The catalytic triad of the influenza C virus glycoprotein HEF esterase: characterization by site-directed mutagenesis and functional analysis

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Influenza C virus is able to inactivate its own cellular receptors by virtue of a sialate 9-O-acetylesterase that releases the acetyl residue at position C-9 of N-acetyl-9-O-acetylenuraminic acid (Neu5,9Ac2). The receptor-destroying enzyme activity is a function of the surface glycoprotein HEF and this esterase belongs to the class of serine hydrolases. In their active site, these enzymes contain a catalytic triad made up of a serine, a histidine and an aspartic acid residue. Sequence comparison with other serine esterases has indicated that, in addition to serine-71 (S71), the amino acids histidine-368 or -369 (H368/369) and aspartic acid 261 (D261) are the most likely candidates to form the catalytic triad of the influenza C virus glycoprotein. By site-directed mutagenesis, mutants were generated in which alanine substituted for either of these amino acids. Using a phagemid expression vector, pSP1D-HEF the HEF gene was expressed in both COS 7 and MDCK I cells. The glycoprotein was obtained in a functional form only in the latter cells, as indicated by its transport to the cell surface and measurable enzyme activity. The low level of expression could be increased by stimulating the NF-kB-binding activity of the cytomegalovirus immediate-earlier promoter/enhancer element of the vector. The esterase activity of the mutant proteins was compared with that of the wild-type glycoprotein. With Neu5,9Ac2 as the substrate, the esterase specific activities of the S71/A mutant and the H368,369/A mutant were reduced by more than 90%. In the case of the D261/A mutant the specific activity was reduced by 64%. From this data we conclude that S71, H368,369 and D261 are likely to represent the catalytic triad of the influenza C virus glycoprotein HEF. In addition, N280 is proposed to stabilize the oxyanion of the presumptive transition state intermediate formed by the enzyme–substrate complex.

Introduction

Influenza viruses are characterized by a receptor-destroying activity, which in the case of influenza C virus is a sialate 9-O-acetylesterase (EC 3.1.1.53) (Herrler et al., 1985). This enzyme releases the acetyl residue from position C-9 of N-acetyl-9-O-acetylenuraminic acid (Neu5,9Ac2), which is the receptor determinant for the binding of influenza C virus to target cells (Rogers et al., 1986; Herrler & Klenk, 1987). Once the 9-O-acetylated sialic acid of a cell surface glycoprotein or glycolipid has been converted to N-acetylenuraminic acid (Neu5Ac), the glycoconjugate can no longer function as a receptor for influenza C virus. Together with the receptor-binding activity (haemagglutination, H) and the fusion activity (F), the receptor-destroying activity (esterase, E) is located on the glycoprotein HEF, which is the only surface protein reported for influenza C virus (Herrler et al., 1988b). The importance of the receptor-destroying activity for the replication cycle of the virus is unknown, but it has been suggested to play a role at virus entry (Muchmore & Varki, 1987; Vlasak et al., 1989; Strobl & Vlasak, 1992). Studies with a sialic acid analogue that is resistant to cleavage by the viral esterase indicated that the fusion activity of influenza C virus is not dependent on inactivation of the virus receptors (Herrler et al., 1992).

The acetylenesterase of influenza C virus belongs to the family of serine hydrolases, which are inhibited by diisopropyl fluorophosphate (DFP). These enzymes include both esterases and proteases (e.g. subtilisin and trypsin). In their active site, a serine, a histidine and an aspartic acid form a catalytic triad which is essential for hydrolysis of the substrate. The exchange of either of these residues in the amino acid sequence of well characterized serine hydrolases, by site-directed mutagenesis, resulted in decreased activity and/or altered substrate specificity (Carter & Wells, 1988).

Serine-71 (S71) of the HEF glycoprotein has been shown to be one of the catalytic residues, as indicated by
covalent binding of the inhibitor DFP (Herrler et al., 1988a; Vlasak et al., 1989). To identify the histidine and aspartic acid residues of the catalytic triad, we compared
the amino acid sequences of the HEF glycoprotein with those of the HE proteins of three coronaviruses which also possess a 9-O-acetylatedesterase, as well as with the amino acid sequence of serine proteases with a known catalytic triad. After changing candidate residues to alanine by site-directed mutagenesis, the recombinant genes were expressed in cultured cells which were analysed for esterase activity. With Neu5,9Ac₂ as substrate the mutant in which the S71 had been changed to alanine showed a decrease in esterase activity by more than 90 %, and no measurable activity was detected with the mutants in which either histidine-368 or -369 (H368/369) was replaced by alanine. The esterase activity of the mutant in which alanine was substituted for aspartic acid 261 (D261) was decreased by 64 %. From our results we conclude that S71, H368/369 and D261 form the catalytic triad of the influenza C esterase.

