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## ISOELECTRIC FOCUSING STUDIES OF A<sub>2</sub>/1957 INFLUENZA NEURAMINIDASE AND ITS SUBUNITS

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### SUMMARY

Purified A<sub>2</sub>/1957 influenza neuraminidase (mucopolysaccharide *N*-acetylneuraminylhydrolase, EC 3.2.1.18) and its subunits were examined by isoelectric focusing ('electrofocusing') in sucrose gradients. Native neuraminidase contained enzymically active components with isoelectric points (pI) of about 5.2, 5.35, 5.5, 5.8, 6.2 and 6.5. The major components were at about pI 5.5 and 5.8. Neuraminidase was dissociated into subunits, whose sulfhydryl groups were blocked with iodo-<sup>14</sup>C-acetamide. 80% of isotope label incorporated was present in a single size of subunits with a molecular weight (*M<sub>r</sub>*) of 51 000 as determined by sodium dodecyl sulfate-acrylamide gel electrophoresis. The pI of denatured subunits was about 3.6 to 4.4. <sup>14</sup>C-labelled peptides of tryptically digested neuraminidase had predominantly acidic isoelectric points. Results are consistent that the pI of native neuraminidase is about 1.5-2 pH units higher than the pI of its structural subunits, suggesting that side chain carboxyl groups are conformationally masked in the native enzyme, and that isoelectric heterogeneity of neuraminidase may result from conformation-dependent variations in the acid-base dissociation of these groups.

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### INTRODUCTION

The enzyme neuraminidase (mucopolysaccharide *N*-acetylneuraminylhydrolase, EC 3.2.1.18) is present on the envelope of influenza viruses<sup>1,2</sup>. Examination of viruses isolated from epidemics in different years has shown that significant variation in antigenic and enzymic properties of neuraminidase occurs<sup>3-5</sup>. The structure of influenza virus neuraminidase is therefore being characterised so as to determine the relation of this viral protein to other viral components, and to elucidate the chemical basis for observed variation amongst neuraminidases.

In previous studies, neuraminidase of Asian (A<sub>2</sub>/1957) influenza virus has been shown to be a glycoprotein of *M<sub>r</sub>* 200 000, consisting of four similar size subunits that are disulfide linked<sup>6,7</sup>. Further information about physico-chemical properties

of A<sub>2</sub>/1957 influenza virus neuraminidase has now been sought by the technique of isoelectric focusing. Preliminary results of these studies have been described<sup>8</sup>.

## METHODS

### *Isolation of neuraminidase*

A<sub>2</sub>/1957 influenza neuraminidase and its [<sup>14</sup>C]carboxamidomethylated subunits were prepared as previously described<sup>7</sup>.

### *Sodium dodecyl sulfate-acrylamide gel electrophoresis*

This was performed in gels of 7.5% acrylamide, 0.2% bisacrylamide, buffered with 0.1 M phosphate, pH 7.4, containing 0.5 M urea and 0.1% sodium dodecyl sulfate. Samples in pH 7.4 buffer, containing 1% sodium dodecyl sulfate, 0.5 M urea and 1% 2-mercaptoethanol were boiled for 2 min prior to electrophoresis. Mercaptoethanol was omitted when determining the presence of disulfide-linked aggregates. Vesicular stomatitis virus was added to samples when protein size markers were required for molecular weight determination<sup>9</sup>. Electrophoresis was for 8 h at 7.7 mA/gel.

### *Electrofocusing*

This was performed using an ISCO Model 212 electrophoresis apparatus (Instrument Specialties Co., Lincoln, Nebr.). Electrodes were repositioned to the innermost wells of anode and cathode reservoirs, which were blocked off from outer wells, and filled with 50 ml of electrolyte solutions. Anode (top) solution was 0.1% phosphoric acid. Cathode (bottom) solution was 1% NaOH in 30% sucrose. Carrier ampholytes (L.K.B. Instruments) were incorporated at 1% concentration into 30-ml gradients of 0–30% sucrose, which were loaded into the electrophoresis column onto chase solution of 35% sucrose, 1% NaOH. When electrofocusing with pH 3–5, or pH 5–7 ampholytes, pH 3–10 ampholytes were incorporated at 0.4% in the cathode solution, and at 1% in the bottom 4 ml of central column solution, thereby preventing a build-up of low conductivity water between alkaline cathode solution and acidic ampholyte central column solution. Samples in 15% sucrose and 1% of appropriate ampholyte solution were gently pipetted into the middle of the gradients used to load the central electrophoresis column. When used, 6 M urea was incorporated in all solutions. Electrofocusing was carried out at 500 V, for at least 40 h, cooling with tap water (about 15–18 °C). Initial experiments using pH 3–10 ampholyte gradients showed that isoelectric points of native neuraminidase and neuraminidase subunits were in the regions of pH 5–6 and pH 3.5–4.5, respectively.

