Characterization of the Carboxypeptidase Involved in the Proteolytic Cleavage of the Influenza Haemagglutinin

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(Accepted 29 June 1983)

SUMMARY

The arginine carboxypeptidase involved in the proteolytic cleavage of the haemagglutinin of influenza A virus has been analysed by an assay employing a Sepharose-bound peptide containing radioactive arginine as a substrate. The enzyme activity has been extracted from purified virus with non-ionic detergents and has been separated from the haemagglutinin and from the neuraminidase by isoelectric focusing and by affinity chromatography. The carboxypeptidase present in virus grown in different host cells shows variations in its isoelectric point. It can be concluded from these observations that the carboxypeptidase is a host component incorporated into the virus envelope. When the enzyme is inhibited by 2-mercaptomethyl-3-guanidinoethylthiopropanoic acid, haemagglutinin with the arginine attached to the carboxy terminus of HA1 can be obtained. The observation that under these conditions the haemagglutinin has retained its haemolytic activity indicates that the carboxypeptidase does not play an essential role in the activation process.

INTRODUCTION

Processing of the haemagglutinin (HA) of influenza virus involves post-translational proteolytic cleavage of the precursor HA into the two fragments HA1 and HA2 (Lazarowitz et al., 1971). This modification is essential for infectivity (Klenk et al., 1975; Lazarowitz & Choppin, 1975), because only cleaved haemagglutinin mediates the fusion of the viral envelope with cellular membranes (Huang et al., 1980, 1981; Maeda & Ohnishi, 1980; White et al., 1981, 1982).

It has long been known that trypsin or a trypsin-like endoprotease furnished by the host is involved in the activation of the haemagglutinin (Lazarowitz et al., 1971). The elucidation of the primary structure of haemagglutinin has shed further light on the cleavage mechanism. The sequence studies indicated that, with most haemagglutinin subtypes, the endoprotease cleaves between the amino terminus of HA2 and an adjacent arginine residue that is lost in the cleavage reaction (for review, see Ward, 1981). In the case of the haemagglutinin of fowl plague virus (FPV), HA1 and HA2 are linked by a basic peptide (Porter et al., 1979) which is also removed during cleavage (Garten et al., 1982). Evidence has been obtained that these differences in the cleavage site play an important role in determining the pathogenicity of avian influenza strains (Bosch et al., 1981). From the observation that the intervening arginine or peptide is eliminated, it had to be concluded that after the trypsin-like protease an arginine carboxypeptidase is involved in the cleavage. Furthermore, the finding that elimination occurs when the haemagglutinin is cleaved in vitro by trypsin as the only enzyme to purified virus, indicated that the carboxypeptidase is a constituent of the virus particle (Garten et al., 1981). It was not clear, however, whether this enzyme is a host component tightly associated with the virion or whether it is encoded by the viral genome.

The amino terminus of HA2 appears to be a critical site for the fusion capacity of the haemagglutinin. This was indicated by the observation that peptides analogous to the amino terminus inhibited infectivity (Richardson et al., 1980). Furthermore, when the amino terminus
is shifted by one or three amino acids in the carboxy-terminal direction, as occurs after cleavage with thermolysin or chymotrypsin, the haemagglutinin is not activated (Garten et al., 1981). Since after cleavage with these enzymes the intervening arginine is not eliminated the possibility exists that the carboxypeptidase also plays a role in activation.

This study was undertaken to throw light on the origin and the biological significance of the arginine carboxypeptidase present in influenza A virus. A new radioassay was developed to identify the enzyme in detergent extracts of virions. The enzyme could be separated from the haemagglutinin and the neuraminidase and from the other viral proteins, and evidence for its host specificity was obtained. Inhibition of the enzyme activity did not prevent the activation of the fusion capacity.