Methods

Cell cultures and media. MDCK I cells were grown in MEM with 10 % fetal calf serum (Gibco BRL).

Plasmids and phagemids. The HEF gene of influenza C virus strain Johannesburg/I/66 (C/JHB/1/66) cloned into pBR 322 (Pfeifer & Compan, 1984) was kindly provided by Dr J. B. Pfeifer. After subcloning into Bluescript KS(+)(Stratagene) the G/C tails were removed by a partial nuclease Bal 31 digestion at the 5' end and by endonuclease digestion at the 3' end. For site-directed mutagenesis and transient expression, the HEF gene was excised with Xhol and XhoI, subcloned between the XhoI and XhoI sites of pcDNA I (Invitrogen) and transfected into Escherichia coli MC1061/P35 cells (Invitrogen). To propagate the vector in a wide range of prokaryotic cells, the Sf10 F selection marker of pcDNA I-HEF1 was cut out by digestion with KspI and MluI and replaced by the β-lactamase gene, which was excised from Bluescript KS (+) by partial digest with DraI and SspI. After filling in the sticky ends, the insert and vector were ligated resulting in the vector pSP1A-HEF. Due to the insertion of the HEF gene into the polylinker of pcDNA I, the restriction sites for HindIII, EcoRV, EcoRI and Pst I upstream of the HEF start codon were doubled. A stretch of 38 nucleotides in the polylinker was deleted by site-directed mutagenesis. In the resulting pSP1D-HEF polynklynert only the HindIII restriction site is present twice (Fig. 1).

Site-directed mutagenesis in vitro. Mutagenesis was performed as described (Kunkel, 1987). The recombinant shuttle phagemid pSP1D-HEF was used to transfet the dut- and ung+ E. coli CJ236 cells (BioRad). This method allows incorporation of uracil into the DNA in place of thymine. Single-stranded DNA (ssDNA) was generated by superinfection with the helper phage M13K07 and filtration of the supernatant (0.2 μm, Schleicher and Schüll). The supernatant was used as a source for isolation of the ssDNA, or it was stored at 4 °C for further use. The mutagenic oligonucleotide primers were annealed to the ssDNA. After second strand synthesis, E. coli T7G1 cells were transfected with the heterogenous double-stranded DNA (dsDNA) for selection of homogenous dsDNA. As most mutations were chosen such that restriction sites were either introduced or deleted, clones were screened for mutations by restriction analysis. Mutations in the selected clones were confirmed by sequencing according to the dyeoxynucleotide chain termination method (Sanger et al., 1977) with an automatic sequencer (Applied Biosystems).

Transfection and expression of the HEF cDNA. The dsDNA of the recombinant shuttle phagemid pSP1D-HEF containing the mutated HEF gene variants was isolated and 40 μg was used to transfect 0.5–1 × 10⁶ MDCK I cells in 6 cm plastic Petri dishes by a modification of the calcium phosphate precipitation procedure (Gorman et al., 1983; Chen & Okayama, 1987). After expression for 48 h, cells were starved for 1 h with MEM deficient in methionine/cysteine (ICN) or leucine (Gibco BRL) and then labelled for 1 h with 50–100 μCi Tran³5S-label (ICN) or [³¹C]leucine (NEF), respectively. Prior to and during labelling of the MDCK I cells, transfection from the cytomegalovirus (CMV) major immediate early (IE)-promoter/enhancer was stimulated through induction of the NF-xβ-binding activity (Wilkinson & Akkrij, 1992) with 50 ng/ml phorbol myristic acid and 4 μg/ml phytosphingosine (PHA). For indirect immunofluorescence analysis cells were transfected with Transfectam (Promega) according to the manufacturer’s protocol.

