Influenza C Virus Esterase: Analysis of Catalytic Site, Inhibition, and Possible Function

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The active site serine of the acetylerase of influenza C virus was localized to amino acid 71 of the hemagglutinin-esterase protein by affinity labeling with ³H-labeled diisopropylfluorophosphate. This serine and the adjacent amino acids (Phe-Gly-Asp-Ser) are part of a consensus sequence motif found in serine hydrolases. Since comparative analysis failed to reveal esterase sequence similarities with other serine hydrolases, we suggest that this viral enzyme is a serine hydrolase constituting a new family of serine esterases. Furthermore, we found that the influenza C virus esterase was inhibited by isocoumarin derivatives, with 3,4-dichloroisocoumarin being the most potent inhibitor. Addition of this compound prevented elution of influenza C virus from erythrocytes and inhibited virus infectivity, possibly through inhibition of virus entry into cells.

Influenza C viruses possess a sialate-*O*-acetylerase activity (12, 32). Such a receptor-destroying activity is also found in human and bovine coronaviruses (39, 40) and may be functionally similar to the receptor-destroying enzyme (neuraminidase) of influenza and parainfluenza viruses (14, 18). The influenza C virus esterase activity was shown to be an integral part of the viral hemagglutinin-esterase (HE) protein (6, 11, 38) and was inhibited by diisopropylfluorophosphate (DFP) (23), which suggested that the virus HE protein is related to the classical B esterases.

Esterases are classified as types A, B, and C according to their reaction with various organophosphate reagents (13). B esterases are inhibited by DFP through irreversible phosphorylation of an active site nucleophile, generally the hydroxyl group of a serine residue. A number of other families of enzymes have active site serine residues which react with DFP and are capable of hydrolyzing various ester, thioester, or amide substrates. Together with B esterases, these enzymes have been called serine hydrolases. The active site serine of a number of serine hydrolases has been identified by labeling with radioactive DFP (2, 16, 28, 37). In addition to the chymotrypsin-trypsin family of serine proteases (26), serine hydrolases include the subtilisin family of serine proteases (42), carboxylesterases (24), thioesterases (43), and viral serine proteases (7, 22).

X-ray crystallographic studies have shown that the active site residues of serine proteases consist of a catalytic triad composed of Ser-195, His-57, and Asp-109. These residues are arranged in an optimal three-dimensional conformation for catalysis. The proposed mechanism of action was confirmed by introduction of single-base changes into cDNAs of serine proteases. These enzymes showed either modified substrate specificities or loss of catalytic activity (4, 5).

In this report, we identify the active site serine residue of the influenza C virus esterase and compare the Phe-Gly-Asp-Ser sequence motif with those found in other serine hydrolases. Furthermore, we have discovered a series of potent mechanism-based isocoumarin inhibitors of the viral

esterase, and we suggest that the influenza C virus esterase (receptor-destroying enzyme) plays a role in the entry of the virus into host cells.

MATERIALS AND METHODS

Viruses and cells. Influenza viruses C/JJ/50 and C/JHG/66 were grown in embryonated eggs. Virus purification has been described previously (40). Influenza virus A/WSN/33 was grown in Madin-Darby canine kidney (MDCK) cells as described previously (40). Erythrocytes derived from chickens (strain, Rhode Island Red; sex-linked chromosome X) were obtained from Pocono Rabbit Farm (Canadensis, Pa.).

Hemagglutination assay. A 22.5- μ l sample of a C/JJ/50 virus suspension was mixed with 2.5 μ l of 3,4-dichloroisocoumarin (final concentration, 50 μ M), incubated at room temperature for 30 min, and used in the assay. Controls were treated in the same way without inhibitor. Hemagglutination assays were performed in V-shaped microdilution plates (Flow Laboratories, Inc., McLean, Va.) as described previously (39).