**METHODS**

**Viruses and cells.** The influenza A strains A/WSN/33 (H1N1), A/chick/Germany/49 (H10N7) (virus N) and A/FPV/Rostock/34 (H7N1) were grown in the allantoic cavities of 11-day-old embryonated eggs for seed stocks. FPV was also propagated in embryonated eggs for isolation of virus. Chick embryo (CE), MDBK, and BHK-21-F cells were grown in Dulbecco's medium, and used for propagation of the viruses.

**Virus purification.** Viruses were collected from the allantoic fluid or from cell culture medium and purified on sucrose gradients as described by Klenk et al. (1972), except for the strain WSN which was purified on potassium tartrate gradients (Compans et al., 1970).

**Haemagglutination titration and neuraminidase assay.** These were carried out according to standard procedures (Davenport et al., 1980). Before haemagglutination titration and neuraminidase assay octylglucoside was removed by dialysis.

**Haemolysis.** This was carried out at acidic pH as described previously (Huang et al., 1981).

**Limited proteolytic cleavage.** Proteolytic cleavage of the haemagglutinins of strains WSN and N was performed in vitro with 10 μg/ml trypsin (TPCK-treated; Worthington) or thermolysin (Boehringer Mannheim), for 30 min at 37 °C as described previously (Klenk et al., 1975; Garten et al., 1981).

**Disruption of virus with non-ionic detergents.** The envelope proteins were solubilized in phosphate buffer (10 mM, pH 7-4) containing 2% n-octylglucoside or Triton X-100 and separated from the other viral proteins by centrifugation (30000 rev/min for 30 min in a SW41 rotor) (Kohama et al., 1981). When virus extracts were measured directly for carboxypeptidase activity by radioassay, the phosphate buffer was replaced by Tris-HCl buffer (10 mM, pH 7-4).

**SDS-polyacrylamide gel electrophoresis (SDS–PAGE).** Electrophoresis was carried out essentially as described by Laemmli (1970) using either 10% gels or 10% to 13% polyacrylamide gradient gels for separation of the proteins. Polyacrylamide gels were stained either with Coomassie Brilliant Blue R-250 or with silver (Oakley et al., 1980) or sequentially with both dyes.

**Isoelectric focusing on sucrose density gradients.** Solubilized viral envelope proteins were separated by isoelectric focusing as described for Newcastle disease virus (NDV) glycoproteins (Kohama et al., 1981). Isoelectrofocusing was carried out in sucrose gradients for 22 h using 1-5% Ampholines (pH 4 to 8; LKB) and 1% n-octylglucoside. The experiments were done on an LKB 8101 column (110 ml). Fractions (3 ml) of the gradient were collected and dialysed against Tris–HCl buffer (10 mM, pH 7-4) to remove sucrose, n-octylglucoside, and ampholytes.

**Two-dimensional isoelectric focusing and electrophoresis.** Isoelectric focusing and electrophoresis of virus proteins were performed as described previously (Garten et al., 1981).

**Radioassay for arginine carboxypeptidase activity.** A simple and highly sensitive assay was developed using matrix-bound ε-aminocaproyl-L-isoleucyl-L-[3H]arginine as a substrate. This was prepared by coupling of ε-aminocaproic acid to cyanogen bromide-activated Sepharose 4B (Cuatrecasas, 1970). L-Isoleucine and L-[3H]arginine were attached by the procedure using N-hydroxysuccinimide esters of the carboxy groups of ε-aminocaproic acid and peptides, which were elongated from the matrix (Cuatrecasas & Parikh, 1972). L-[3H]Arginine, which was added at the concentration of 10^{-5} M (1 Ci/mol), was coupled at 0-5 μmol per ml of packed agarose. Samples to be tested for carboxypeptidase activity (0-1 ml) were mixed in microvials (Eppendorf) with 0-3 ml Tris–HCl buffer (10 mM, pH 7-4) containing cobalt acetate (5 mM), n-octylglucoside (0-1%), and a suspension of ε-aminocaproyl-L-isoleucyl-L-[3H]arginine–Sepharose 4B (0-01 μCi to 0-1 μCi). The agarose beads were agitated during incubation at 37 °C for 1 h and then pelleted by centrifugation at 2000 g for 2 min. Aliquots (0-1 ml) of the supernatants were withdrawn. Each sample was mixed with 10 ml Bifluor emulsifier cocktail (New England Nuclear), and radioactivity was measured in a scintillation counter. All values were derived from double determinations.