Immuno precipitation. After labelling the cells were washed once with cold PBS and lysed with 46 mM-N-octyl glucoside, 20 mM-dodecylglucoside and 30 mM-N-dodecyl-β-N-dimethyl-3-ammonio-1-propanesulphonate (in PBS). The lysates were sonicated for 30 s and the cellular debris was precipitated at 15000 g for 30 min. Polyclonal rabbit antiserum directed against C/JHB/1/66 or monospecific rabbit antiserum directed against the HEF protein was added (1:500) to the supernatant. After incubation for 4 h at 4 °C, the antibodies were absorbed to protein A-Sepharose. The precipitates were washed three times with wash buffer 1 (0.5% NP40, 1 mM-NaCl, 1 mg/ml BSA and 50 mM-Tris-HCl, pH 7.4) and twice with wash buffer 2 (50 mM-Tris-HCl, pH 6.8). The precipitated proteins were either solubilized by heating in electrophoresis sample buffer for 5 min at 95 °C, or used for the esterase assay. Prior to determination of the esterase activity, the HEF protein was isolated from the immunocomplexes as described below.

Isolation of enzymatically active HEF protein and esterase assay with Neu5,9Ac₂. The immunoprecipitates obtained from eight dishes were pooled and incubated three times sequentially in 1 vol. glycine buffer (0.2 M-glycine, pH 2.8) for 10 min at room temperature with constant shaking. The supernatants were combined and, after addition of 1/20 vol. of Tris buffer (25 mM-Tris-HCl, pH 8.0), were stored on ice. The eluate was concentrated in a Microcon concentrator (Amicon) at 15000 g at 4 °C and washed once with cold esterase buffer (100 mM-MES-KOH, 100 mM-TESS-KOH, pH 7.5, 100 mM-NaCl and 5 mM-MgCl₂). In a pilot test, it had been shown that the pH of the elution buffer did not inactivate the enzyme and that more than 90 % of the esterase activity was eluted from the immunocomplexes. For this purpose, samples were incubated with 60 μM-4-methylumbelliferylacetate in 0.5 ml of esterase buffer at 37 °C for 1 h. The reaction was stopped with 0.5 ml of 100 % ethanol and cleavage of the substrate was measured with a fluorescence photometer (Perkin-Elmer) operating at 365 nm for excitation and 450 nm for emission. Addition of detergent was not required during elution, as previous studies have indicated that in the absence of detergent the glycoprotein forms rosettes that are enzymatically active (Herrler et al., 1988b).

The concentrated eluate was combined with esterase buffer to achieve a total volume of 50 μl containing 1 mM-Neu5,9Ac₂, synthesized according to the method of Ogura et al. (1987), and 0.015 mM-BSA. An aliquot of 10 μl was taken out and incubated with 1 vol. DMB reagent (7 mM-1,2-diamino-4,5-methylene-dioxynbenzene, 14 mM-acetic acid, 0.75 mM-mercaptoethanol and 18 mM-sodium hydrogensulphate), mixed and stored at -20 °C. The residual sample was incubated at 37 °C for 24 h. Next 10 μl were taken out and mixed with DMB reagent and stored at -20 °C. The DMB mixtures were
influenza C virus HEF catalytic triad

Fig. 1. The phagemid pSP1D-HEF1 contains the coding region of the HEF gene (segment 4 of influenza C virus) under the control of the CMV IE promoter/enhancer. The β-lactamase gene (bla) allows selection in a wide range of E. coli cells. Single-stranded DNA for mutagenesis is made from the M13 origin after superinfection with a helper phage. In COS 7 cells the SV40 origin allows for high replication numbers.

Incubated at 56 °C for 2.5 h and stored at -20 °C prior to HPLC analysis. The remainder of the sample was separated by 10% SDS-PAGE (Laemmli, 1970) and the relative amount of HEF protein was determined after exposure of the gel on a phosphorimager screen (Molecular Dynamics). The DMB reagent-treated samples were analysed by HPLC (Merck/Hitachi) using a RP-18 column (4 mm2, 250 mm; Merck) and acetonitrile-methanol-H2O (9:7:84) as the mobile phase (1 ml/min). The eluate was analysed for the presence of sialic acids with a fluorescence detector operating at 375 nm for excitation and 445 nm for emission. The percentage of Neu5Ac was calculated with a D-6000 HPLC Manager program (Merck). The relative specific activity was calculated as follows:

$$\frac{\Delta \text{Neu5Ac(mol)}}{\Delta t(\text{min}) \times \text{relative amount of HEF}} = \text{relative specific activity}$$

Three to five determinations were performed on the wild-type and each of the different mutant glycoproteins and there was no indication that the specific activity was changed by differential cellular dilution.