Plaque assays. Influenza viruses C/JJ/50 and A/WSN/33 were pretreated with 3,4-dichloroisocoumarin (final concentration, 50 μ M) for 1 h at room temperature and grown in MDCK cells by standard procedures (39). Mock-treated virus was used as a control.

Labeling of the HE protein with [1,3-³H]DFP. Concentrated influenza virus C/JHG/66 (25 mg of protein) was incubated in 1.2 ml of phosphate-buffered saline containing 200 μ Ci of [1,3-³H]DFP (4.4 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) for 12 h at 4°C. Labeled virus was purified over a 30 to 60% sucrose gradient, pelleted by ultracentrifugation for 2 h at 25,000 rpm in an SW 27 rotor, and suspended in 0.5 ml of phosphate-buffered saline. The HE protein was solubilized by incubation with 2% Triton X-100 and separated from viral cores by ultracentrifugation for 30 min at 35,000 rpm in an SW 50.1 rotor. The labeled HE protein was precipitated by the supernatant by adding 15 volumes of butanol, and the precipitate was dried and dissolved in 50 mM Tris buffer (pH 7.5) containing 0.05% Triton X-100.

Purification and amino acid sequencing of [1,3-³H]DFP-labeled tryptic peptides. [1,3-³H]DFP-labeled HE protein

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was digested for 16 h with 50 μ g of tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin (Worthington Diagnostics, Freehold, N.J.), adjusted to 50 mM triethylammonium acetate (TEAA) buffer (pH 7.2), and directly injected into a reverse-phase high-pressure liquid chromatography column (μ -BondPak; Millipore Corp., Bedford, Mass., and Waters Associates, Inc., Milford, Mass.). Tryptic fragments were eluted in 90 min by using a linear gradient of 0 to 60% acetonitrile–50 mM TEAA (pH 7.2) at a flow rate of 1.5 ml/min. Fractions (1 ml) were collected, and radioactivity was measured by scintillation counting of 5- μ l samples. Fractions containing the 3 H-labeled peptide were lyophilized, dissolved in 0.1% trifluoroacetic acid, and rechromatographed on the same column using an acetonitrile-trifluoroacetic acid gradient. After fractions containing the radioactive peptide were collected, the samples were pooled, lyophilized, and sequenced at the Rockefeller University Protein Sequencing Facility (New York, N.Y.) with an automated gas phase sequencer (model 470A; Applied Biosystems). Forty percent of the phenylthiohydantoin (PTH)-amino acid derivatives of each degradation step was injected on line into a PTH analyzer (model 120A; Applied Biosystems). The remaining 60% was used for detection of incorporated radioactivity by liquid scintillation counting.

Enzyme and inhibitors. The stock solution of the influenza virus C/JHG/66 esterase (suspended in 10 mM sodium-phosphate buffer, pH 7.1, containing 150 mM NaCl) consisted of purified virus at a concentration of 0.3 μ g/ μ l. Inhibitors were dissolved in dimethylsulfoxide, and the stock solution of *p*-nitrophenylacetate (100 mM) was prepared in acetonitrile.

Trifluoroacetophenone was purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis.; *m*-amino-phenylboronic acid was purchased from Alfa. The synthesis of the majority of isocoumarins has been previously reported (8, 17). The benzoxazinones were prepared by the method of Teshima et al. (36). Boronophthalide derivatives were prepared as reported by Lennarz and co-workers (20). The synthesis of the remaining isocoumarins, boronic acid derivatives, and trifluoromethyl ketones will be published elsewhere (J. C. Powers et al., manuscript in preparation).

Enzyme inhibition. Enzyme stock solution (20 μ l) was added to 1.94 ml of sodium phosphate buffer (pH 7.1) containing 150 mM NaCl in a cuvette at 25°C. This was followed by the addition of inhibitor (20 μ l) and *p*-nitrophenylacetate (20 μ l), and the A_{400} was monitored. Percent inhibition was calculated as $(1 - V_i/V_o) \times 100$. V_i and V_o represent the hydrolysis rates of *p*-nitrophenylacetate in the presence and absence of inhibitor, respectively. The final assay buffer contained 1 mM *p*-nitrophenylacetate, 1% dimethylsulfoxide, 1% acetonitrile (CH_3CN), and 3 μ g of influenza C virus protein per ml.

