The disulphide bonds of an Asian influenza virus neuraminidase

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Received 4 January 1983

The arrangement of the disulphide bonds in the pronase-released neuraminidase heads of the Asian influenza virus A/Tokyo/3/67 have been examined by cyanogen bromide fragmentation, enzymic digestion and diagonal peptide mapping. There are 9 intrachain disulphide bridges and one interchain bridge which links pairs of monomers at the distal end of the stalk region of the neuraminidase tetramer. The disulphide bond arrangements of the remaining 3 half-cystine residues in the membrane-embedded stalk region of the neuraminidase were not examined.

Influenza virus neuraminidase *Disulphide bond*

1. INTRODUCTION

The neuraminidase (NA) is the second surface antigen of influenza virus. It represents about 10% [1] of the total surface spikes on the virus and exhibits major (antigenic shift) and minor (antigenic drift) changes in structure during the evolution of new virus strains [2,3].

The influenza NA is a tetramer [4–8] with the characteristic morphology of a mushroom-shaped peplomer having a box-like head (100 × 100 × 60 Å), a narrow, central stalk (15 × 100 Å) and a hydrophobic base which serves to anchor the NA in the viral membrane [9]. It is an integral membrane glycoprotein that can be released from virus particles by detergents, lipid solvents or proteolytic enzymes (review [1]). Proteolytic digestion removes the enzymically-active heads from the membrane-attached stalk region of the enzyme [6,9].

Nucleotide sequence studies [10–13] together with the N- and C-terminal amino acid sequence data [7] show that the N2 neuraminidase monomer consists of a single polypeptide chain of 469 amino acids including 22 half-cystine residues. Comparison of this sequence data for the intact enzyme with that for the pronase-released NA heads [14] shows that the membrane-embedded stalk region of the NA comes from the N-terminal end of the protein and that the head region comprises residues 74–469. Thus of the 22 half-cystine residues in the N2 NA, one (residue 21) occurs in the trans membrane region of the protein, two (residues 42 and 53) occur in the stalk region and nineteen (residues 78, 92, 124, 129, 175, 183, 193, 230, 232, 237, 278, 280, 289, 291, 318, 337, 417, 421 and 447) occur in the head.

Here, we have examined the disulphide bond arrangements for the neuraminidase heads of A/Tokyo/3/67, by cyanogen bromide fragmentation, enzymic digestion and diagonal peptide mapping. The results establish 6 of the 9 intrachain disulphide bridges in the NA heads and indicate that Cys_{78} is involved in an interchain bridge that links pairs of NA monomers at the distal end of their stalk region. This chemical study was carried out to assist the resolution of the 2.9 Å electron density map of the N2 NA being carried out concurrently in this laboratory [8].

2. MATERIALS AND METHODS

Viral neuraminidase (NA) was prepared from A/Tokyo/3/67 influenza virus as in [15]. NA (300 nmol) was digested with CNBr (300 mg) in 4.0 ml of 70% (v/v) formic acid for 18 h at room

Published by Elsevier Biomedical Press
00145793/83/0000–0000/$3.00

temperature. The digest was rotary evaporated, dissolved in 2.0 ml 50% (v/v) formic acid and the disulphide-bonded fragments separated by gel filtration on a column (1.0 cm × 150 cm) of Sephacryl S300 in 50% (v/v) formic acid.

Cyanogen bromide fragments were suspended in 2.0 ml 0.1 M *N*-ethylmorpholine-acetate buffer (pH 7.5). Fragment CN2 was digested with thermolysin (50 μg) for 4 h at 37°C; fragment CN3 and the disulphide-linked complex of CN1–CN4 were each digested with a mixture of trypsin (200 μg) and chymotrypsin (200 μg) for 8 h at 37°C.

Disulphide-bonded peptides were identified and isolated by diagonal peptide mapping at pH 6.5 on Whatman 3MM paper as in [16]. The neutral disulphide-bonded peptides were resolved by diagonal mapping at pH 1.9. Peptides were visualized by staining side strips with fluorescamine [17] or ninhydrin. Mobilities are expressed relative to aspartic acid, $m = -1.0$ [18].

Amino acid analysis was carried out after hydrolysis in 6 N HCl–0.004 N thioglycollic acid at 108°C in vacuo. Dansyl end-group determinations were done as in [16]. Manual Edman degradations were done in 5.0 ml conical quick-fit tubes as in [19], except with 100 μl 50% (v/v) pyridine–0.05% dithiothreitol as coupling buffer, a coupling period of 30 min and a single 1.0 ml *n*-heptane/ethyl acetate (2/1) extraction [20] before drying and cleavage. Phenylthiohydantoins were identified by HPLC on a Hewlett-Packard 1084 B using a Zorbax ODS 4.6 mm × 25 cm column [21].