Indirect immunofluorescence. Cells were grown on cover slips and, after transfection and expression, washed once with cold PBS. For cell surface immunofluorescence, cells were incubated on ice for 0.5-1 h with polyclonal rabbit antiserum directed against influenza virus C/JHB/1/66 (1:1000 in PBS, 3% BSA). After three washes with cold PBS, the samples were fixed for 15 min with acetone-methanol (1:1, -20 °C) and washed twice with PBS. Finally, the samples were incubated for 1 h at room temperature with fluorescein-conjugated mouse anti-rabbit serum (1:100 in PBS, 3% BSA) followed by three washes with PBS and two washes with H2O. For studying intracellular immunofluorescence, the cells were first fixed, washed twice with PBS and then incubated with polyclonal rabbit antiserum directed against influenza virus C/JHB/1/66. After subsequent washes, the samples were incubated with fluorescein-conjugated mouse anti-rabbit serum, washed again and analysed with a fluorescence microscope (Zeiss).

Computer analysis. Computer analysis of the amino acid sequences was performed with the help of HUSAR (Heidelberg Unix Sequence Analysis Resources) (German Cancer Research Centre, Heidelberg, Germany).

Results

Sequence comparison

To identify the residues of the catalytic triad we first compared the amino acid sequence of the HEF protein of influenza virus C/JHB/1/66 with the HE protein sequences of three coronaviruses which also contain a 9-O-acetyesterase activity: bovine coronavirus (BCV) (Parker et al., 1989; Kienzle et al., 1990), human coronavirus (HCV-OC43) (Zhang et al., 1992) and mouse hepatitis virus (MHV-JHM) (Shieh et al., 1989). The overall similarity between the HE and the HEF proteins has been reported to be about 30% (Luytjes et al., 1988). There are a few short stretches of amino acids that are completely conserved in both proteins. One of the conserved sequences comprises the active site serine
Table 1. Comparison of amino acids around the key residues of the catalytic triad in different serine hydrolases

<table>
<thead>
<tr>
<th>Protein</th>
<th>Serine</th>
<th>Histidine</th>
<th>Aspartic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subtilisin Carlsberg*</td>
<td>NGTSMAS</td>
<td>.HG.‡</td>
<td>G...KVAV.-DT-GIQASHPD</td>
</tr>
<tr>
<td>Thermitase*</td>
<td>SGTSMAT</td>
<td>.HG.</td>
<td>G.G.KIAIV-DT-GVQ...HPD</td>
</tr>
<tr>
<td>Proteinase K*</td>
<td>SGTSMAT</td>
<td>.HG.</td>
<td>G.G..VYVI-DT-GI.ASHPD</td>
</tr>
<tr>
<td>Lactococcal proteinase*</td>
<td>SGTSMAS</td>
<td>.HG.</td>
<td>G.G.VVSVI-DS-GI...TH.D</td>
</tr>
<tr>
<td>Yeast proteinase*</td>
<td>SGTSMAC</td>
<td>.HG.</td>
<td>G....VSTVVD-GIR.TH...</td>
</tr>
<tr>
<td>HEF (C/JHB/1/66)</td>
<td>PGDSRTD</td>
<td>HHGD</td>
<td>FGDQYVNK-DT-GVYGLNS</td>
</tr>
<tr>
<td>HE (MHV)†</td>
<td>PGDSRTD</td>
<td>HHGD</td>
<td>FDSQYVNK-DT-GVYGLNS</td>
</tr>
<tr>
<td>HE (BCV)†</td>
<td>PGDSRTD</td>
<td>NHGD</td>
<td>FDSQYVNK-DT-GVYGLNS</td>
</tr>
<tr>
<td>HE (HCV-OC43)†</td>
<td>PGDSRTD</td>
<td>NHGD</td>
<td>FDSQYVNK-DT-GVYGLNS</td>
</tr>
</tbody>
</table>

* Serine hydrolases with characterized catalytic triad (Vos et al., 1989).
† HE protein sequences of different coronaviruses (Zhang et al., 1992).
‡ Non-conserved amino acids.

