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O-Acetylation and De-*O*-acetylation of Sialic Acids

SIALIC ACID ESTERASES OF DIVERSE EVOLUTIONARY ORIGINS HAVE SERINE ACTIVE SITES AND ESSENTIAL ARGININE RESIDUES*

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We and others have recently described 9-*O*-acetylsialic acid esterase (9-*O*-Ac-SA esterase) activities that appear to be specific for removal of *O*-acetyl esters from the 9-position of naturally occurring sialic acids. We have now examined a variety of species for such enzymes and found them in vertebrates and higher invertebrates, but not in plants or in lower invertebrates. This evolutionary distribution correlates well with that of the sialic acids themselves. All of the 9-*O*-Ac-SA esterase activities tested were inhibited by diisopropyl fluorophosphate (DFP) in a dose-dependent fashion. This indicates that each of these enzymes has a serine active site similar to the well known serine esterases and serine proteases.

Methyl esterification of the carboxyl group of 9-*O*-acetyl-*N*-acetylneuraminic acid significantly reduced the activity of all of the 9-*O*-Ac-SA esterases against the *O*-acetyl group. This indicates that each of these enzymes may recognize the negatively charged carboxyl group of the sialic acid. Enzymes that recognize anionic substrates frequently have an essential arginine residue (Riordan, J. F., McElvany, K. D., and Borders, C. L., Jr. (1977) *Science* 195, 884–886). We therefore studied the effects of the arginine-specific modifying reagents 2,3-butanedione and phenylglyoxal on 9-*O*-Ac-SA esterase activities from influenza C virus, human erythrocytes, rat liver, starfish gonads, and sea bass brain. All of these enzymes were inhibited in a dose-dependent fashion by both reagents, under conditions previously known to avoid nonspecific modification. In contrast, the typical serine proteases trypsin and kallikrein and the serine esterase acetylcholinesterase were not significantly affected, even by the highest concentrations of these reagents used. These data indicate that five 9-*O*-Ac-SA esterase activities from evolutionarily distinct origins all have serine active sites and essential arginine residues. We postulate that the arginine residue is involved in substrate recognition via the negatively charged carboxyl group of the sialic acids. Thus, these 9-*O*-Ac-SA esterase activities may be members of a previously undescribed class of serine esterase.

Sialic acids are the outermost sugar residues on many glycoconjugates. There are more than 25 known naturally occurring derivatives of the parent *N*-acetylneuraminic acid molecule, several of which are *O*-acetylated at the 9-position (1, 2). In the absence of lactone formation, each of these molecules has a net negative charge at physiologic pH. The species distribution of the sialic acids has been extensively studied in the past (3) and has been summarized well by Schauer (1). The sialic acids seem to have appeared late in evolution, being present only in vertebrates and higher invertebrates, but not in plants or in lower invertebrates. Certain strains of bacteria with a pathogenic or commensal relationship to mammals are also known to express sialic acids (1).

Enzyme activities capable of removing acetyl groups from the 9-position of sialic acids have recently been described in certain mammalian viruses (4–6), in human erythrocytes (7), and in murine and equine livers (1, 8, 9). Each of these organisms either contains sialic acid itself or is hosted by an organism that does. Studies of these enzymes have suggested that while they can cleave small synthetic esters, they are otherwise specific for their sialic acid substrate (7, 8). The basis of specificity of these 9-*O*-acetylsialic acid esterases (9-*O*-Ac-SA esterases)[1] is not yet understood. In those cases studied, the 9-*O*-Ac-SA esterases were inhibited by diisopropyl fluorophosphate (DFP), a covalent modifier of active site serine residues in the classical serine hydrolases (10, 11). However, the derived amino acid sequence of the 9-*O*-Ac-SA esterase from influenza C (12, 13) does not contain the active site sequence Gly-*X*-Ser-*X*-Gly commonly found in other serine hydrolases (14). This suggested to us that the serine active site of the 9-*O*-Ac-SA esterases might be different from those of previously studied serine hydrolases.