Progressive inhibition of the influenza C virus esterase was further investigated for the three most potent isocoumarin inhibitors. Enzyme and inhibitor were added to the phosphate buffer (pH 7.1 at 25°C) and allowed to stand for various time periods (1 to 25 min). Substrate was then added, and the residual enzyme activity was measured. Kinetic parameters for the inhibition of the influenza C virus esterase (see Table 2) were determined from plots of the ln of activity versus time (see Fig. 3).

RESULTS

Purification and amino acid sequence analysis of a [1,3- 3 H]DFP-labeled tryptic HE fragment. In order to identify the

FIG. 1. Purification of [3 H]DFP-labeled HE fragment by reverse-phase high-pressure liquid chromatography. (a) The 3 H-labeled HE protein was digested with tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin, diluted 1:2 in 50 mM TEAA, and separated on a C-18 column (μ -Bondpak) with a linear gradient (0 to 60% acetonitrile in 50 mM TEAA, pH 7.2). (b) The two fractions of the first purification run containing 3 H counts were pooled, lyophilized, and rechromatographed on the same column using a linear 0 to 60% acetonitrile–0.1% trifluoroacetic acid gradient. Flow rates for both column separations were 1.5 ml/min. OD, optical density.

active site of the influenza C virus acylesterase, concentrated egg-grown influenza virus C/JHG/66 was labeled with [1,3- 3 H]DFP. Labeled virus was separated from unincorporated [1,3- 3 H]DFP by centrifugation over a 30 to 60% sucrose gradient, and virions obtained from the gradient were pelleted and suspended in phosphate-buffered saline. Triton X-100 was added to a final concentration of 2%, and after incubation for 15 min, viral cores were pelleted by ultracentrifugation. The solubilized HE protein was recovered from the supernatant and digested with tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin as described in Materials and Methods.

The digestion products were injected directly into a C-18 reverse-phase high-pressure liquid chromatography column (μ -Bondpak) and separated by using a linear acetonitrile-TEAA gradient (Fig. 1a). Two 3 H-containing fractions, which eluted after approximately 52 min, were collected, pooled, and rechromatographed by using a linear acetonitrile-trifluoroacetic acid gradient. Fractions containing material detected by A_{210} were collected, and the peak which eluted after approximately 56 min (Fig. 1b) was found to contain 3 H. This fraction was lyophilized and used for automated Edman degradation. Analysis of the amino acid sequence revealed a peptide which could be aligned (Fig. 2)

▼

predicted: A-G-A-S-V-L-N-Q-S-T-W-I-G-F-G-D-S-R

observed: X-G-A-S-V-L-X-Q-S-X-X-I-G-F-X-X-X-X

FIG. 2. Amino acid sequence of ³H-labeled peptide. ³H-labeled peptide recovered from the high-pressure liquid chromatography separation (Fig. 1b) was subjected to microsequence analysis. PTH amino acid derivatives of each Edman degradation step were analyzed by using a PTH-amino acid analyzer. Positive identification of several amino acids permitted alignment of the peptide with the amino acid sequence of a tryptic peptide predicted from the cDNA sequence (3, 25). The amounts of positively identified PTH amino acids ranged from 2 to 12 pmol. The position of the [³H]DFP-labeled serine residue is indicated (▼) and corresponds to amino acid position 57 in the mature HE protein. X, Unidentified PTH amino acid derivative.

with a tryptic fragment predicted from the HE sequence (3, 25). Liquid scintillation counting of fractions of each degradation cycle revealed the presence of radioactivity in cycle 17. In this position a serine residue which corresponds to Ser-71 of the HE protein is predicted. This serine residue is part of the sequence Phe-Gly-Asp-Ser, which is a consensus sequence around active site serine residues in serine hydrolases (see Discussion).