**Affinity chromatography on d-arginine coupled to agarose.** D-Arginine was coupled to ε-aminocaproyl-L-isoleucyl-agarose which is identical to the component used for preparing the substrate of the radioassay for the carboxypeptidase. In this case, L-[3H]arginine was substituted by D-arginine for preparing the affinity beads. The
amount of coupled D-arginine was roughly 0.7 μmol per ml of settled wet gel. The affinity gel (5 ml in a 10 ml plastic syringe) was equilibrated with Tris–HCl buffer (10 mM, pH 7-4) containing 0.1% octylglucoside. Samples containing carboxypeptidase were dissolved in the equilibration buffer and passed 10 times through the column. Unbound material was washed away with the equilibration buffer. Carboxypeptidase bound to the column was eluted by adding 50 mM-L-arginine to the equilibration buffer. Fractions of 1.5 ml were collected, and dialysed against 10 mM-Tris–HCl pH 7-4 prior to enzyme assays. The whole procedure was performed at 4 °C.

Affinity chromatography on sulphanilic acid coupled to agarose. The method described previously (Huang, 1974; Bosch et al., 1980) was modified as follows. Three ml of settled gel was equilibrated with Tris-HCl buffer (0.01 M, pH 7.4) containing 0.1% n-octylglucoside. Octylglucoside extracts of virions clarified by ultracentrifugation (30 min, 100000 g) were passed through the column. The unbound proteins were eluted with the equilibration buffer in fractions of 2 ml until haemagglutinin could no longer be detected. Then the pH was raised to 9.2 (Tris-HCl, 0.01 M) for elution of the neuraminidase.

Materials. 1,10-Phenanthroline, iodoacetamide, 4-hydroxymercuribenzoic acid (sodium salt), phenylmethylsulphonyl fluoride (PMSF), N-α-tosyl-L-lysylchloromethyl ketone (TLCK) and z-aminocaproic acid were obtained from Serva (Heidelberg, F.R.G.); leupeptin was from Boehringer Mannheim; L-arginine, D-arginine, spermidine, agmatine sulphate, L-canañanine sulphate and L-argininic acid were from Sigma; 2-mercaptomethyI-3-guanidinoethyl-thiopropanoic acid was from Calbiochem; porcine carboxypeptidase B was from Worthington; [5(n)-3H]arginine monohydrochloride (28 Ci/mmol) was from Amersham Buchler. α-N-Bromoacetyl-D-arginine was prepared according to Plummer (1971).

RESULTS

The origin of the arginine carboxypeptidase associated with virus particles

In a previous study, we were not able to detect carboxypeptidase activity in virions by a conventional spectrophotometric assay with hippuryl-L-arginine as substrate (Garten et al., 1981). Therefore, we developed a radioassay for this enzyme, which was 100- to 1000-fold more sensitive. An arginine carboxypeptidase associated with the virus was found using this assay in the presence of the non-ionic detergents Triton X-100 or n-octylglucoside. Virus was disrupted by the detergents and the soluble viral envelope proteins were separated from the nucleocapsid and M protein by ultracentrifugation. More than 90% of the arginine carboxypeptidase could be solubilized by this procedure; less than 10% remained in the solid virus material. The enzyme activity could also be detected in intact virus, when [3H]arginine_labelled peptide not bound to a matrix was used as a substrate (W. Garten & H.-D. Klenk, unpublished data). The kinetics of the enzyme reaction using virus disrupted with n-octylglucoside are shown in Fig. 1. Arginine was released at the same rate regardless of whether virus contained uncleaved haemagglutinin or haemagglutinin that was cleaved by different proteases. The envelope proteins of virus N solubilized with n-octylglucoside were separated by isoelectric focusing (Fig. 2). The carboxypeptidase activity is well separated from the haemagglutinin, but overlaps with the fractions that contain neuraminidase activity. The protein pattern of each fraction was analysed by polyacrylamide gel electrophoresis. Only the haemagglutinin and the neuraminidase were detected in the respective fractions when the gels were stained with Coomassie Brilliant Blue. However, in each fraction additional bands could be seen when the gels were subsequently stained by the silver stain technique (data not shown).