3. RESULTS AND DISCUSSION

N2 neuraminidase heads contain no free sulphydral groups as judged by their absence of reaction with iodo[^{14}C]acetamide in the native state and after heat denaturation in 6 M urea [22]. Thus all the half-cystine residues in the NA heads are involved in disulphide bonds.

Cyanogen bromide digestion of non-reduced Tokyo/67 heads yielded 3 fragments as shown in fig.1. Amino acid analysis and dansyl-end group analysis showed that the first peak contained CN1 (which has 4 half-cystine residues at positions 78, 92, 124 and 129) and CN4 (which has 3 half-cystines at positions 417, 421 and 447) suggesting at least one disulphide bridge between these two

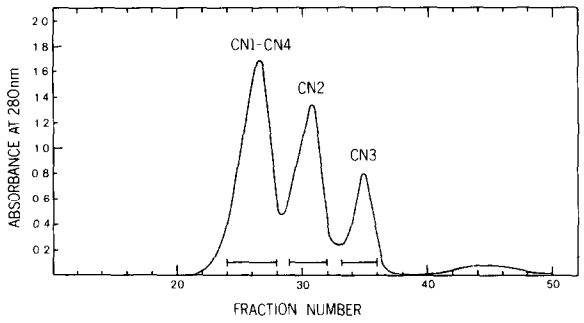

Fig.1. Separation of disulphide-bonded CNBr fragments of A/Tokyo/3/67 neuraminidase heads. The digest was fractionated on a column (1.0 cm × 150 cm) of Sephacryl S300 in 50% (v/v) formic acid at a flow rate of 4.0 ml/h, fraction size 2.0 ml. The eluate was monitored by A_{280}.

peptides. The middle peak consisted of CN2 only, indicating that the 10 half-cystine residues in this region of the molecule (residues 175, 183, 193, 230, 232, 237, 278, 280, 289 and 291) were disulphide bonded within this fragment. Similarly the third peak consisted of peptide CN3 only suggesting that the two half-cystine residues in this fragment, Cys_{318} and Cys_{337}, are involved in one disulphide bond.

Each of these fragments was subjected to enzymic digestion and diagonal peptide mapping. The CN1–CN4 complex (peak 1 in fig.1) and the CN3 fragment (peak 3) were digested with a mixture of trypsin and chymotrypsin to avoid the production of large insoluble peptides [14]. Fragment CN2 (peak 2) was digested with thermolysin. The amino acid compositions, electrophoretic mobilities and amino acid sequences of the disulphide-linked peptides isolated from these three digests are summarized in table 1. The disulphide-bonding arrangements for 13 of the 19 half-cystine residues were established by this study. The bonding arrangements for the remaining 6 were obtained from the recently completed three-dimensional structure of the N2 neuraminidase [8].

The disulphide bonded pairs established chemically were Cys_{92}–Cys_{417}, the only link between the N-terminal peptide CN1 and the penultimate C-terminal peptide CN4; three of the five bonds in CN2, Cys_{183}–Cys_{230}, Cys_{278}–Cys_{291} and Cys_{280}–Cys_{289}, and the single disulphide bond in CN3 involving Cys_{318}–Cys_{337}. Several overlapp-

Table 1

Amino acid compositions, mobilities and sequences of disulphide-bonded peptides from A/Tokyo/3/67 NA heads

Fragment	Peptide	Mobility		Sequence	Cys residue
		Before	After		
CN1–CN4	1	+0.02	−0.36	E I C P K	78
	2a	+0.21	0	S K P Q C Q I(T G F)	92
	2b		0	S C I N R	417
	3	+0.02	−0.48	V S C D P V K C Y	124,129
CN2	4	+0.02	−0.66	V C	175,193 or 232
	5a	+0.12	+0.07	S S S S(C H D G K A W)	183
	5b		−0.30	I L R T(Q E S E C)	230
	$6a_1c$	+0.25	−0.19	L A G S(A Q H V E E C S C Y P R Y P G)	278,280
	$6a_2c$		−0.22	S A Q H(V E E C S C Y P R Y P G)	278,280
	$6a_3c$		−0.22	A Q H V(E E C S C Y P R Y P G)	278,280
	6b		+0.06	I C R D(N W K G)	291
	6d		+0.03	V R C	289
	$6a_4c$	+0.17	−0.48	V E E C(S C Y P R Y P G)	278,280
	6b		+0.06	I C R D(N W K G)	291
	6d		+0.03	V R C	289
	6c	+0.37	+0.03	S C Y P(R Y P G)	280
	6d		+0.03	V R C	289
CN3	7a	+0.25	−0.37	V C S G L	318
	7b		+0.02	S S N S N C R	337