FGDSRS/TD (aa 68–74) (Table 1). Among the other conserved regions, histidine residues were found to be present only in the sequence HHGD (aa 368–371). A position next to a glycine residue is characteristic for the histidine in the catalytic triad of the subtilisin family of serine hydrolases. Therefore, amino acids 368 and 369 are prime candidates for being involved in the catalytic triad of the influenza C esterase. A candidate for the aspartic acid in the catalytic triad of HEF protein is less obvious from the sequence comparison. However, the aspartic acid residue in the catalytic triad of the subtilisin family of serine proteases is part of the sequence motif DSG or DTG. The latter motif is also present in the coronavirus HE protein. When HE and the HEF proteins are aligned the DTG motif of coronavirus corresponds to the DSSG (aa 261–264) sequence of the influenza C esterase. Therefore, a likely candidate for the aspartic acid of the catalytic triad of HEF protein is amino acid 261. In subtilisin, asparagine-155 in the sequence motif GNSG (aa 154–157) is thought to stabilize the transition state (Robertus et al., 1972) (see Fig. 6). This motif is also present in the HEF protein (aa 279–282).

Expression of HEF protein in MDCK I cells

Due to the simian virus 40 (SV40) replicon present in the pSP1D-HEF phagemid, high levels of expression were achieved in COS 7 cells, which constitutively express the large T antigen of SV40. However, HEF protein expressed from COS cells was neither transported to the cell surface nor enzymatically active (data not shown). Because of the presence of a CMV promoter, pSP1D can be used for expression of foreign genes in a wide range of eukaryotic cells. The vector cannot replicate in these cells and, therefore, the amount of protein expressed is much lower than that expressed in COS 7 cells. As we grow influenza C virus routinely in MDCK I cells, we tried to express the HEF gene in this subline of canine kidney cells. After intracellular staining of transfected MDCK I cells fluorescence signals were detected for both the wild-type and the mutant glycoproteins (Fig. 2, shown only...
Fig. 3. Transient expression of the HEF gene in cultured cells. Cells were transfected with pSP1D-HEF1 and the NF-kB-binding activity was induced by treatment with PHA and phorbol ester. Proteins were labelled with [35S]methionine, and the HEF protein was immunoprecipitated with anti-virion antiserum and analysed by SDS-PAGE. Lane A, Purified virions; lane B, mock transfected cells; lane C, cells transfected with pSP1D-HE and lane D, marker proteins.

Fig. 4. Inhibition of the esterase activity of recombinant wild-type HEF protein by DFP.

for the wild-type). In contrast to COS 7 cells, HEF protein synthesized in the canine kidney cell line was also detected on the cell surface. This is shown in Fig. 2 for the wild-type HEF protein, but was also observed with the different mutant glycoproteins (not shown). When the polypeptides of transfected MDCK I cells were analysed by SDS-PAGE the HEF1 subunit was the major influenza C virus-specific protein detected (Fig. 3). The expression shown in Fig. 3 was obtained after induction of the NF-kB-binding activity of the major IE promoter/enhancer. This was achieved by a combined treatment with PHA and phorbol ester that resulted in an increase by a factor of 18 of the HEF protein synthesized. When immunoprecipitated wild-type glycoprotein was incubated with the substrate p-nitrophenyl acetate, esterase activity was detected indicating that the glycoprotein synthesized in MDCK I cells was enzymatically active.

Analysis of mutants

Similar to other serine hydrolases, the influenza C virus esterase is inactivated by DFP, which covalently binds to the active site serine. Inhibition of the acetyl esterase activity by DFP in a dose-dependent manner was also observed with HEF protein expressed from the pSP1D-HEF vector in MDCK I cells (Fig. 4). This indicates that the vector-expressed protein has retained the characteristics of a serine hydrolase. Therefore, this system was suitable for analysing the importance of selected amino acids for the esterase activity of HEF protein. For this purpose, the nucleotide sequence of the HEF gene was altered by site-directed mutagenesis. In the proteins derived from the mutated genes, the presumptive amino acids of the catalytic triad were replaced by alanine. The wild-type as well as the mutant HEF proteins were immunoprecipitated from lysates of MDCK I cells and analysed for esterase activity. To compare the different HEF proteins, it was necessary to correlate the enzyme activity with the amount of protein available. Because of the low level of expression a conventional protein assay was not feasible. The relative amount of protein was determined by analysing each [35S]methionine-labelled sample by SDS-PAGE and comparing the relative radioactivity of the HEF protein bands. These values were used to calculate the relative specific activity of the wild-type and mutant proteins. The mutants were analysed for enzyme activity with the specific substrate Neu5,9Ac2. To optimize the conditions of the enzyme assay, the HEF protein was isolated from the immuno-
Fig. 5. Vector expressed wild-type and mutated HEF proteins were immunoprecipitated from MDCK I cells and analysed for esterase activity as described in Methods. The enzyme activity indicated is the mean value of three to six determinations performed on the wild-type and each of the different mutants. The single-letter code was used to designate the amino acids affected by the mutation. The first letter indicates the amino acid in the wild-type protein at the indicated amino acid position, the second letter indicates the amino acid generated by site-directed mutagenesis. The esterase activity was determined with the specific substrate Neu5,9Ac₂.