Other enzymes that act on negatively charged substrates frequently contain arginine residues that are essential for activity and that may be involved in substrate recognition (15). In this study, we have examined the evolutionary distribution of the 9-*O*-Ac-SA esterases and their sensitivity to DFP and asked if these enzymes all contain arginine residues essential for activity.

EXPERIMENTAL PROCEDURES

Materials—Diisopropyl fluorophosphate (Aldrich) was prepared as 1 M and 100 mM stocks in isopropyl alcohol and stored in a desiccator at −20 °C. Diazomethane in ether was kindly provided by Drs. Alan Hoffman and Steve Rossi, Department of Medicine, UCSD. Phenyl-

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[1] The abbreviations used are: SA, sialic acid; DFP, diisopropyl fluorophosphate; [^{3}H]Neu5,9Ac$_2$, 9-*O*-[*acetyl*-^{3}H]acetyl-*N*-acetylneuraminic acid; [^{3}H]Neu1Me5,9Ac$_2$, methyl ester of 9-*O*-[*acetyl*-^{3}H]acetyl-*N*-acetylneuraminic acid; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, 4-morpholine propane sulfonic acid.

glyoxal and 2,3-butanedione were from Sigma. All other chemicals were obtained from commercial sources and were of reagent grade.

Porcine kallikrein was obtained from Sigma; bovine tosylphenylalanyl chloromethyl ketone-treated trypsin was obtained from Worthington Biochemical Co.; *Torpedo californica* acetylcholinesterase and [*acetyl*-^{3}H]acetylcholine were kindly provided by Dr. Palmer Taylor, UCSD; influenza C virus from embryonated eggs was generously provided by Dr. Richard O'Callaghan and Pam Wagaman, LSU. European medicinal leeches were provided by Dr. William Kristan, UCSD, and soybean and tobacco cells grown in suspension were furnished by Dr. Christopher Lamb, Salk Institute. *Dictyostelium discoideum* amoebae were provided by Dr. Hudson Freeze, La Jolla Cancer Research Foundation.

Enzyme Preparations—The partial purification of the human erythrocyte esterase has been previously published (7). The rat liver-glycosylated 9-*O*-Ac-SA esterase was purified as previously described (16) except that the final S-200 and Procion Red chromatographic steps were omitted. The gonads of the starfish *Pisaster brenispinous* and the brain of the sea bass *Paralabrax nebulifer* were dissected out and sonicated in PBS containing 1 mM EDTA. Following sonication, the suspensions were centrifuged at 100,000 × *g* for 1 h. The resulting supernatants were used without further purification. Five leeches (6–7 weeks old) of the species *Hirudo medicinalis* were first minced and then subjected to sonication in PBS. Sonication was performed for 10 min using a Sonics & Materials sonicator at a power setting of 4, operated in the pulse mode, with a duty cycle of 70%. The resulting suspension was then clarified by centrifugation at 12,000 × *g* for 10 min.

Soybean (*Glycine max*) and tobacco (*Nicotiana tobacum*) cells that had been grown in suspension were washed twice in PBS and resuspended in PBS. Cells were lysed using a nitrogen cavitation apparatus. Lysis was confirmed microscopically, and the lysates were used without further purification. The vacular contents of the plant cells that are released during cell lysis are potential inhibitors of endogenous enzymes. In order to control for this, a separate aliquot of cells was washed twice in PBS and resuspended in PBS to which was added β-mercaptoethanol to 10 mM and decolorizing charcoal. These cells were then lysed by sonication in a fashion similar to that used above for the disruption of leech tissue. Cell lysis was again confirmed by microscopy. The resulting sonicate was then clarified by centrifugation at 12,000 × *g* for 10 min, and the supernatant was used without further purification. There was no difference in the results obtained with these preparations compared with the lysates prepared with nitrogen cavitation.

Amoebae of the species *D. discoideum* were washed twice in PBS and then resuspended in PBS. This cell suspension was then sonicated as above except that the time was decreased. Lysis was again confirmed microscopically. The resulting sonicate was then centrifuged at 12,000 × *g* for 10 min. The resulting supernatant was then used without further purification. Sf-9 cells, a cell line derived from the fall army worm *Spodoptera frugiperda*, were washed twice in PBS and then resuspended in PBS. These cells were disrupted by sonication in a fashion similar to that used for *D. discoideum*. The resulting suspension was used without further purification.

