Influenza Virus Haemagglutinin: Estimation of Tryptophan and Tyrosine Content and Localization of Tryptophan Residues

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SUMMARY

The tryptophan and tyrosine content of the bromelain-released subtype H3 haemagglutinin (H3 BHA) of influenza virus were measured by u.v. absorption and fluorescence techniques. The values obtained (8 and 18 residues, respectively) are in close agreement with those derived from amino acid analysis. Essentially all of the tryptophan residues are demonstrated to be localized on the surface of the BHA molecule.

The haemagglutinin represents the major surface glycoprotein of the influenza virion. It plays a dominant role in the adsorption of virus particles on the mucopolysaccharide receptors of cellular membranes and an, as yet, not completely clear function in the process of the virus reproduction in the infected cell (reviewed by Schulze, 1975). The glycoprotein molecule (HA) involved in the formation of haemagglutinin is synthesized as a primary gene product with a mol. wt. of 75,000 to 80,000. However, HA is usually cleaved (presumably by one of the host cell proteolytic enzymes) to yield two polypeptides: HA₁ (mol. wt. approx. 55,000) and HA₂ (mol. wt. approx. 25,000). Both HA₁ and HA₂ are glycoproteins with carbohydrate side chains which are synthesized and covalently linked to the HA polypeptide by host cell transferases. The HA₁ and HA₂ portions of HA are held together by disulphide bonds. According to recent data (Wiley et al. 1977; Siniakov et al. 1980), the haemagglutinin molecule is a trimer of HA or HA₁+HA₂ subunits. However, the details of its secondary and tertiary structure are still largely unknown.

This paper describes the determination of the tyrosyl and tryptophanyl content of H3 influenza virus haemagglutinin and the localization of the latter residues in the HA molecule by optical methods.

The high-egg-yielding recombinant of the prototype strain A/Port Chalmers/73 (H3N2) (MRC-11 recombinant) was propagated, isolated and purified as previously described (Kharitonenkov et al. 1977). BHA (bromelain-released haemagglutinin) was isolated from virus particles by the method described by Brand & Skehel (1972). The purity of BHA preparations was determined by polyacrylamide gel electrophoresis (Laemmli, 1970), electron microscopy and neuraminidase assay (Warren, 1959). After purification, the BHA solution was exhaustively dialysed against double-distilled water and then lyophilized.

The u.v. absorption spectra of BHA and reference protein solutions were recorded with a Pye-Unicam SP-1800 spectrophotometer. The tyrosine/tryptophan ratios (Ctyr/Ctrp) for BHA and reference proteins were calculated from the u.v. absorption spectra (Goodwin & Morton, 1946). Correction for light scattering was made by the extrapolation method (England & Epstein, 1957).

The tryptophan content of BHA and reference proteins was determined from the fluorescence spectra (Pajot, 1976). The method is based on measuring the fluorescence of denatured protein molecules with reduced hydrogen bonds in 6 M-guanidine hydrochloride in the presence of 2-mercaptoethanol. Fluorescence measurements were made with a Hitachi
Fig. 1. The fluorescence properties of H3 BHA. (a) Fluorescence spectra of BHA (164 μg/ml in 1 M-KCl + 2 mM-HEPES + 2 mM-Na-citrate, pH 7.5) excited at 280 nm (---) and at 297 nm (----) and the fluorescence spectrum of BHA in the same buffer in the presence of 6 M-guanidine hydrochloride (○—○); (b) determination of tryptophan content of reference proteins by fluorescence measurements and the correlation of these data with tryptophan content known from amino acid sequencing data. The abscissa represents the magnitudes obtained from amino acid sequencing data, and the ordinate the data for the same proteins measured by the fluorescence method: 1 = cytochrome c; 2 = bovine serum albumin; 3 = soybean trypsin inhibitor; 4 = deoxyribonuclease; 5 = trypsin; 6 = pepsin; 7 = lysozyme; 8 = α-chymotrypsinogen; 9 = α-chymotrypsin; 10 = aldolase. Filled circles = the data reported in Pajot's paper (1976); open circles = the data obtained in this work. The dotted line limits the values for H3 BHA; (c) modified Stern-Volmer plot of the quenching of H3 BHA fluorescence by iodide ions in 2 mM-HEPES + 2 mM-Na-citrate solution, pH 7.5. The fluorescence was measured at 340 nm for six different BHA samples (0.22 mg/ml) with different concentrations of KI (the ionic strength was compensated by KCl solution).

MPF-2 fluorometer using 0.5 cm diam. quartz cuvettes. Protein and tryptophan concentrations were measured using the values of molar extinction coefficients reported by Pajot (1976). Studies on the intrinsic fluorescence quenching of the BHA tryptophan residues by iodide ions were performed in the presence of 10^{-4} M-Na_{2}S_{2}O_{3} to prevent I^{-} formation (Pelley & Horowitz, 1976). The details of these experiments are given in the corresponding figure legend.