It was next of interest to find out whether or not the carboxypeptidase is associated with the neuraminidase. To this end, n-octylglucoside extracts of virus N were passed through an affinity column containing agarose-linked sulphanilic acid that binds specifically the neuraminidase at neutral or acidic pH (Huang, 1974; Bosch et al., 1980). Haemagglutinin and carboxypeptidase were not bound to the ligand whereas neuraminidase was selectively retained at pH 7-4 and was only released by raising the pH to 9.2 (Fig. 3). The results of the experiments shown in Fig. 2 and 3 indicate that the arginine carboxypeptidase is not identical with the viral glycoproteins. We then made an attempt to isolate the enzyme by affinity chromatography specific for arginine carboxypeptidases. This was done by passing an n-octylglucoside extract from virus N through a column containing D-arginine coupled to agarose (Fig. 4). Unbound material was eluted until haemagglutinin and neuraminidase activities decreased to background level. The arginine carboxypeptidase was specifically eluted by adding L-arginine to the elution buffer. A fraction of
Fig. 1. Time course of hydrolysis of ε-aminocaproyl-L-isoleucyl-L-[3H]arginine–Sepharose 4B by virus-associated arginine carboxypeptidase and pancreatic carboxypeptidase B. n-Octylglucoside-solubilized viral envelope proteins from virus N which was grown in CE cells and either treated with trypsin (△), thermolysin (■) or left untreated (▲) were incubated with the substrate–agarose beads for different time intervals. In control experiments, pancreatic carboxypeptidase (0-2 units/ml) (○) or as a blank Tris-HCl buffer (10 mM, pH 7.4) (●) were added to the substrate. Aliquots of the samples were withdrawn, acidified with acetic acid, and analysed for released arginine as described in Methods.

Fig. 2. Separation of the envelope proteins of virus N by isoelectric focusing on density gradients. Virus was grown in CE cells. Viral envelope proteins were solubilized in 2% n-octylglucoside. Isoelectric focusing was carried out as described in Methods. The pH (...) was determined in each fraction. Aliquots of each fraction were taken for haemagglutination titration (○) and for the enzyme assays determining neuraminidase activity (absorbance at 549 nm, ■) and arginine carboxypeptidase activity measured by the release of L-[3H]arginine (▲). For other details see Methods.
Fig. 3. Separation of virus envelope proteins on sulphanilic acid coupled to agarose. Virus N was grown in CE cells. Viral envelope proteins were solubilized in 2% n-octylglucoside and applied to the affinity column (1.3 × 3.5 cm) at pH 7.4. The equilibration buffer, 10 mM-Tris–HCl pH 7.4 containing 0.1% n-octylglucoside, was used for elution of all viral envelope proteins except for the neuraminidase. At fraction no. 8, the pH of the equilibration buffer was adjusted to 9.2. Each fraction (1.5 ml) was analysed for haemagglutination (○), neuraminidase activity (■) and carboxypeptidase using the [3H]arginine release assay described in Methods (▲).