D261 also contributes to the enzyme activity of the HEF protein. A dramatic effect on the esterase activity was also observed when asparagine-280 was replaced by alanine.

Discussion

The aim of this study was to identify the key residues in the catalytic triad of the esterase epitope on the HEF glycoprotein. In previous studies SV40 and vaccinia virus expression systems were used to elucidate the different functions of the HEF protein (Vlasak et al., 1987; Szepanski et al., 1994). Only the wild-type protein was analysed for esterase activity. To assign the role in the catalytic triad to distinct amino acids, we analysed different mutants with a transient expression system. The pSP1D vector was used for this purpose because it is suitable for both site-directed mutagenesis and gene expression. HEF protein expressed in MDCK I cells was transported to the plasma membrane and showed measurable esterase activity. In contrast, no enzyme activity and surface expression were detectable with COS 7 cells. Failure of HEF protein to be transported to the plasma membrane was also observed when two viral expression systems, recombinant SV40 or vaccinia virus, respectively, were used to analyse the glycoprotein of influenza virus C/JHB/1/66 in CV 1 cells (Szepanski et al., 1994). The latter cells are the parental cell line of COS 7 cells. It appears that monkey cells lack a factor that promotes the processing of HEF protein in MDCK I cells.

As the pSP1D-HEF vector does not replicate in MDCK I cells, the HEF gene was expressed only at a low level. The amount of protein obtained from these cells was insufficient for a detailed enzymatic analysis. By stimulating the NF-κB-binding activity of the CMV major IE promoter/enhancer, it was possible, however, to increase the esterase activity recovered from transfected cells to an extent that enabled us to analyse the amino acids serine, histidine and aspartic acid of the catalytic triad.

The role of the catalytic triad in the course of the enzyme reaction has been established for several serine hydrolases, e.g. proteases and esterases (Fig. 6). The nucleophilic hydroxyl oxygen of the reactive serine attacks the carbonyl group carbon of the substrate leading to an acyl enzyme intermediate. The hydroxyl proton is transferred to the imidazol of the histidine. This residue functions as a catalytic base by assisting in the transfer of a proton from the serine hydroxyl to the substrate leaving group. The aspartate is believed to position the histidine tautomer in the initial enzyme substrate complex and to stabilize the protonated form of histidine in the subsequent transition state complex (Craik et al., 1987). The assumed acyl enzyme in-
Fig. 6. Schematic diagram of the involvement of the catalytic triad (Ser, His and Asp) in the hydrolysis of the substrate (S) by serine hydrolases (E). The rate limiting step of the enzyme reaction is the formation of the tetrahedral transition state intermediate (E-S*).

The functions of the individual amino acids are described in the text. In the case of subtilisin, the oxyanion of the transition state complex is stabilized by Asn. Only the acetyl group of the substrate is indicated. The complete structure of the substrate Neu5,9Ac₂ is shown below.

The intermediate is then hydrolysed by the reversed mechanism with the difference that water instead of serine serves as the nucleophile.

Comparative sequence analysis suggested that, in addition to S71, H368/369 and D261 are the most likely candidates for being part of the catalytic triad of HEF protein. In order to confirm the importance of these amino acids for the esterase activity of the influenza C virus glycoprotein, mutants were generated with amino acid exchanges at either of these positions. To minimize unfavourable steric contacts and to avoid new charge interactions, alanine was chosen to replace the amino acids of interest.