Synthesis and Purification of the Methyl Ester of 9-O-[^{3}H]Acetyl-N-acetylneuraminic Acid—[^{3}H]Neu5,9Ac$_2$ was prepared as described in the preceding paper (16), except that the label released by neuraminidase was collected by dialysis rather than by centrifugation. The purified labeled material (800,000 cpm) was dissolved in 200 μl of anhydrous methanol at room temperature in a sonicating water bath for 6 h. Diazomethane in ether was added sufficient to maintain the yellow color (200 μl). The esterification reaction was allowed to proceed at room temperature for 60 min, spotted directly onto Whatman No. 3MM paper, and chromatographed in 95% ethanol:1 M ammonium acetate, pH 5.0 (7:3), for 12 h. The paper was dried and cut into 1-cm strips, each of which was soaked in 1 ml of water on ice, and aliquots (10 μl) from each tube were monitored for radioactivity. The majority of the label was converted to the methyl ester ([^{3}H]Neu1Me5,9Ac$_2$), which migrates with an R_f of 1.24 relative to [^{3}H]Neu5,9Ac$_2$. The peak of radioactivity was pooled, lyophilized, and taken up in water prior to use. The purified compound was found to be >95% intact upon rechromatography.

Enzyme Assays—The assay of 9-*O*-acetylsialic acid esterases was performed using *O*-[*acetyl*-^{3}H]Neu5,9Ac$_2$ as previously described (16) with the following modifications: reaction volumes were typically 50 μl, and the entire reaction volume was subjected to scintillation counting following quenching with an equal volume of "stopping solution." In this assay, the released [^{3}H]acetate enters the toluene-based scintillation mixture where it is counted, whereas the substrate remains in the aqueous phase and is not counted (16). Release of radioactivity from the methyl ester of [^{3}H]Neu5,9Ac$_2$ ([^{3}H]Neu1Me5,9Ac$_2$) could not be monitored in this manner, because the substrate itself is hydrophobic enough to enter the toluene phase. The activity of the 9-*O*-Ac-SA esterases against this substrate was therefore monitored by evaporation of the released [^{3}H]acetate (7). These reactions were carried out in 100 mM MOPS buffer, pH 6.8, to minimize breakdown of the methyl ester during the incubation. The reactions (100 μl) were quenched by addition of 10 μl of glacial acetic acid, evaporated on a Speed-Vac concentrator, and taken to complete dryness on a lyophilizer. Following addition of 1 ml of water, each tube was mixed well, and any particulate matter was spun down at 10,000 × *g*. Aliquots (900 μl) of the supernatant were removed for monitoring of residual radioactivity. Parallel assays under identical incubation conditions with similar amounts of [^{3}H]Neu5,9Ac$_2$ were carried out and monitored in the same fashion. One blank reaction was spotted directly onto Whatman No. 3MM paper and chromatographed in 95% ethanol:1 M ammonium acetate, pH 5.0 (7:3), for 13 h, to monitor breakdown of the methyl ester during the incubation. In both of these assays, 1 unit of activity is defined as the release of 1% of [^{3}H]acetate per h. All assays were performed under conditions where product formation was linear with time and added enzyme.

Acetylcholinesterase was assayed essentially according to Johnson and Russell (18) except that the reaction volumes were typically 50 μl, and the reaction was stopped by the addition of an equal volume of stopping solution. Kallikrein and trypsin were assayed essentially according to Morita *et al.* (19) except that the substrates, L-prolyl-L-phenylalanyl-L-arginine-7-amido-4-methylcoumarin, and *N*-benzoyl-L-arginine-7-amido-4-methylcoumarin, respectively, were used at 8 μM final concentration, and fluorescence was monitored at 60-s intervals to confirm the linearity of the assay.