Fig. 4. Chromatography of the virus-associated carboxypeptidase on ε-aminocaproyl-L-isoleucyl-D-arginine-Sepharose 4B. Virus N was grown on CE cells. The virus envelope proteins were dissolved by 2% n-octylglucoside and the sample was applied to the affinity gel. For more details, see Methods. Aliquots of the fractions were analysed for haemagglutinin (○), neuraminidase (■) and arginine carboxypeptidase (▲) as described in Methods. Inset: (a) SDS-PAGE of the total n-octylglucoside extract from virus N applied to the column and stained by Coomassie Brilliant Blue; (b) fraction no. 13 containing carboxypeptidase activity analysed electrophoretically on a 10 to 13% acrylamide gradient gel and stained by silver. Protein markers used for mol. wt. determination were myosin (220K), β-galactosidase (130K), phosphorylase B (94K), catalase (60K), carboxypeptidase A (34K) and myoglobin (17K).
Table 1. Isoelectric points of the haemagglutinin and of the carboxypeptidase depending on the virus strain and the host cell

<table>
<thead>
<tr>
<th>Virus*</th>
<th>Host</th>
<th>Haemagglutinin</th>
<th>Carboxypeptidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>CE</td>
<td>5-7</td>
<td>6-7</td>
</tr>
<tr>
<td></td>
<td>MDBK</td>
<td>5-4</td>
<td>8-5</td>
</tr>
<tr>
<td></td>
<td>BHK</td>
<td>5-4</td>
<td>8-2</td>
</tr>
<tr>
<td>WSN</td>
<td>CE</td>
<td>6-0</td>
<td>6-7</td>
</tr>
<tr>
<td></td>
<td>MDBK</td>
<td>6-1</td>
<td>8-5</td>
</tr>
<tr>
<td>FPV</td>
<td>CE</td>
<td>6-3-6-6†</td>
<td>6-7</td>
</tr>
<tr>
<td></td>
<td>Egg</td>
<td>6-0-6-7†</td>
<td>6-2-6-7‡</td>
</tr>
<tr>
<td></td>
<td>MDBK</td>
<td>6-3-6-8‡</td>
<td>8-5</td>
</tr>
</tbody>
</table>

* Influenza strains A/chick/Germany/49 (H10N7)(N), A/WSN/33 (H1N1) and A/FPV/Rostock/34 (H7N1) were propagated in different cells and in embryonated eggs. The purified virions were disintegrated with 2% n-octylglucoside. The solubilized viral envelope proteins were subjected to isoelectric focusing in sucrose gradients as shown in Fig. 2 for virus N.

† The wide range of the isoelectric points of the FPV haemagglutinin has also been found in electrofocusing studies on polyacrylamide gels (Bosch et al., 1981). These observations reflect the structural heterogeneity at the C termini of HA, that still contain a variable number of charged amino acids derived from the intervening peptide (Garten et al., 1982).

‡ When FPV grown in embryonated eggs was analysed for the carboxypeptidase two distinct isoelectric points were found at pH 6-2 and 6-7. This finding could be explained by the heterogeneity of host cells in the embryonated egg which might be equipped with arginine carboxypeptidase isoenzymes.

Inhibition of the carboxypeptidase prevents elimination of the arginine at the cleavage site

To prove that the enzyme activity analysed in the experiments described so far is involved in the processing of the haemagglutinin we have searched for specific inhibitors and we have analysed whether such an inhibitor has an effect on the elimination of the arginine in the cleavage reaction. Carboxypeptidase isolated from purified virions by extraction with n-octylglucoside and isoelectric focusing was incubated with a number of substances that were expected to act as enzyme effectors. These included divalent metal ions, chelating agents, sulphydryl reagents, alkylating reagents, inhibitors of serine proteases, and competitive inhibitors of arginine peptidases (Table 2). Some of the metal ions, notably cobalt, slightly enhanced the enzyme activity (pH optimum between pH 5-5 and pH 8-0), whereas others, particularly cadmium, had an inhibitory effect. The inhibiting heavy metal ions interfered with haemagglutination and haemolysis and were therefore not suitable for assays of the biological function of the haemagglutinin to be described below. Another effective inhibitor, the alkylating reagent α-N-bromoacetyl-D-arginine (Plummer, 1971; Juillerat-Jeanneret et al., 1982), could also not be used in these studies, because the haemagglutinin was partially
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Table 2. *Influence of effectors on the virus-associated arginine carboxypeptidase*.