Inhibition of influenza C virus esterase by isocoumarin derivatives. The results obtained by DFP labeling of the HE protein suggested that other serine hydrolase inhibitors might be used to inhibit the viral enzyme. Purified preparations of influenza virus C/JJ/50, which readily forms plaques in MDCK tissue culture cells, were used as a source for the enzyme.

A variety of compounds were surveyed with the virus esterase, including heterocyclic compounds (benzoxazinones and isocoumarins), boronic acid derivatives, and trifluoromethyl ketones (Table 1). Three isocoumarins—3,4-dichloroisocoumarin, 3-chloroisocoumarin, and 4-chloro-3-[(3-isothioureido)propoxy]-isocoumarin—were the only compounds to show significant reactivity towards the influenza C esterase during the 3- to 5-min assay period. The general serine protease inhibitor 3,4-dichloroisocoumarin was the most potent inhibitor, completely inactivating the enzyme during the assay period. The monochloro compound, 3-chloroisocoumarin, was also highly reactive toward the influenza C virus esterase (95% inhibition). The enzyme was less effectively inhibited (21% inhibition) by the 4-chloro-3-[(3-isothioureido)propoxy]-isocoumarin, which is a potent inhibitor for many trypsin-like enzymes (17). None of the other heterocyclic compounds were effective inhibitors of the influenza C esterase during the 3- to 5-min assay period. Most are potent irreversible inhibitors for a variety of serine proteases, including elastases and chymotrypsin-like enzymes. The most surprising result was the lack of reactivity of 3,4-dibromoisocoumarin. Changing the two chlorine atoms to bromines converted our most potent inhibitor into an inactive compound in this assay.

Since several irreversible serine protease inhibitors were effective at inhibiting the viral esterase, we decided to test a number of the transition state inhibitors for serine proteases. Transition state inhibitors typically inhibit serine proteases by interaction with the active site serine to form stable tetrahedral structures which resemble the tetrahedral intermediate formed during ester or peptide bond hydrolysis. Two classes of compounds which are potent transition state inhibitors for serine proteases are boronic acid derivatives (19) and trifluoromethyl ketones (15). A number of boronic

TABLE 1. Inhibition of viral esterase activity by isocoumarins, benzoxazinones, boronic acid derivatives, and trifluoromethyl ketones^a

Compound	Concn (μM)	% Inhibition ^b
3,4-Dichloroisocoumarin	100	100
3-Chloroisocoumarin	100	95
4-Chloro-3-[(3-isothioureido)propoxy]-isocoumarin	52	21
4-Chloro-3-propoxyisocoumarin	100	NI ^c
4-Chloro-3-ethoxy-7-[ethylcarbamoylamino]-isocoumarin	100	NI
7-Amino-3,4-dichloroisocoumarin	100	NI
4-Carboxy-3-methylisocoumarin	110	NI
3,4-Dibromoisocoumarin	49	NI
3-Ethoxy-7-[(3-nitrobenzoyl)amino]-isocoumarin	13	NI
4-Chloro-3-ethoxyisocoumarin	100	NI
4-Bromo-3-methylisocoumarin	97	NI
3-Acetoxisocoumarin	90	NI
2-Trifluoromethyl-4H-3,1-benzoxazine-4-one	95	NI
2-Propyl-4H-3,1-benzoxazine-4-one	100	NI
Boronophthalide	73	NI
5-Nitroboronophthalide	100	NI
3-Aminophenylboronic acid	98	NI
2-Butoxyphenylboronic acid	120	NI
5-[(3-Phenylpropionyl)amino]-boronophthalide	43	NI
Trifluoroacetophenone	100	NI
3-[(3-Phenylpropionyl)amino]-trifluoroacetophenone	100	NI

^a Inhibition reactions were measured in 10 mM phosphate buffer (pH 7.1) containing 1% dimethylsulfoxide and 1% acetonitrile at 25°C with 1 mM *p*-nitrophenylacetate as a substrate. The final concentration of the influenza C virus was 3 μg/ml.