Inhibition by Diisopropyl Fluorophosphate—A stock solution of DFP in isopropyl alcohol (100 mM) was used to prepare serially diluted 20× stock solutions in water. Aliquots of the various enzymes previously determined to cause less than 20% hydrolysis of the [^{3}H]Neu5,9Ac$_2$ substrate in 60 min at 37°C (in the linear range of the assay) were placed in polypropylene tubes. PBS was added to a final volume of 50 μl, and to this was added 2.6 μl of 20× inhibitor. The inhibition reaction was allowed to proceed for 20 min at room temperature. Following this incubation the [*acetyl*-^{3}H]Neu5,9Ac$_2$ substrate was added to the tubes, and the standard assay was performed.

Inhibition by 2,3-Butanedione or Phenylglyoxal—2,3-Butanedione was dissolved in water, and phenylglyoxal was dissolved in 95% ethanol. Both reagents were prepared as serially diluted 20× stock solutions to ensure constant final solvent concentration in the reactions. An amount of enzyme previously determined to cause less than 20% hydrolysis of the [^{3}H]Neu5,9Ac$_2$ substrate in 60 min at 37°C (in the linear range of the assay) was placed into a polypropylene tube. To this was added 25 μl of either 200 mM HEPES, 60 mM NaBO$_4$, pH 8.0 (if 2,3-butanedione was to be used), or 25 μl of 200 mM HEPES, pH 8.0 (if phenylglyoxal was used). Water was added to a final volume of 50 μl, and to this was added 2.6 μl of 20× inhibitor. The inhibition reaction was allowed to proceed for 20 min in the dark at room temperature. Following this incubation, the [*acetyl*-^{3}H]Neu5,9Ac$_2$ substrate was added to the tubes, and the standard assay was performed. The presence of either inhibitor did not affect the background of the enzyme assay. The borate which was added to prevent nonspecific reactions with 2,3-butanedione also decreased enzyme activity, even in the absence of the reagent. This was controlled with an appropriate buffer blank.

The "control" serine hydrolases acetylcholinesterase, trypsin, and kallikrein were similarly treated with each reagent, using a quantity of enzyme required for linear conditions. Since the ethanol itself inhibited the acetylcholinesterase, the effect of phenylglyoxal on this enzyme could not be tested.

RESULTS AND DISCUSSION

Species Distribution of 9-O-Acetylsialic Acid Esterase Activity—We have developed a rapid and simple radiometric assay that permits the sensitive and specific detection of 9-*O*-Ac-SA esterases, even in crude extracts of tissues (16, 17). We used this assay to look at extracts from a variety of tissues and cells (see Table I). We identified such activities in the gonads of the starfish *Pisaster brenispinous* and in the brain of the sea bass *Paralabrax nebulifer*. In contrast, significant

TABLE I
Species distribution of 9-O-acetylsialic acid esterases

Enzyme source	Activity (*A*)	-Fold purified over 100,000 × *g* supernatant (*B*)	*A*/*B*
	units/mg		
Influenza C virus	1040	NA[a]	NA
Human erythrocyte	3660	860	4
Rat liver	1210	45	27
Starfish gonad	36	1	36
Sea bass brain	65	1	65
Sf-9 cells	ND[b]	NA	NA
Leech	ND	NA	NA
D. discoideum	ND	NA	NA
Tobacco[c]	ND	NA	NA
Soybean	ND	NA	NA

[a] NA, not applicable.
[b] ND, not detectable. Values of less than twice the buffer control are considered insignificant. Final protein concentrations in these negative samples were similar to those used in the assays of starfish gonad and sea bass brain. A mixing experiment with human erythrocyte esterase (a 60-min preincubation with each of the negative extracts) did not show evidence for inhibitors. Also, 10-fold dilution of these extracts did not result in the appearance of measurable activity in any of the samples.
[c] In some experiments, tobacco cell extracts showed just detectable levels of activity.

9-*O*-Ac-SA esterase activity was not found in extracts of the medicinal leech (*H. medicinalis*), Sf-9 insect cells (derived from the fall army worm *S. frugiperda*), amoebas of *D. discoidium*, or in cultured cells from soybean (*Glycine max*) and tobacco (*N. tobacum*). Thus, the evolutionary distribution of these 9-*O*-Ac-SA esterase activities appears to parallel the previously described distribution of the sialic acids in nature, being present in higher invertebrates and in vertebrates (1, 3). This also implies that the hydrolysis of the [^{3}H]Neu5,9Ac$_2$ substrate by the extracts from higher animals is not due to the action of "nonspecific esterases."