### *Tryptic digestion*

[<sup>14</sup>C]Carboxamidomethylated neuraminidase subunits (about 10–50 µg) were incubated for 5 h at pH 8.5 and 35 °C with 10 µg of L-(tosylamido 2-phenyl)ethyl-chloromethyl ketone-treated trypsin (Worthington) in the presence of 2·10<sup>-4</sup> M CaCl<sub>2</sub>.

### *Determinations*

The pH of undiluted electrofocusing fractions was determined with a combi-

nation fibre junction electrode (Beckman Instruments No. 39013) and digital pH meter. Values from experiments using 6 M urea were corrected by the factor of 0.56 pH unit, this representing the elevation in pH of 1% ampholyte solution measured in the presence of urea.

Neuraminidase activity was determined with fetuin substrate<sup>7</sup>. 5- $\mu$ l aliquots of electrofocusing fractions were incubated with substrate at 37 °C for 1 h before determining released sialic acid.

Radioactivity in aqueous samples was determined using an emulsion liquid scintillation sample preparation system<sup>7</sup>. Acrylamide gels were frozen to -70 °C, thawed and sliced into 1.5-mm segments. These were prepared for scintillation counting by heating to 50 °C for 2 h with 0.5 ml of 90% NCS solubiliser (Amersham-Searle) and then adding 10 ml of PPO-POPOP-toluene scintillator.

## RESULTS

### *Dissociation of neuraminidase into subunits*

To verify for the present experiments that neuraminidase was being adequately dissociated into subunits by the [<sup>14</sup>C]carboxamidomethylation procedure used<sup>7</sup>, samples were examined by sodium dodecyl sulfate-acrylamide gel electrophoresis. Fig. 1 shows that under non-reducing conditions about 80% of the <sup>14</sup>C-labelled neuraminidase electrophoresed as a single sharp peak of  $M_r$  51 000, with a minor peak of  $M_r$  about 100 000 also present. When samples were prepared for electrophoresis under reducing conditions, the minor peak was virtually eliminated, but mobility of

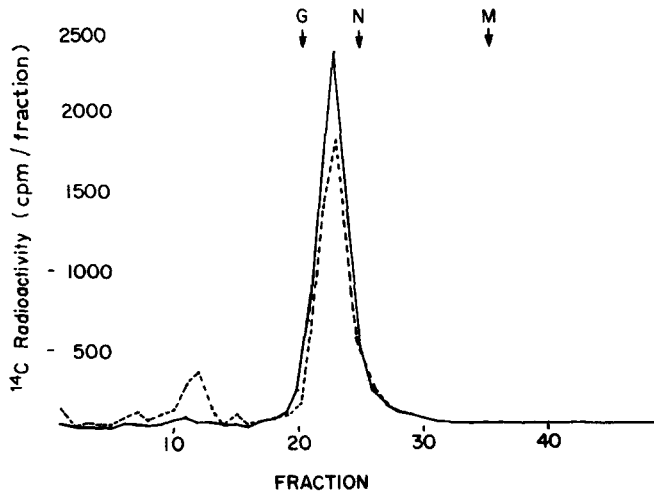


Fig. 1. Dodecyl sulfate-acrylamide gel electrophoresis of neuraminidase subunits. <sup>14</sup>C-Labelled neuraminidase samples were prepared for electrophoresis in the presence (—) or absence (---) of reducing agent, and analyzed on parallel gels. Similar results were obtained with three preparations of labelled neuraminidase. Arrows indicate the positions of <sup>3</sup>H amino acid labelled glycoprotein (G), nucleoprotein (N) and matrix protein (M) of vesicular stomatitis virus, included as internal size marker. Most recent estimates of the molecular weights of G, N and M proteins are about 60 000, 45 000, and 25 000, respectively (ref. 16; Kiley, M. P. and Kendal, A. P., unpublished).

the major component was unaffected (Fig. 1). As previous studies indicated a size of about 50 000 daltons for neuraminidase subunits<sup>7</sup>, it was concluded that 80% of the <sup>14</sup>C-labelled neuraminidase was in the subunit form.

### Isoelectric focusing

*Native neuraminidase.* This was examined using pH 5–7 gradients. The main peak of enzymic activity was consistently between pH 5.5 and 5.9, with lesser peaks in the ranges pH 4.9–5.3, and pH 6.2–6.8 (Fig. 2A). Shoulders to these peaks suggested that neuraminidase preparations contained overall at least six components, with isoelectric points (pI) of about 5.2, 5.35, 5.5, 5.8, 6.2 and 6.5 or higher. Each isoelectric component of neuraminidase was chromatographed on a Sephadex G-200 column, and eluted with the same  $K_{AV}$  (0.33) as unfractionated neuraminidase, indicating that neuraminidase had not formed stable aggregates or enzymically active subunits during electrofocusing.