<table>
<thead>
<tr>
<th>Effector (10^-4 M)</th>
<th>Activity (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Mg^{2+}</td>
<td>110</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>120</td>
</tr>
<tr>
<td>Co^{2+}</td>
<td>170</td>
</tr>
<tr>
<td>Zn^{2+}</td>
<td>10</td>
</tr>
<tr>
<td>Cd^{2+}</td>
<td>5</td>
</tr>
<tr>
<td>Mn^{2+}</td>
<td>10</td>
</tr>
<tr>
<td>EDTA</td>
<td>30</td>
</tr>
<tr>
<td>1,10-Phenanthroline</td>
<td>20</td>
</tr>
<tr>
<td>Iodacetamide</td>
<td>100</td>
</tr>
<tr>
<td>p-Hydroxymercuribenzoate</td>
<td>100</td>
</tr>
<tr>
<td>α-N-Bromoacetyl-D-arginine</td>
<td>0</td>
</tr>
<tr>
<td>Phenylmethylsulphonyl fluoride (PMSF)</td>
<td>100</td>
</tr>
<tr>
<td>N-α-Tosyl-L-lysylchloromethyl ketone (TLCK)</td>
<td>100</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>100</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>30</td>
</tr>
<tr>
<td>D-Arginine</td>
<td>10</td>
</tr>
<tr>
<td>ε-Aminocaproic acid</td>
<td>30</td>
</tr>
<tr>
<td>Spermidine</td>
<td>80</td>
</tr>
<tr>
<td>Agramine</td>
<td>40</td>
</tr>
<tr>
<td>Canavanine</td>
<td>20</td>
</tr>
<tr>
<td>L-Argininic acid</td>
<td>5</td>
</tr>
<tr>
<td>2-Mercaptomethyl-3-guanidinoethyl-thiopropanoic acid</td>
<td>0</td>
</tr>
</tbody>
</table>

* The carboxypeptidase was isolated from virus N grown on CE cells by solubilization with n-octylglucoside and isoelectric focusing as demonstrated in Fig. 2. The activity was measured by the radioassay described in Methods.

...denatured by this reagent and yielded atypical fragments when cleaved by trypsin (data not shown). The most potent inhibition substance was 2-mercaptomethyl-3-guanidinoethyl-thiopropanoic acid which had been shown before to be an effective competitive inhibitor of human carboxypeptidase N (Plummer & Ryan, 1981). It had no apparent denaturing effects on the viral glycoproteins.

The shift in the isoelectric point of the haemagglutinin resulting from the removal of arginine in the cleavage reaction (Bosch et al., 1981; Garten et al., 1981) should not occur when the carboxypeptidase is inhibited. That this is indeed the case is illustrated in Fig. 5. When 2-mercaptomethyl-3-guanidinoethyl-thiopropanoic acid was added to the incubation mixture before trypsin treatment, cleavage occurred, but the isoelectric point of the cleaved haemagglutinin was identical to that of the uncleaved haemagglutinin (Fig. 5a). In a control experiment the inhibitor was added after trypsin treatment. In this, a shift in the isoelectric point was seen (Fig. 5b). These results directly demonstrate that the virus-associated carboxypeptidase is responsible for the elimination of the arginine in the processing of the haemagglutinin.

Is the carboxypeptidase essential for the activation of the haemagglutinin?