Inhibition of All 9-O-Acetylsialic Acid Esterases by DFP—The 9-*O*-Ac-SA esterase activities from starfish and sea bass were then studied in parallel with those previously described from influenza C virus (4, 6, 20), human erythrocytes (7), and rat liver (16). Some of the enzymes were assayed in a partially purified state, while others were studied as extracts. As shown in Table I, comparison of the activity of these preparations relative their respective 100,000 × *g* supernatants shows that per mg of protein, the sea bass brain contained the highest amount of activity, followed by starfish gonads, rat liver, and human erythrocytes. Influenza C virus contained a higher specific activity than any of the other preparations in the crude state. However, since the virus is prepared from embryonated eggs, it cannot be compared directly with the tissue extracts. As shown in Fig. 1, all of these activities were inhibited in a dose-dependent fashion by DFP. This indicates that each of these 9-*O*-Ac-SA esterases has a serine active site mechanism akin to the classic serine hydrolases (10, 11).

Methyl Esterification of the Carboxyl Group of 9-O-Acetyl-N-acetylneuraminic Acid Decreases the Activity of the Esterases—Each of the five 9-*O*-Ac-SA esterases, from influenza C virus, human erythrocytes, rat liver, starfish gonads, and sea bass brain was tested for its ability to remove the acetyl group from the 9-position of sialic acids in comparison with their activities against the corresponding 1-*O*-methyl ester derivative. The results are summarized in Table II. All of the enzymes hydrolyzed the methyl ester derivative at a significantly lower rate when compared to the free acid (range 6–52% of control). These results indicate that each of these enzymes may recognize the negatively charged carboxyl group of the sialic acid substrate. Of course, it cannot be ruled out that the introduction of the methyl group hinders access to the substrate.

The Arginine-modifying Reagents 2,3-Butanedione and Phenylglyoxal Inhibit 9-O-Acetylsialic Acid Esterases—It has previously been reported that enzymes that recognize anionic substrates frequently have an essential arginine residue whose positively charged side chain may contribute to substrate recognition (15, 21). All of the sialic acid derivatives known in nature have a net negative charge at physiologic pH. Since the negatively charged carboxyl group on the [^{3}H]Neu5,9Ac$_2$ substrate seemed to be important for optimal activity of all of the 9-*O*-Ac-SA esterases, we reasoned that they might have arginine residues involved in substrate recognition. We therefore studied the effects of 2,3-butanedione and phenylglyoxal, two well known covalent modifiers of arginine residues, on each of the five 9-*O*-Ac-SA esterase activities. The conditions chosen were those previously used by others to avoid nonspecific modification (21–24). As shown in Fig. 2, each of these enzymes exhibited dose-dependent inhibition of activity with either of the two modifying reagents. These results indicate that all of these enzymes have 1 or more arginine residues that are essential for their activity.

The Arginine-modifying Reagents Do Not Inhibit Other Serine Hydrolases—Because of the varying degrees of purity of the 9-*O*-Ac-SA esterase enzyme preparations (and hence the actual amounts of protein in each inhibition reaction), we had deliberately chosen to use relatively high concentrations of the modifiers, for short periods of time. To ensure that the inhibitions seen were not due to nonspecific effects upon nonessential arginine residues, we examined the effects of the same concentrations of these reagents on purified preparations of the well known serine active site enzymes trypsin, kallikrein, and acetylcholinesterase. As shown in Fig. 3, bovine trypsin and porcine kallikrein were essentially unaffected by either 2,3-butanedione or phenylglyoxal when preincubated with these inhibitors in a manner identical with that used for the 9-*O*-Ac-SA esterases. Likewise, as shown in Fig. 3, *Torpedo californica* acetylcholinesterase was essentially unaffected by 2,3-butanedione. The ethanol used to dissolve the phenylglyoxal inhibited the acetylcholinesterase, making assays with this reagent impossible. It should be noted that these three enzymes (trypsin, kallikrein, and acetylcholinesterase) contain 2, 3, and 27 arginine residues, representing 0.9, 1.3, and 4.5% of the protein by weight, respectively.