The tyrosine/tryptophan ratio for the H3 BHA molecule was determined by the Goodwin & Morton (1946) procedure based on the analysis of the u.v. absorption spectrum of the protein solution under study, in 0.1 M-NaOH. The accuracy of the method used was tested for several proteins with known primary structure, i.e. with known C_{tyr}/C_{trp} ratios. The
Table 1. Tyrosine and tryptophan content of H3 HA and H3 BHA

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>H3 HA</th>
<th></th>
<th></th>
<th>H3 BHA</th>
</tr>
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<tbody>
<tr>
<td>Tyrosine</td>
<td>HA₁</td>
<td>11·0 (11)</td>
<td></td>
<td>18·9 (19)</td>
</tr>
<tr>
<td></td>
<td>HA₂</td>
<td>7·9 (8)</td>
<td></td>
<td>8·5 (9)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>18·9 (19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tryptophan</td>
<td>HA₁</td>
<td>4·8 (5)</td>
<td></td>
<td>3·7 (4)</td>
</tr>
<tr>
<td></td>
<td>HA₂</td>
<td>3·7 (4)</td>
<td></td>
<td>8·5 (9)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>8·5 (9)</td>
<td></td>
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</tr>
</tbody>
</table>

* Data from Ward & Dopheide (1976).
† Data from the present work.
‡ The integral values are presented in parentheses.

The content and localization of tryptophan residues in H3 BHA was estimated by the fluorescence techniques. Fig. 1(a) represents the fluorescence spectra (excited at 280 and 297 nm) of H3 BHA. The wavelength 280 nm is known to excite the fluorescence of both tyrosine and tryptophan residues, whereas only the tryptophan fluoresces at 297 nm. The spectrum of BHA fluorescence has an intensity maximum at 343 nm ($\lambda_{\text{max}} = 343$ nm) and half-width ($\Delta \lambda$) equal to 60 nm. These values imply that the majority of tryptophan residues in the haemagglutinin of influenza virus are located at the surface of the BHA molecule since the fluorescence spectra of proteins with external localization of all tryptophan residues have almost identical parameters: $\lambda_{\text{max}} = 340$ to 342 nm and $\Delta \lambda = 53$ to 55 nm. It seems unlikely that the tryptophan residues are mostly situated in the internal hydrophobic regions of the BHA molecule (for this kind of protein $\lambda_{\text{max}} = 330$ to 332 nm and $\Delta \lambda = 48$ to 50 nm) or in the regions with random coil structure ($\lambda_{\text{max}} = 350$ to 353 nm, $\Delta \lambda = 59$ to 61 nm; Vedenkina & Burstein, 1970). The fact that under the denaturation conditions employed (6 M-guanidine hydrochloride) the fluorescence maximum displaces to 354 nm (Fig. 1(a)) indicates the native structure of the original H3 BHA.

The tryptophan content of H3 BHA was determined according to Pajot (1976). The number of tryptophan residues per protein molecule was determined after extrapolation of the dependence of the fluorescence intensity observed at 340 nm (excited at 297 nm) on the concentration of free tryptophan residues to zero fluorescence. Using the calibration curve shown in Fig. 1(b) it was possible to calculate the tryptophan residues content of H3 BHA; this was found to be equal to 8·0 ± 0·5. Using this value and a tyrosine/tryptophan ratio of 2·28 it was possible to estimate the tyrosine content of H3 BHA (18·2 ± 1·1 per BHA molecule). It was of interest to compare our results with those obtained by Ward & Dopheide (1976) for 'complete' H3 haemagglutinin (H3 HA) isolated from A₉/Memphis/102/72 (see Table 1). Their values for tryptophan content were derived from the extrapolation of amino acid analysis data to zero time. The difference in tryptophan content between H3 HA and H3 BHA seems real and probably reflects a loss of one tryptophan residue during bromelain treatment. For tyrosine, the error limits of ± 1·1 make the comparison less certain, but still suggest a loss of one tyrosine residue during bromelain digestion of influenza virus H3 haemagglutinin.

The quenching of protein fluorescence by ions and radicals is widely used for investigation of the aromatic amino acid localization in the protein molecule (Shinitzky & Rivnay, 1977; Ingham et al. 1976). In this work we used iodide ions to quench the tryptophan fluorescence. The quenching of the tryptophan fluorescence of BHA by iodide gives a linear plot in the modified Stern-Volmer relationship (Lehrer, 1971):
where $F_0$ and $F$ are the fluorescence intensities in the absence and presence of a quencher at a molar concentration $[I^-]$, $f_a$ is the fraction of tryptophan residues accessible to quenching by iodide and $K_0$ is the quenching constant; $f_a$ can be estimated from the intercept of the straight line $(F_0/F_0 - F)$ v. $[I^-]$ with the ordinate scale (Fig. 1 c). The H3 BHA at pH 7.5 has an $f_a$ close to 1.0. It appears that almost 100% of tryptophan fluorescence is accessible to quenching by iodide and hence that most of the tryptophanyl residues of BHA are located on the surface of the molecule. The same conclusion had been made after analysis of BHA fluorescence parameters presented in this work.

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REFERENCES


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