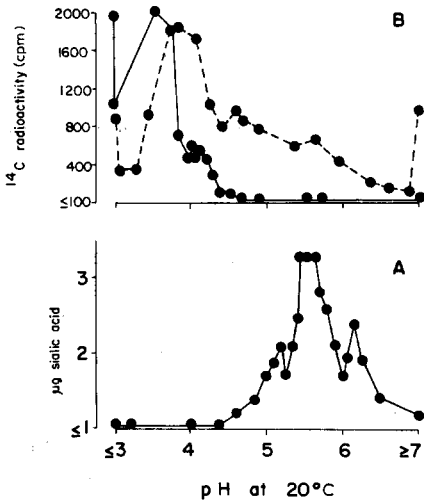


Fig. 2. Electrofocusing of A<sub>2</sub>/1957 influenza neuraminidase and its subunits. (A) Native neuraminidase enzymic activity (●—●). (B) [<sup>14</sup>C]Carboxamidomethylated neuraminidase subunits, in the presence of 6 M urea (●—●); tryptically digested [<sup>14</sup>C]carboxamidomethylated subunits (●---●).

*Neuraminidase subunits.* [<sup>14</sup>C]Carboxamidomethylated subunits were electrofocused in pH 3–5 gradients containing 6 M urea to minimize polypeptide folding. Components were consistently identified with pI values of about 4.4, 4.0, 3.8 and 3.6, and often also in the acidic solution (pH ≤3) adjacent to the anode (Fig. 2B). The subunits clearly had a lower pI than native neuraminidase. Sephadex G-200 chromatography indicated that the denatured subunits, recovered after electrofocusing, had aggregated. The amount of protein present in these experiments (10–50 μg) was too small to precipitate during electrofocusing, since the aggregated subunits were found at a pI near the top of the electrofocusing column. However, formation of micro-aggregates may have contributed to isoelectric heterogeneity of the denatured subunits. To examine whether aggregation might also have caused the low pI observed

for the subunits, trypsin-digested  $^{14}\text{C}$ -labelled subunits were electrofocused. The labelled cysteinyl peptides (about 40% of the total peptides predicted from trypsin-digested neuraminidase<sup>7</sup>), also had predominantly acidic isoelectric points, between pH 3 and pH 5 (Fig. 2B). This is consistent with the structural subunits of neuraminidase having a lower isoelectric point than the native enzyme.

#### DISCUSSION

The present results, and those of Neurath *et al.*<sup>10</sup>, indicate that isolated neuraminidases from several strains of influenza virus are isoelectrically heterogeneous. This cannot readily be attributed to digestion by proteolytic enzymes during the procedure for isolating neuraminidase since, (1) detergent-solubilized neuraminidase is also isoelectrically heterogeneous<sup>10</sup>, and (2) neuraminidase isolated with the aid of proteolytic enzymes has a quite homogeneous subunit size, judged by dodecyl sulfate-acrylamide gel electrophoresis (ref. 7, and Fig. 1).

Isoelectric heterogeneity of neuraminidase is also unlikely to be due to the presence of varying amounts of sialic acid on the molecule, as found with many other glycoproteins. Neuraminidase itself liberates sialic acid from glycoproteins, including those at the membrane where virions mature from influenza-infected cells<sup>11</sup>.

However, isoelectric heterogeneity might result from variable degrees of conformation-dependent interactions which can cause anomalous acid-base dissociation of ionizing groups in native proteins<sup>12</sup>. Indirect evidence to support this possibility is provided by calculating the theoretical net electrical charge on neuraminidase from its amino acid composition. Table I shows that if normal intrinsic dissociation constants ( $pK_{\text{int}}$ ) are assumed for ionizing amino acid side chains, there is a large theoretical net negative charge on the molecule at pH 5.5. Since pH 5.5 corresponds to the major isoelectric region of native neuraminidase (Fig. 2A) it appears that many side-chain carboxyl groups are masked in the native enzyme, as also suggested experimentally by the large drop in isoelectric point when neuraminidase is denatured.

Several bacterial neuraminidases are isoelectrically homogeneous<sup>10,13</sup>. Since these bacterial enzymes have a molecular weight of only 55 000–70 000, comprising a single subunit<sup>13,14</sup>, it is possible that the isoelectric heterogeneity of many native

TABLE I

THEORETICAL NET ELECTRIC CHARGE ON INFLUENZA NEURAMINIDASE ( $A_2/1957$ ) AT pH 5.5

Amino acid ionizing group*	$pK_{\text{int}}$ **	Number/subunit ( $n_i$ )	Degree of dissociation at pH 5.5 ( $x_i$ )	Total charge/subunit***
Side chain carboxyl	4.7	114	0.86	-98
Imidazole	6.5	10	0.09	+ 9
$\epsilon$ -Amino	> 10	20	0	+ 20
Guanidyl	> 10	30	0	+ 30
Net charge/subunit = $\sum n_i z_i$		-39		

\* N- and C-terminal amino acids were considered insignificant. Amino acid composition was from ref. 7.

\*\* Ref. 12.

\*\*\*  $z_i = 1-x_i$  for cationic groups, or  $z_i = -x_i$  for anionic groups.

influenza neuraminidases is a consequence of their larger size and more complex tetrameric subunit structure<sup>6,7,15</sup>.

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