As pointed out above, it was not clear whether tryptic cleavage alone was sufficient for activation of the haemagglutinin or whether elimination of the intervening arginine was also necessary. Since we could obtain trypsin-cleaved haemagglutinin with the arginine still present at the carboxy terminus of HA₁, it was possible to answer this question. We therefore analysed the fusing capacity of virus N derived from chick embryo cells by measuring haemolysis at acidic pH (Table 3). To obtain cleaved haemagglutinin with the intervening arginine still attached to HA₁, virus was first exposed to the carboxypeptidase inhibitor and then to trypsin. Controls were a virus sample in which the arginine had been eliminated by inhibitor treatment following trypsin incubation and a sample that was neither incubated with inhibitor nor with trypsin and, thus, contained uncleaved haemagglutinin. The results in Table 3 demonstrate clearly that cleaved haemagglutinin exhibits haemolytic activity regardless of whether or not the
Fig. 5. Effect of a carboxypeptidase inhibitor on the isoelectric points of the haemagglutinin of virus N. Virus grown in CE cells was cleaved by in vitro treatment with trypsin before (b) or after (a) treatment with 2-mercaptomethyl-3-guanidinoethyl-thiopropanoic acid. Trypsin-treated virions were mixed with virus containing uncleaved haemagglutinin which was also obtained from CE cells. Viral proteins were analysed by two-dimensional isoelectric focusing and electrophoresis. SDS–PAGE was carried out on 10% acrylamide gels. Isoelectric focusing was done under non-reducing conditions, while electrophoresis was done under reducing conditions. A virus sample which was analysed by the SDS–PAGE only is also shown (c). The gels were stained with Coomassie Brilliant Blue. The neuraminidase (NA) cannot be distinguished from the haemagglutinin (HA) in this experiment; other proteins identified are nucleoprotein (NP) and membrane protein (M).

Table 3. Effects of 2-mercaptomethyl-3-guanidinoethyl-thiopropanoic acid on the carboxypeptidase activity and on the haemolytic capacity of virus N*

<table>
<thead>
<tr>
<th>Virus</th>
<th>Carboxypeptidase activity (release of $[^{3}H]$arginine; d/min)$\dagger$</th>
<th>Haemolysis ($A_{540}$) with amount of virus (HAU):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>Inhibitor treatment before</td>
<td>30/51</td>
<td>0.075</td>
</tr>
<tr>
<td>trypsin treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibitor treatment after</td>
<td>28/35</td>
<td>0.068</td>
</tr>
<tr>
<td>trypsin treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No inhibitor, no trypsin</td>
<td>8806/8543</td>
<td>0.000</td>
</tr>
</tbody>
</table>

* Virus was treated with the inhibitor (1 mM) for 30 min at 37 °C and with trypsin (10 μg/ml) for 20 min at 37 °C. Trypsin treatment was stopped by the addition of TLCK (20 μg/ml).

$\dagger$ Double determinations.
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intervening arginine is present, whereas uncleaved haemagglutinin has very little activity. Thus, the carboxypeptidase is not essential for the activation process.

DISCUSSION

Influenza virus haemagglutinin is activated in vivo and in vitro by the same general mechanism: a trypsin-like protease of cellular origin cleaves between the intervening arginine and the glycine residue forming the amino terminus of HA. Subsequently, the arginine is eliminated by a virus-associated carboxypeptidase. The origin and the biological role of the carboxypeptidase was the subject of this study. The basic question whether the carboxypeptidase is identical with the haemagglutinin itself or whether it is another virus-coded or a host component required an assay which is sensitive and not affected by interference with other viral components as was the case when hippuryl-L-arginine was used as a substrate (Folk et al., 1960). These conditions were met by the radioassay described here. It was designed following the basic principles of an assay for prolyl-carboxypeptidase (angiotensinase C) (Skidgel et al., 1981) except that the radioactive peptide was coupled to agarose to facilitate the separation of the cleavage product from the substrate. This method was successfully employed as an assay not only for the virus-associated enzyme, but also for pancreatic carboxypeptidase B (Fig. I) and carboxypeptidase N from blood serum (data not shown). Because of this broad specificity, the assay is useful for screening studies. It is therefore reasonable to assume that the identified enzyme is the only carboxypeptidase present in the virus. Furthermore, the observation that 2-mercaptomethyl-3-guanidinoethyl-thiopropanoic acid is a specific inhibitor both for the virus-associated carboxypeptidase activity and for the elimination of the arginine from the carboxy terminus of HA, strengthens the concept that the carboxypeptidase activity which we have identified here is involved in the cleavage of the haemagglutinin.