^b Compounds progressively inhibited the enzyme, and the values reported were measured 5 min after the addition of *p*-nitrophenyl acetate.

^c NI, No detectable inhibition.

acids and trifluoromethyl ketones were tested with the influenza C virus esterase (Table 1). However, none of these compounds inhibited the influenza C virus esterase.

Kinetic parameters of isocoumarin inhibitors. The three most potent inhibitors of the influenza C virus esterase were further studied. Time-dependent inactivation kinetics were measured for the three isocoumarins cited above. The reaction of the viral esterase was clearly time dependent, as shown in Fig. 3 for the reaction of dichloroisocoumarin with the influenza C virus esterase. Pseudo-first-order inactivation rate constants (k_{obsd}) were determined from plots of the ln of activity versus time (Fig. 3). The corresponding half-lives for the inactivation and the apparent second-order rate constants (k_{obsd}/I) were calculated and are reported in Table 2. The 3,4-dichloroisocoumarin was the most potent inhibitor, with a k_{obsd}/I value of 410 per s per M. The 4-chloro-3-[(3-isothioureido)propoxy]-isocoumarin was 12 times less reactive than the 3,4-dichloroisocoumarin, and the 3-chloroisocoumarin was intermediate in reactivity, with a k_{obsd}/I value of 240 per s per M. The inactivation of the viral esterase was concentration dependent and took place at a higher rate as the inhibitor concentration increased. The half-life at any inhibitor concentration I' can be calculated from the equation $t_{1/2} = .693/I'/(k_{\text{obsd}}/I)$ which for dichloroisocoumarin would be $t_{1/2} = (0.693/I')/(410 \text{ per s per M})$. The enzyme could be considered to be dead (>99% inactivated) after 10 half-lives. With dichloroisocoumarin at a concentration of 100 μM, this would occur after 170 s (2.8 min).

FIG. 3. Inhibition of influenza C virus esterase by isocoumarins. Conditions were 10 mM phosphate buffer (pH 7.1) at 25°C containing 1 mM *p*-nitrophenylacetate, 1% dimethylsulfoxide, and 1% CH₃CN. The final concentration of viral proteins was 3 µg/ml. Isocoumarins tested were 6.3 µM 3,4-dichloroisocoumarin (◆), 13 µM 3-chloroisocoumarin (□), and 46 µM 4-chloro-3-[(3-isothioureido)propoxy]-isocoumarin (■).

Inhibition of elution of influenza virus C/JJ/50 from erythrocytes by 3,4-dichloroisocoumarin. A suspension of influenza virus C/JJ/50 was treated with 3,4-dichloroisocoumarin (0.1 mM) for 30 min at room temperature, and the virus was then used for hemagglutination assays. Elution was effectively inhibited in the row containing drug-treated virus (Fig. 4), while control virus readily eluted from erythrocytes after incubation for 3 h at 37°C. This finding indicated that the isocoumarin derivative inhibits not only hydrolysis of synthetic substrates by the viral enzyme but also the removal of viral receptors from intact cell membranes.

Inhibition of influenza C virus plaque formation. Previously, we had shown that inhibition of the receptor-destroying enzyme (esterase) of coronaviruses results in the loss of virus infectivity in a plaque assay and that the coronavirus enzyme is most likely necessary for entry of the virus (39). A similar plaque inhibition experiment was performed with the isocoumarin inhibitor and influenza C virus. Pretreatment of the virus with 3,4-dichloroisocoumarin resulted in a marked reduction of virus titer but had no effect on the hemagglutination titer (Table 3). The latter result suggested that attachment of influenza C virus to cell receptors is not inhibited in the presence of the inhibitor and that an active esterase is required for virus replication. On the other hand, no significant effect on the infectivity of influenza virus A/WSN/33 was shown when the virus was incubated under the same conditions with the isocoumarin derivative (Table 3).