The data obtained with the highest concentrations of each of the inhibitors with all of the enzymes are summarized in Fig. 4. It can be seen that each of the five 9-*O*-Ac-SA esterases, but none of the three tested control serine active site esterases, was inhibited by the arginine-modifying reagents. Taken together, the results presented above indicate that there is at least 1 arginine residue in each of the 9-*O*-Ac-SA esterases that is necessary for enzyme activity. We postulate that such an arginine residue could participate in the recognition of the negatively charged sialic acid substrate.

Support for the Hypothesis from Currently Known Sequences—In order for the essential arginine residue to be involved in substrate recognition, it must be physically close to the serine active site pocket. However, the location of the arginine residue on the polypeptide chain itself obviously cannot be predicted from these results. At present, the only relevant sequence data available are the derived amino acid sequences of the influenza C hemagglutinin esterase protein. We therefore examined the predicted amino acid sequences of all 11 influenza C hemagglutinin esterase found in the EMBL (Release 17.0) and GenBank (Release 58.0) data bases

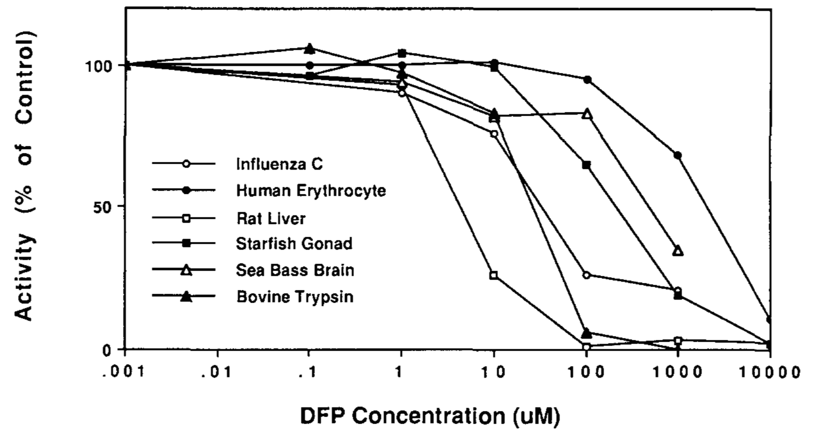

FIG. 1. **Effects of DFP on the activity of various enzymes.** The various enzymes were preincubated with the indicated concentrations of DFP and analyzed for residual 9-*O*-Ac-SA esterase activity, exactly as described under "Experimental Procedures."

TABLE II

Comparison of activity of 9-O-acetylsialic acid esterases against 9-O-acetyl-N-acetylneuraminic acid and its methyl ester

Amounts of each enzyme sufficient to cause cleavage of about 20% of the [^{3}H]Neu5,9Ac$_2$ substrate was incubated with 10,000 cpm of either [^{3}H]Neu5,9Ac$_2$ or the methyl ester [^{3}H]Neu1Me5,9Ac$_2$, and the release of [^{3}H]acetate was monitored as described under "Experimental Procedures." All values are the mean of duplicate determinations. To check for spontaneous breakdown of the methyl ester during the reactions, a similarly incubated buffer control was spotted on Whatman No. 3MM paper and chromatographed as described under "Experimental Procedures." The resulting profile showed 4% breakdown of the methyl ester during the incubation.

Enzyme source	Rate of cleavage of *O*-acetyl esters from [^{3}H] Neu5,9Ac$_2$	Rate of cleavage of *O*-acetyl esters from [^{3}H] Neu1Me5,9Ac$_2$	Relative activity against the methyl ester
	units/μl enzyme	*units/μl enzyme*	*% control*
Influenza C virus	0.295	0.018	6
Human erythrocyte	1.340	0.255	19
Rat liver	0.433	0.223	52
Starfish gonad	0.800	0.184	23
Sea bass brain	0.222	0.080	36