Our experiments demonstrate clearly that the carboxypeptidase is a minor constituent of the virus envelope. It can be discriminated from the haemagglutinin and the neuraminidase and from the other viral proteins. Furthermore, it exhibits host-dependent variations in isoelectric point. These findings taken together support the concept that the enzyme is of host origin. It should be noted here that incorporation of a cell-specific carboxypeptidase into the viral envelope is not restricted to influenza virus, since we have observed carboxypeptidase showing the same host-dependent variation in its isoelectric point in other lipid-containing viruses, such as NDV, Semliki Forest virus and vesicular stomatitis virus (data not shown). Since evidence has been obtained for the involvement of a carboxypeptidase in the cleavage of the F protein of NDV (Kohama et al., 1981) and of the E2 protein of Semliki Forest virus (Garoff et al., 1980), it appears that these enzymes are also derived from the host.

Various carboxypeptidases which release lysine or arginine from peptides and proteins have been identified. They include pancreatic carboxypeptidase B (Folk & Gladner, 1958; Folk et al., 1960), carboxypeptidase N (kininase I) from blood plasma (Erdös & Sloane, 1962; Oshima et al., 1975) and a carboxypeptidase involved in the processing of enkephalin precursors from adrenal glands (Hook et al., 1982). When these enzymes are compared to the virus-associated carboxypeptidase, the following picture emerges. The enkephalin processing enzyme can be distinguished from the virus-associated enzyme by its insensitivity to 2-mercaptomethyl-3-guanidinoethyl-thiopropanoic acid and its sensitivity to sulphhydryl reagents (Hook et al., 1982). Unlike the enkephalin processing enzyme, the virus-associated carboxypeptidase is metal-dependent, as are carboxypeptidases B and N. On the other hand, zinc inactivates the virus-associated carboxypeptidase, in contrast to carboxypeptidase B which is activated by this metal. Thus, in its response to these and the other effectors tested, the virus-associated enzyme resembles carboxypeptidase N (Jeanneret et al., 1976; Plummer & Ryan, 1981). It should also be mentioned that, similar to the virus-associated enzyme, several subunits of carboxypeptidase N have been identified with molecular weights ranging between 23000 and 70000 (Plummer & Hurwitz, 1978; Levin et al., 1982; Jeanneret et al., 1976). However, carboxypeptidase N as well as carboxypeptidase B and the enkephalin processing enzyme are soluble proteins, whereas the virus-associated carboxypeptidase appears to be a membrane protein.

Arginine-specific carboxypeptidases are found in many tissues and body fluids and may have important regulatory functions. Thus, carboxypeptidase N inactivates in the serum potent
effector peptides such as kinins, fibrinopeptides (Teger-Nilsson, 1968; Erdős, 1979) and anaphylatoxins (Bokisch & Müller-Eberhard, 1970). On the other hand, because of their ubiquity these enzymes may act in concert with trypsin-like endoproteases even if removal of arginine (or lysine) is not necessary for the biological function of the cleavage product. This appears to be the case with the influenza haemagglutinin. We have found here that elimination of the arginine is not essential for the expression of the fusion activity of the haemagglutinin. Thus, trypsin cleavage alone is sufficient for activation. This finding strengthens the concept derived from previous observations (Richardson et al., 1980; Garten et al., 1981) that the fusion activity of the haemagglutinin depends on a specific amino acid sequence exclusively at the amino terminus of HA2.

We thank Professor R. Rott for his encouragement and his interest and Drs R. T. C. Huang and H. Niemann for many helpful discussions. We also thank Mr W. Berk for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 47, Virologie).

REFERENCES


Carboxypeptidase of influenza virus


(Received 17 May 1983)