TABLE 2. Kinetic parameters for the inhibition of influenza C virus esterase by isocoumarin derivatives

Compound	Concn (µM)	Half-life (s)	k_{obsd} (10 ⁻³ per s)	k_{obsd}/I (per s per M)
3,4-dichloroisocoumarin	6.3	270	2.6	410
3-chloroisocoumarin	13	230	3.0	240
4-chloro-3-[(3-isothioureido)propoxy]-isocoumarin	46	460	1.5	33

FIG. 4. Inhibition of elution of influenza virus C/JJ/50 from erythrocytes by 3,4-dichloroisocoumarin. Influenza virus C/JJ/50 was incubated in the presence of 100 µM 3,4-dichloroisocoumarin for 30 min at room temperature. Hemagglutination was then performed with chicken erythrocytes. (A) Microdilution plate incubated at 4°C for 60 min and photographed. (B) The same plate further incubated at 37°C for 180 min and rephotographed. +, Influenza virus C/JJ/50 incubated in the presence of 3,4-dichloroisocoumarin; -, mock-treated influenza virus C/JJ/50.

DISCUSSION

In this study, the active site of the influenza C virus acetylsterase was analyzed. DFP labeling of the enzyme identified the active site serine in position 71 of the predicted amino acid sequence of the HE protein. Assuming a signal cleavage at Ala-14 (25), this position corresponds to amino acid 57 of the mature HE protein. Earlier, sequence comparison studies showed that the N-terminal portion of the influenza C virus glycoprotein molecule cannot be easily aligned with the hemagglutinin molecules of either influenza A or B virus (25), which may indicate a unique functional domain for the influenza C virus HE in this N-terminal region. Beyond the first 120 to 150 amino acids, there are sites including cysteines and prolines which are conserved among the influenza A, B, and C virus hemagglutinin and HE molecules. This suggests (i) a common evolutionary relationship for these proteins and (ii) that these proteins possess corresponding functional domains responsible for receptor-binding, fusion, and membrane-anchoring functions.

Once the active site serine had been located in the influenza C virus HE protein, we decided to analyze the active site region surrounding the serine in greater detail and to compare its amino acid sequence with those of other serine hydrolases (Table 4). The amino acids adjacent to the active site serine of the influenza C virus esterase are identical to the consensus motif sequences (Gly-Asp-Ser)

TABLE 3. Plaque formation of influenza virus C/JJ/50 after treatment with 3,4-dichloroisocoumarin^a

Virus and treatment	Hemagglutination	Esterase activity (%)	Titer (PFU/ml)
Influenza virus C/JJ/50	256	100	2.6×10^6
Influenza virus C/JJ/50 plus 3,4-dichloroisocoumarin	128–256	<1	5.0×10^4
Influenza virus A/WSN/33 (no treatment)	8	NA ^b	6.4×10^6
Influenza virus A/WSN/33 plus 3,4-dichloroisocoumarin	8	NA	3.5×10^6

^a Virus was pretreated for 60 min at room temperature with 3,4-dichloroisocoumarin (50 µM). Hemagglutination and esterase assays and plaque titrations in MDCK cells were performed as described in Materials and Methods.

^b NA, Not applicable.

TABLE 4. Comparisons of sequences around active site serine residues in serine hydrolases

Enzyme (source)	Active site sequence ^a	Reference
Serine esterases		
HE (influenza virus C/JHG/66)	<u>G</u> F <u>G</u> D <u>S</u> R T	25, this report
Acetylcholinesterase (torpedo)	<u>I</u> F <u>G</u> E S A G	33
Butyrylcholinesterase (human)	<u>L</u> F <u>G</u> E S A G	21
Serine proteases		
Chymotrypsin-trypsin family		
Neutrophil elastase (human)	<u>C</u> F <u>G</u> D S G S	34
Chymotrypsin (bovine A)	<u>C</u> M <u>G</u> D S G G	10
Trypsin (bovine)	<u>C</u> Q <u>G</u> D S G G	41
Capsid protease (Semliki Forest virus)	<u>G</u> R <u>G</u> D S G R	22
Subtilisin family		
Subtilisin (Carlsberg)	<u>L</u> N <u>G</u> T S M A	35