Values are expressed as residues per mole. Mobilities are given for the disulphide-linked peptides before performic acid oxidation and for the constituent cysteic acid-containing peptides after oxidation. Mobilities are given with respect to the mobility of aspartic acid ($m = -1.0$). The amino acid sequence for A/Tokyo/3/67 NA heads is given in [14]

ing thermolytic peptides ($6a_1c$ to $6a_4c$) which contained the two half-cystine residues at 278 and 280 were found linked to peptides 6b and 6d leaving two possible arrangements for the disulphide bonds involving these four half-cystine residues. The isolation of the peptide pair 6c/6d established the bond between Cys_{280}–Cys_{289}. Therefore Cys_{278} should be linked to Cys_{291}.

The CN1 linkage between Cys_{124} and Cys_{129} was not established with certainty since enzymic digestion did not cleave the Lys_{128}–Cys_{129} bond and the two Cys residues remained in a single peptide. However, no other Cys containing peptides were isolated after oxidation of this peptide and the three-dimensional structure [8] confirms the 124–129 linkage.

The two disulphide bonds from CN2 involving Cys residues 175–193 and 232–237 plus the final disulphide bond in CN4 involving residues 421–447 were not established chemically but have been readily identified from the three-dimensional structure [8]. The peptide Val–Cys was isolated in high yield from the neutral peptide fraction of the thermolytic digest of CN2 (table 1) and is consistent with the disulphide linkage between Val–Cys_{175} and Val–Cys_{193}. It could represent Val–Cys_{232} although no other Cys-containing peptide was recovered after oxidation of this fraction.

The N1 [23,24] and type B [25] NA sequences contain 16 of the 19 half-cystine residues present in the N2 heads. They do not contain Cys residues 175 and 193 and thus lack the disulphide bond between these two residues [8]. They also lack the nineteenth Cys residue at position 78 in the N2 structure. This residue is not found in any of the intra-chain disulphide bonds (table 1) but appears

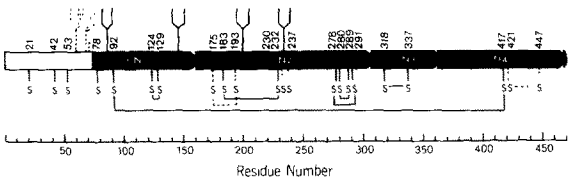

Fig.2. Schematic representation of A/Tokyo/3/67 neuraminidase showing the N-terminal stalk region (residues 1–73) removed by pronase digestion, the location of the five CNBr peptides from the NA heads and the arrangement of the disulphide bonds. The bonds confirmed by chemical analysis are shown by solid lines, the bonds inferred from the three-dimensional structure of the NA [8] are shown by dotted lines. For completeness the location of the four oligosaccharide side chains on the heads and the three potential glycosylation sites on the stalk are also shown.

to be involved in interchain disulphide bonds between adjacent monomeric units in the NA tetramer. This was first suspected when we found [7] different preparations of pronase-released heads contained variable proportions of monomer to dimer when examined on SDS gels without reduction. Since pronase digestion was known to produce ragged-end cleavage in front of residues 74 and 76 [14] it seemed possible that in some preparations pronase digestion could include some cleavage after Cys_{78} resulting in the absence of disulphide-linked dimers in these NA head preparations. In this study no partner was found for peptide 1 (which contains Cys_{78}) in the enzymic digest of CN1–CN4 (table 1) and the elution position of the CN1–CN4 complex on Sephacryl S300 (fig.1) suggests it is present as the dimer. In addition the comparative sequences [10,14,23–25] and three-dimensional structure [8] suggest that Cys_{78} occurs at the top of the stalk region of N2 NA where it can participate in disulphide linkage with the homologous residue on another monomer. The B/Lee/40 NA, which does not contain Cys_{78} and which contains only a single Cys residue in the stalk region at position 54 [25], consists of two disulphide linked dimers when isolated by detergents, but consists of four non-covalently-linked monomers when isolated as trypsin-released heads [5,26]. Other A sub-type NA structures show variable numbers of half-cystine residues in this N-terminal stalk region [27]. These could contribute to multiple disulphide bridges between the extended polypeptide chains of the four identical monomeric units that make up the functional NA peplomer.

ACKNOWLEDGEMENTS

We wish to thank Mr N.W. Cook and Dr N.M. McKern for details of the rapid manual sequencing procedure and the PTH identifications and Dr C.M. Roxburgh for the amino acid analyses.

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