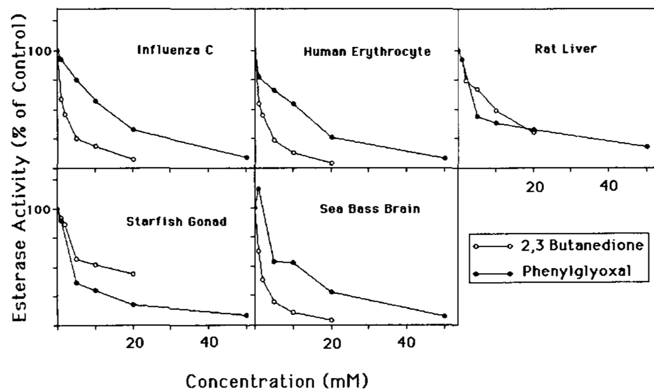

FIG. 2. **Effects of arginine-modifying reagents on the activity of various enzymes.** The various enzymes were preincubated with the indicated concentrations of 2,3-butanedione or phenylglyoxal and analyzed for residual 9-*O*-Ac-SA esterase activity, exactly as described under "Experimental Procedures."

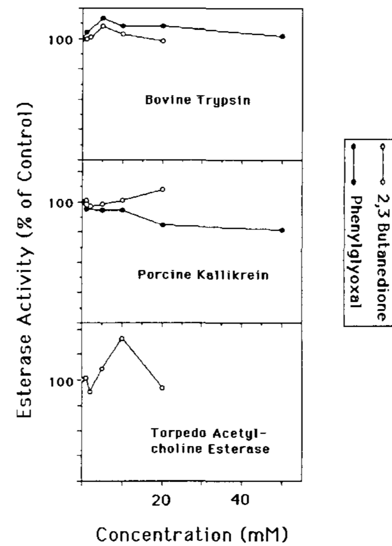

FIG. 3. **Effects of arginine-modifying reagents on the activity of various enzymes.** The various enzymes were preincubated with the indicated concentrations of 2,3-butanedione or phenylglyoxal and analyzed for residual activity, as described under "Experimental Procedures." For reasons described in the text, the effect of phenylgloxal on the acetylcholinesterase could not be tested.

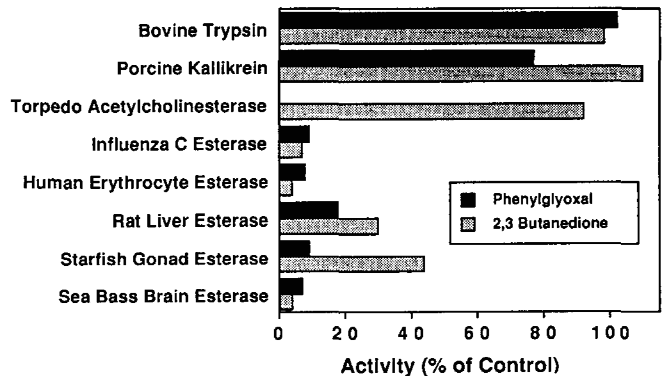

FIG. 4. **Summary of the effects of arginine-modifying reagents on the activity of various enzymes.** The effects of the highest concentrations of 2,3-butanedione (20 mM) or phenylglyoxal (50 mM) on all of the enzymes tested are presented for direct comparison.

(25, 26). In no case did we find the Gly-*X*-Ser-*X*-Gly motif found in many of the other prototypic members of the families of serine esterases (see recent compilation of serine active sequences by Brenner (14)). Vlasak *et al.* (27) have very recently used [^{3}H]DFP to covalently radiolabel the active site serine of the influenza C/California/78 hemagglutinin esterase protein. Tryptic digestion, purification, and sequencing demonstrated the incorporation of ^{3}H into a residue corresponding to the serine at position 71. When we further examined this region of the hemagglutinin esterase proteins' derived amino acid sequence, we noted that there is an argi-

TABLE III

Nucleotide and derived amino acid sequences surrounding the putative active site serine of the hemagglutinin esterases of several independent isolates of influenza C

All available influenza C hemagglutinin esterase sequences from the EMBL/GenBank data bases were examined. Nine of the eleven sequences surrounding the active site serine are shown. Two additional sequences (influenza C/pig/Beijing/115/81 and influenza C/pig/Beijing/439/82) are not shown because they are identical with influenza C/pig/Beijing/10/81 in the region of interest. The active site serine is *italic* in each sequence. The adjacent arginine and its corresponding codons are **boldface**.