^a Bold letters represent catalytic serine residues; underlined letters represent conserved residues between the influenza C virus esterase and serine hydrolases.

found in serine proteases such as trypsin, chymotrypsin, and elastase. In other serine esterases, such as the acetylcholine esterase, the Gly-Asp-Ser motif is changed to Gly-Glu-Ser, and in the subtilisin serine protease family it is replaced by Gly-Thr-Ser (Table 4). Interestingly, the third position upstream from the active site serine is phenylalanine in the influenza C virus esterase, the acetylcholinesterase, butyrylcholinesterase, and the neutrophil elastase. It should also be noted that the active site sequences Phe-Gly-Asp-Ser are conserved in all 11 influenza C virus HE proteins sequenced so far (3, 25, 30).

The catalytic site of serine proteases comprises three key residues (the reactive serine, an aspartic acid, and a basic histidine residue) which form a hydrogen-bonding system often referred to as the catalytic triad. All serine proteases have similar catalytic mechanisms in which the reactive hydroxyl group of the active site serine attacks the carbonyl of the substrate scissile bond, with the aspartic acid and histidine mediating the proton transfer during hydrolysis. The sequences of influenza C virus esterase and serine hydrolases do not have extensive sequence identities, and it was not possible to unambiguously identify the active site histidine or aspartic acid residue in the virus esterase by sequence comparison alone. In this context, it should be noted that the chymotrypsin-trypsin and subtilisin families of serine proteases lack extensive regions of sequence identity and that only the three-dimensional geometries of the catalytic residues are similar in these two families of serine hydrolases (31). Thus, it is possible that even in the absence of sequence identities the active site geometries of other B esterases are similar to those of the chymotrypsin-trypsin and subtilisin families. Clearly, a definitive answer to the question of involvement of additional amino acids in the active site of the influenza C virus esterase must await further experimentation. Overall, the analysis of the consensus motif sequences supports the idea that the influenza C virus esterase is a serine hydrolase. Since it lacks extensive sequence identities shared with other serine hydrolases, it appears that this viral enzyme is a member of a new family of serine esterases.

Since the influenza C virus esterase was inhibited by DFP, we decided to test a variety of mechanism-based and transition state inhibitors of serine proteases with this esterase.

FIG. 5. Structure of the isocoumarin inhibitors. For 3,4-dichloroisocoumarin, X and Y = Cl; for 3-chloroisocoumarin, X = H and Y = Cl; for 4-chloro-3-[(3-isothioureido)propoxy]-isocoumarin, X = Cl and Y = OCH₂CH₂CH₂SC(=NH₂⁺)NH₂.

Isocoumarins (Fig. 5) are inhibitors of serine proteases; they inactivate this class of enzymes by acylation of the active site serine, often followed by the unmasking of other reactive groups in the active site of the enzyme (8, 9, 17). The influenza C virus esterase was rapidly inhibited, in a time-dependent fashion, by several isocoumarins, including 3-chloroisocoumarin, 3,4-dichloroisocoumarin, and 4-chloro-3-[(3-isothioureido)propoxy]-isocoumarin (Table 1). The most potent inhibitor for the esterase is 3,4-dichloroisocoumarin (Table 2). This result suggests that the active site of the influenza virus esterase is similar to that of enzymes in the serine protease family. Interestingly, 3,4-dichloroisocoumarin reacts very poorly with acetylcholinesterase ($k_{\text{obsd}}/I = 0.5$ per s per M), suggesting that there are significant differences in the active site structures of these two enzymes and that the viral esterase belongs to a new serine esterase family.

