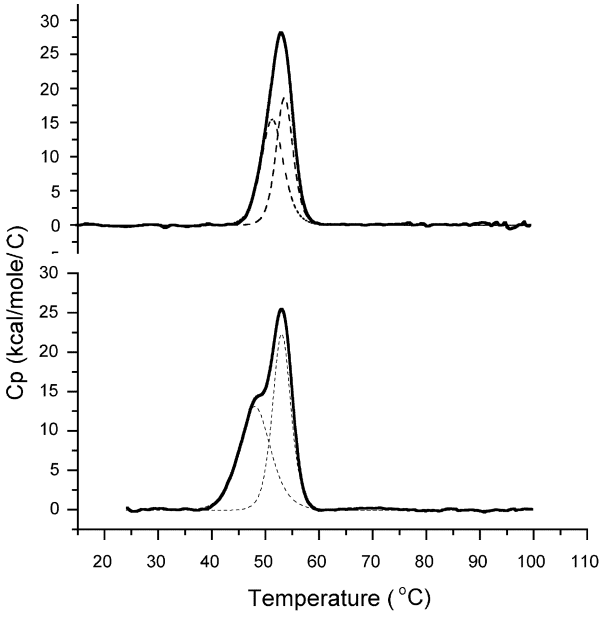

Fig. 1. Thermal denaturation of cHN. The excess heat capacity was recorded at 60°C/h in PBS. Baseline-subtracted DSC recording of cHN (0.4 mg/ml) (lower trace) and cHN in the presence of 0.4 mM DNANA (upper trace). In both cases the best fit of the experimental data is obtained assuming two non-two-state transitions. Solid line: experimental data; dashed line, fitted data.

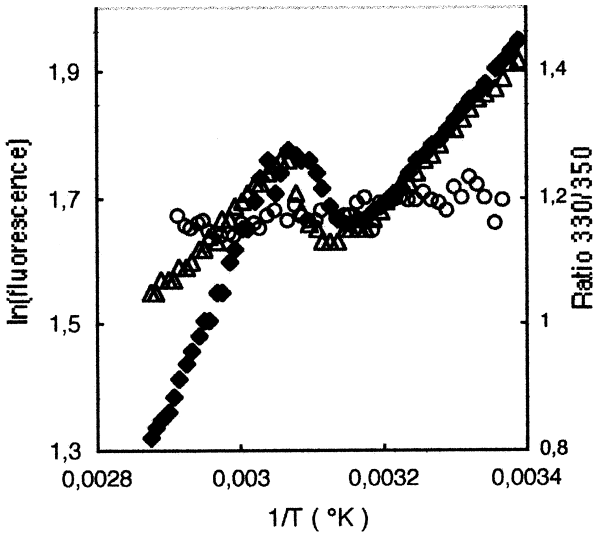

Fig. 2. Temperature dependence of the intrinsic fluorescence of cHN. Fluorescence emission intensity of cHN (0.05 mg/ml) at 330 and 350 nm was recorded in PBS at a heating rate of 1°C/min. Empty circles (right ordinate): ratio of emission at 330/350; filled diamonds (left ordinate): fluorescence intensity at 330 nm in the absence of DNANA; empty triangles: fluorescence intensity at 330 nm in the presence of DNANA.

The denaturation of cHN can also be observed by measuring the intensity of the intrinsic fluorescence of the protein. When the fluorescence emission intensity was measured (Fig. 2), a sharp increase was observed between 35°C and 55°C, and the presence of DNANA shifted the apparent T_m from 49°C to 51°C. This means that, in the 35–55°C range, there is a thermal transition sensitive to the presence of the substrate analogue.

The transition could not be detected during a second heating cycle; however, when the first heating cycle was stopped at temperatures below 50°C, a second heating cycle still detected the thermal transition. The transitions observed during the first and the second heating cycles are nearly identical if the temperature of the first cycle did not exceed 47°C. These data suggest that while complete unfolding occurs at temperatures higher than 75°C, cHN undergoes a reversible conformational transition(s) at about 49°C, followed by an irreversible inactivation. The changes in the fluorescence intensity are clearly correlated with the first transition observed by DSC, since in both cases the presence of the substrate analogue shifts the T_m to higher temperatures. In addition the T_m obtained by fluorescence measurements is in quite good agreement with that measured by DSC both in the absence and in the presence of DNANA (Table 1).

3.3. Selective proteolysis of cHN

Attempts to investigate the cHN denaturation by selective proteolysis with commercial proteases (trypsin, *Staphylococ-*

Table 1
Thermodynamic parameters of the denaturation process of cHN

	T_m (°C)			
	Peak 1		Peak 2	
	Calorimetric	Fluorescence	Calorimetric	Fluorescence
cHN	48.3	49.8	53.2	nd
cHN+DNANA	50.5	51.1	53.1	nd

nd: not detected.

cus aureus V8 protease, pronase) were unsuccessful. A host-derived protease [17], which is separated from cHN in the last step of purification, selectively degrades cHN as the temperature rises to 45–50°C (Fig. 3). At 28°C, the proteolytic activity measured on azocasein was one third of the activity measured at 48°C. However, incubation of cHN at 28°C for up to 60 min did not cause appreciable digestion, while at 48°C a 5 min digestion showed the appearance of degradation products. This means that the protease is effective on cHN only after the thermal transition observed both by DSC and by fluorescence measurements. The presence of the substrate analogue DNANA protects against the proteolytic digestion, suggesting that the protease acts on the cHN domain involved in the binding of the substrate. When the digestion was carried out at 55°C, a temperature higher than the second transition detected by DSC, cHN was completely degraded and DNANA did not exert any protective effect.