Using 9-O-acetylated sialic acid as substrate, the relative specific activity of the S71/A mutant was reduced by more than 90%. This result confirms the important role of S71 in the esterase activity of HEF protein. It may appear surprising that the enzyme activity is not completely lost after removal of the reactive serine, because in this mutant no covalent bond can be formed between the enzyme and the substrate acyl group and the reaction cannot proceed by the usual serine acyl-enzyme intermediate (Carter & Wells, 1988). In this case, it is assumed that, unlike the normal reaction mechanism, the nucleophilic attack of a water molecule on the scissile bond results in a single tetrahedral intermediate that collapses causing hydrolysis of the substrate. One should keep in mind that the active site of serine hydrolases comprises not only the catalytic triad, but also other elements, e.g. a binding site for hydrogen bonding to a tetrahedral oxyanion and a site for specific recognition of the substrate (Kraut, 1977). If the catalytic triad is inactivated by site-specific mutagenesis, the other elements of the active site are still functional and account for the residual enzyme activity as has been shown for serine proteases (Carter & Wells, 1988). The extent to which the individual elements of the active site are dispensable should depend on the type of linkage to be hydrolysed by the enzyme. In the case of a stable amide linkage, the interplay of the different elements of the active site is expected to be more critical than in the case of a labile ester linkage. With respect to the S71/A mutant, these considerations provide an explanation for the residual activity of 4.1%.

With the H368/A, H369/A and H368,369/A mutants,
the specific activity was below the level of detection. Thus, replacing either of the two histidine residues by alanine severely affects the esterase activity. Such a strong effect is expected for the histidine of the catalytic triad which enables the nucleophilic attack of the reactive serine on the substrate by serving as an acceptor for the proton from the hydroxyl group of the serine. From our results and the sequence data we conclude, therefore, that the second component of the catalytic triad, the histidine, is located at amino acid positions 368/369 of the HEF protein. In order to exactly determine the role of both histidine residues for the esterase activity of HEF protein, one has to await the establishment of a more efficient expression system that allows determination of the various kinetic parameters of the mutant enzymes.

Sequence comparison suggested that D261 is the third component of the catalytic triad. The D261/A mutant was indeed shown to have a reduced esterase activity. The reduction was, however, less pronounced than in the case of the S71/A or H368,369/A mutants, respectively. We conclude, therefore, that the aspartic acid residue is less important for the enzyme activity than the two other constituents of the catalytic triad. This finding is not unexpected, because the aspartic acid residue contributes only indirectly to the enzyme reaction (Fig. 6). A comparable result has been reported for mutants of the serine protease subtilisin (Carter & Wells, 1988).

Even though there is no information available about the three-dimensional structure of the HEF protein, we conclude from the data presented here that S71, H368/369, and D261 form the catalytic triad of the influenza C virus glycoprotein. As mentioned above, there are additional elements involved in the active site of serine hydrolases. A well-characterized element is an oxyanion binding site that stabilizes the transition state by forming hydrogen bonds to the carboxy oxygen of the substrate (Fig. 6). Replacement of N280 which is part of the GNSG motif present in the HEF protein (amino acid positions 279–282) by aspartic acid resulted in a mutant protein that was devoid of measurable enzyme activity. This decrease is more pronounced than in the case of the D261/A mutant suggesting that N280 is involved in the active site of the influenza C virus esterase.

A complex picture of the functional domains of the influenza C virus glycoprotein is emerging. The HEF protein is already known to be unique among viral glycoproteins, because it is responsible for three viral activities: receptor-binding, receptor-inactivation and fusion. At least in the case of the esterase activity it is obvious now, that this functional domain consists of several subunits. In addition to the functional domains the HEF protein has several antigenic epitopes, which may overlap with the receptor-binding site and the active site of the esterase (Matsuzaki et al., 1992; Szepanski et al., 1992; Umetsu et al., 1992). The considerable structural constraints imposed in order to accommodate these functional and antigenic domains on a single glycoprotein may explain in part why antigenic variation with influenza C viruses is much less pronounced than with influenza A and B viruses (Homma et al., 1982).

Neu5,9Ac₂ was kindly provided by G. Zimmer. This work was conducted by S. Pleschka in partial fulfillment of the requirements for the Doctor rerum naturarum degree from FB17, Philippus-Universität, Marburg, Germany. Financial support was provided by a grant from Deutsche Forschungsgemeinschaft (SFB 286).

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(Received 4 May 1995; Accepted 6 June 1995)