Isolate/strain	Sequence
Influenza C/California/78	GGCTTTGGAGATTCA**AGA**ACTGACCAAAGCAATTCA GlyPheGlyAsp*Ser***Arg**ThrAspLysSerAsnSer
Influenza C/Ann Arbor/1/50	GGCTTTGGAGATTCA**AGG**ACTGACAAAAGCAATTCA GlyPheGlyAsp*Ser***Arg**ThrAspLysSerAsnSer
Influenza C/England/892/83	TGCTTTGGAGATTCA**AGA**ACTGACCAAAGCAATTCA CysPheGlyAsp*Ser***Arg**ThrAspGlnSerAsnSer
Influenza C/Great Lakes/1167/54	GGCTTTGGAGATTCA**AGA**ACTGACAAAAGCAATTCA GlyPheGlyAsp*Ser***Arg**ThrAspLysSerAsnSer
Influenza C/Mississippi/80	GGCTTTGGAGATTCA**AGG**ACTGACAAAAGCAATCAA GlyPheGlyAsp*Ser***Arg**ThrAspLysSerAsnPro
Influenza C/Yamagata/10/81	GGCTTTGGAGATTCA**AGA**ACTGACAAAAGCAATTCA GlyPheGlyAsp*Ser***Arg**ThrAspLysSerAsnSer
Influenza C/Johannesburg/66	GGCTTTGGAGATTCA**AGG**ACTGACAAAAGCAATTCA GlyPheGlyAsp*Ser***Arg**ThrAspLysSerAsnSer
Influenza C/pig/Beijing/10/81	TGCTTTGGAGATTCA**AGA**ACTGACCAAAGCAATTCA CysPheGlyAsp*Ser***Arg**ThrAspGlnSerAsnSer
Influenza C/Taylor/1233/47	GGCTTTGGAGATTCA**AGG**ACTGACAAAAGCAATTCA GlyPheGlyAsp*Ser***Arg**ThrAspLysSerAsnSer

nine residue immediately following the proposed active site serine. The resulting sequence Phe-Gly-Asp-Ser-**Arg**-Thr-Asp was not found in any other previously known serine active site enzyme. Furthermore, we found that the identical motif is preserved in the derived amino acid sequences of all of the isolates of the influenza C virus hemagglutinin esterase protein reported to date in the EMBL/GenBank data base. Table III shows all nine unique sequences of the influenza C hemagglutinin esterase protein found in the data bases. The isolates span nearly 4 decades and are from many different geographic regions. As with the envelope proteins of most RNA viruses, there is significant drift in the nucleotide sequence encoding these hemagglutinin esterase proteins. Such changes can be seen in the regions flanking the proposed active site sequence in several cases (see Table III). Conservative changes can even be seen in the codon usage for the arginine residue (AGA and AGG). However, in every case, the sequence Gly-Asp-Ser-Arg-Thr is present. This conservation of the proposed active site sequence over 4 decades of drift and substantial geographic separation suggests that the arginine residue immediately adjacent to the active site serine may be the one whose modification leads to loss of enzyme activity.

CONCLUSIONS

In this study, we have shown that the evolutionary distribution of the 9-O-Ac-SA esterases appears to correlate with the distribution of the sialic acids themselves. Furthermore, we have shown that several of these enzymes from diverse evolutionary origins not only have a DFP-sensitive serine active site but, further, have at least 1 essential arginine residue. Since the enzymes all appear to recognize the carboxyl group of the acetylated sialic acid, we postulate that such an arginine residue is involved in substrate recognition. These 9-O-acetylsialic acid esterases may therefore be members of a previously undescribed family of serine active site esterases. It remains to be seen whether the motif Gly-Asp-Ser-Arg-Thr, found to be conserved in the influenza C esterase, represents a unique active site consensus sequence for all of these enzymes.

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