The 3,4-dichloroisocoumarin is an easily handled, stable, crystalline material which does not react with other classes of proteases (8). These properties make it a useful alternative to the more toxic DFP for future studies of the influenza C virus esterase. In addition, the inhibition rate of the viral esterase by 3,4-dichloroisocoumarin is faster than the rates at which DFP inhibits similar enzymes such as chymotrypsin and acetylcholinesterase ($k_{\text{obsd}}/I = 150$ and 183 per s per M, respectively [1]).

A few other isocoumarins, such as 3-chloroisocoumarin and 4-chloro-3-[(3-isothioureido)propoxy]-isocoumarin (17), also inhibited the influenza C virus esterase, although they were generally less potent inhibitors than dichloroisocoumarin. The fact that the 4-chloro-3-[(3-isothioureido)propoxy]-isocoumarin was reasonably reactive towards the esterase, whereas other 3,4-disubstituted isocoumarins such as 4-chloro-3-propoxyisocoumarin showed no inhibition, possibly suggests a preference for basic residues in the active site of the enzyme. Many other substituted isocoumarins, benzoxazinones, and transition state inhibitors, including boronic acid derivatives and trifluoromethyl ketones, were inactive toward the viral enzyme. Surprisingly, 3,4-dibromoisocoumarin did not show any reactivity toward the esterase, whereas its dichloro analog was the most potent inhibitor of the influenza C virus esterase. These results suggest a very restricted active site and a high degree of substrate specificity.

The most potent inhibitor was then used to study the possible role of the esterase in cellular attachment and infection of influenza C virus. Pretreatment of influenza C virus with the isocoumarin inhibitor drastically reduced the infectivity titer concomitant with a complete inhibition of the esterase activity. In contrast, the receptor-binding activity was not affected in the presence of the esterase inhibitor, as

evidenced by an essentially unchanged hemagglutination titer, and elution from erythrocytes was prevented in the presence of the esterase inhibitor (Fig. 4). Although we cannot rigorously exclude the possibility that the isocoumarin derivative affects influenza C viruses in ways not mediated through the inhibition of the esterase activity, several lines of evidence suggest that this is not the case. First, influenza A viruses lacking an esterase activity were not inhibited by the isocoumarin derivative. Second, treatment of influenza C virus with DFP, another serine hydrolase reagent, prevented replication in embryonated eggs (23), presumably through a similar mechanism. Third, only those isocoumarin derivatives that completely inhibited esterase activity inhibited virus replication (unpublished data). Furthermore, the infectivity of coronaviruses which possess a similar esterase activity is also inhibited by esterase inhibitors (39). Since the esterase inhibitor is not present during virus replication, we postulate that inhibition of the receptor-destroying activity of influenza C viruses results in the inhibition of an early event, possibly virus entry. Further experiments should help to precisely define the early step that is impaired in the absence of a functioning esterase. In addition, we will address the question of whether the influenza C virus receptor-destroying enzyme-esterase plays a role at the end of the replication cycle similar to the function identified for the neuraminidase of influenza A viruses (29).

On the basis of the comparative analysis of the active site sequences of the influenza C virus esterase and those of other serine hydrolases and their reactivity toward serine protease inhibitors, we conclude that the influenza C virus esterase is a serine hydrolase. It appears to be a member of the Ser-Asp-His group of enzymes, whose active site geometries and catalytic mechanisms are highly conserved. However, additional studies will be necessary to define the precise catalytic site of the enzyme. Specifically, it will be important to identify and localize the histidine and aspartic acid residues which may participate in the charge relay system (catalytic triad) of this serine hydrolase. The inactivation of the influenza C virus esterase by isocoumarins probably involves the acylation of the active site serine to form stable acyl enzyme derivatives, a process which is similar to the first step in substrate hydrolysis. The isocoumarin inhibitors of the virus esterase should be helpful in elucidating the biological functions of the esterase, and they represent a useful first step in the design of more potent and specific inhibitors for viral esterases.

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