The digestion of cHN yielded two main peptides, with apparent MWs, determined by SDS–PAGE, of 30 and 27 kDa. Amino-terminal sequencing of the two peptides gave the sequence QELT (the N-terminal sequence of cHN) for the 27 kDa peptide, and the sequence ITWL (sequence 371–374 of HN) for the 30 kDa peptide, meaning a protease cut at the lysine 370.

4. Discussion

DSC data show that two different domains, undergoing a thermal transition at different temperatures, are present in cHN. The thermal transition of the first domain, centered at a lower temperature, is accompanied by an increase of the intrinsic fluorescence of the protein. This domain is involved in the binding of the substrate, since the presence of the substrate analogue DNANA shifts its T_m at higher temperatures. The thermal transition of the second domain is irreversible, and this domain does not appear to be involved in the binding of the substrate, since DNANA does not modify the T_m of the transition.

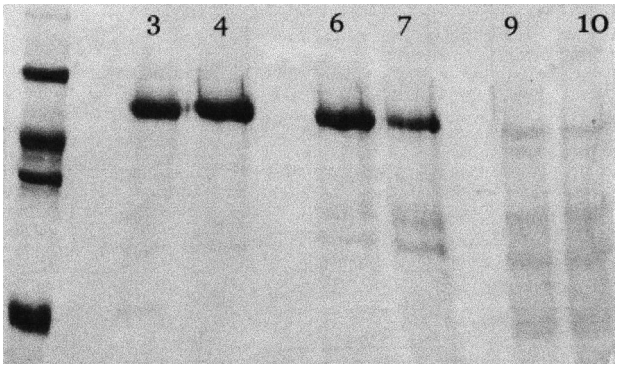

Fig. 3. Limited proteolysis of cHN. MW standards (lane 1) are: bovine serum albumin (67 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), soybean trypsin inhibitor (20.4 kDa). Partially purified cHN was incubated: for 30 min at 28°C in the presence (lane 3) and in the absence of DNANA (lane 4); for 5 min at 48°C in the presence (lane 6) and in the absence (lane 7) of DNANA; for 5 min at 55°C in the presence (lane 9) and in the absence of DNANA (lane 10).

The possibility that the DSC is detecting two sequential structural changes on a single domain must be ruled out, since the two transitions are very close, but only the one occurring at the lower temperature is affected by the presence of the substrate analogue DNANA.

The denaturation analysis shows that cHN is composed of different folding units. This is not surprising, because a 51 kDa protein can easily account for two or even three folding units. Two folding units are likely present also in the HN of other paramyxoviruses [18]. However, only one domain is involved in the binding of the substrate. This raises the question of the compatibility with the three-dimensional structure of paramyxovirus neuraminidases [1–3,19]. The known bacterial and viral neuraminidases all have the same structure [20–23], the so-called β6-propeller. This structure is composed of six four-stranded β-sheets, arranged like the blades of a propeller, with the C- and N-terminal parts forming the sixth β-sheet. Since the amino acids involved in the binding of the substrate, or in the catalytic activity, are located on five of the six β-sheets of the propeller, the substrate binding is expected to affect the stability of the whole domain. The known β-propeller structures are composed of about 380 amino acids, while cHN is composed of 444 amino acids, meaning that if the β-propeller structure is present, the second folding unit should comprise about 100 amino acids. The alignment of the sequence of Sendai virus with that of Newcastle disease virus shows that the β-propeller structure should span from the N-terminal to the C-terminal part of the cHN, which comprises only the residues 132–575 of HN [1,19]. Thus the structure does not allow a second domain at the N- or C-terminal end of the protein. A second, substrate-independent, folding unit should be inserted within a loop of the β-propeller, in a way similar to that suggested for the measles hemagglutinin [3] or for the *Vibrio* neuraminidase [22]. However, sequence alignment does not allow a loop within the β-propeller large enough to account for a second folding domain. The largest domain outside the β-propeller structure is a 40 amino acid α-helical domain between amino acids 340 and 380; however, limited proteolysis shows that this domain, containing Lys 370, can be unambiguously assigned to the substrate binding domain, because the presence of the substrate protects against proteolytic digestion. The limited proteolysis at Lys 370 shows that the only site allowed for a second, substrate-independent, domain is at or near the substrate binding domain. The second domain could be the sub-

unit interface domain; however, the X-ray structure of Newcastle disease virus shows large changes of the subunit interface upon binding of the substrate analogue. The assignment of a transition to changes at the subunit interface cannot be experimentally tested, because the subunits are covalently linked by a disulfide bridge.

While the denaturation experiments are unexpected on the basis of the three-dimensional structure, the evidence of two different folding domains accounts well for the different biological activities of cHN. We have already shown that cHN is composed of two functional domains, a catalytic domain and an inhibitory domain [24], and the presence on Sendai virus of different functional domains has been also suggested by Portner et al. [6,25]. The evidence here reported of two independent folding domains gives a structural basis for the different functions.

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