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Inhibition of Influenza Virus Fusion by Polyanionic Proteins

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ABSTRACT. Anionic charge-modified human serum albumin (HSA) has previously been shown to exert potent *in vitro* activity against human immunodeficiency virus type 1 (HIV-1). In these studies, introduction of the additional negative charges was performed by derivatizing the ϵ-amino groups of lysine residues with succinic (Suc-HSA) or *cis*-aconitic anhydride (Aco-HSA), by which primary amino groups are replaced with carboxylic acids. The anti-HIV-1 activity was related to inhibition of gp41-mediated membrane fusion. Here, we investigated the activity of aconitylated and succinylated proteins on influenza virus membrane fusion, which is mediated by the viral membrane glycoprotein hemagglutinin (HA). Aco-HSA and Suc-HSA markedly inhibited the rates and extents of fusion of fluorescently labeled virosomes bearing influenza HA, with target membranes derived from erythrocytes. The inhibitory activity was dependent on the overall negative-charge density; HSA modified with 36 or less extra negative charges failed to inhibit fusion. The inhibition of fusion showed a certain degree of specificity for the protein carrying the negative charges: polyanionic HSA and β-lactoglobulin A derivatives had fusion-inhibitory activity, whereas succinylated BSA, lactalbumin, lactoferrin, lysozyme, and transferrin were inactive. Aco_{60}-HSA and Aco-β-lactoglobulin A inhibited influenza virus membrane fusion in a concentration-dependent manner, IC_{50} values being about 4 and 10 μg/mL, respectively. HA-mediated membrane fusion is pH dependent. Aco_{60}-HSA did not induce a shift in the pH threshold or in the pH optimum. Fusion with liposomes of another low pH-dependent virus, Semliki Forest virus, was not specifically affected by any of the compounds reported here. In view of some structural and functional similarities between influenza HA and the HIV-1 gp120/gp41 complex, it is tempting to postulate that the current results might have some implications for the anti-HIV-1 mechanism of polyanionic proteins. BIOCHEM PHARMACOL 53;7:995–1003, 1997. © 1997 Elsevier Science Inc.

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Human serum albumin (HSA§), derivatized with succinic anhydride (Suc-HSA) or *cis*-aconitic anhydride (Aco-HSA), has potent *in vitro* anti-human immunodeficiency virus type 1 (HIV-1) activity [1–4]. Succinylation of lysine residues modifies the net charge per reacted residue from +1 to −1, whereas aconitylation modifies the net charge per residue from +1 to −2. Thus, completely derivatized HSAs are highly negatively charged, Aco-HSA being relatively more negatively charged than Suc-HSA. Previously, it has been shown that the anti-HIV-1 activities of Suc-HSA and Aco-HSA predominantly result from inhibition of virus cell binding and fusion through interactions with the gp120/gp41 complex of the HIV-1 envelope [1, 2, 5, 6]. Recently, a number of other plasma and milk proteins, i.e. lysozyme, transferrin, and lactoferrin, were also investigated for anti-HIV-1 activity [4]. Lysozyme and transferrin had no appreciable anti-HIV-1 activity, but unmodified lactoferrin inhibited HIV-1 replication. After succinylation the anti-HIV-1 activity of lactoferrin increased further [4, 6].

Enveloped viruses can be classified in two categories: the low pH-independent viruses, like HIV-1, which fuse with the plasma membranes of cells, and the low pH-dependent ones that fuse with the membranes of acidic endosomes after cellular uptake by receptor-mediated endocytosis. It is of pharmacologic interest to investigate whether (negative charge-modified) proteins also inhibit membrane fusion of viruses other than HIV-1. Influenza virus and Semliki Forest virus (SFV) are two classical examples of low pH-

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§ *Abbreviations:* HA, hemagglutinin; HSA, human serum albumin; Suc-HSA and Aco-HSA, HSA derivatized with succinic anhydride or *cis*-aconitic anhydride, respectively; HIV-1, human immunodeficiency virus type 1; SFV, Semliki Forest virus; $C_{17}E_8$, octaethyleneglycol monododecyl ether; pyrPC, pyrene-labeled phosphatidylcholine; HBS, HEPES-buffered saline.

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dependent viruses. Infection of cells by influenza virus is mediated by the viral hemagglutinin (HA) [7–9]. The three-dimensional structure of HA has been known for 15 years [10]. The distal globular domains each contain a receptor-binding pocket, while the stem region of the spikes bears the so-called fusion peptides. At neutral pH, the hydrophobic fusion peptides are located at the interfaces of the subunits in the stem region. At low pH, between pH 5 and pH 6, conformational changes are induced, which lead to exposure of the fusion peptides and subsequent merging of the viral membrane with the limiting membrane of the endosomal cell compartment (see [11–13] for further details).

Here, we provide a detailed characterization of the effect of negative charge-modified HSA on HA-mediated membrane fusion. The marked fusion–inhibitory activity of negative charge-modified HSA prompted us to also investigate whether similar charge modifications of other proteins, such as lysozyme, transferrin, and some milk proteins, would result in compounds with antifusion activity as well. To this end, a well-defined model system was used, based on pyrene-labeled HA-containing vesicles (virosomes) and target membranes derived from erythrocytes [14]. Pyrene forms fluorescent dimers, composed of an excited and a nonexcited monomer (excimer), in a concentration-dependent fashion. Upon membrane fusion, the pyrene-labeled lipids mix with the target membrane lipids, which results in probe dilution and subsequent loss of excimer fluorescence. Our results show that polyanionic HSA and β-lactoglobulin A do not interfere with HA-dependent binding of influenza virosomes to erythrocyte ghosts at neutral pH, but inhibit a specific step in HA-mediated membrane fusion.

MATERIALS AND METHODS

Materials

HSA was obtained from the Central Laboratory of the Dutch Red Cross Blood Transfusion Service (Amsterdam, The Netherlands); other proteins were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). The monomeric fraction of the proteins was at least 95% as determined by FPLC. *Cis*-Aconitic anhydride and succinic anhydride were obtained from Janssen Chimica (Beerse, Belgium). Octaethyleneglycol monododecyl ether ($C_{12}E_8$) was from Fluka Chemie AG (Buchs, Switzerland), Bio-Beads SM-2 (bead size 300–1,180 μm) were obtained from Bio-Rad (Hercules, CA, USA), and 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (pyrPC) and 1-hexadecylpyrene were from Molecular Probes (Eugene, OR, USA). Egg phosphatidylcholine, phosphatidylethanolamine (prepared by transphosphatidylation of egg phosphatidylcholine), brain sphingomyelin, and cholesterol were from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). All other chemicals were of the highest grade available.

Influenza and Semliki Forest Viruses

The X47 recombinant strain of influenza virus, carrying the HA of influenza A/Victoria/3/75 (subtype H3N2), and influenza A/Shangdong/9/93 virus (subtype H3N2) were grown in the allantoic cavity of 10-day-old embryonated hens' eggs. The virus was purified as described [15]. After the resuspension of sedimented virus in 5.0 mM HEPES (pH 7.4), containing 0.15 M NaCl (HEPES-buffered saline; HBS), virus samples were loaded atop linear 10–60% (w/v) sucrose gradients in HBS, and centrifuged at 90,000 × *g* (for 36 hr at 4°C). The virus-containing bands were sampled and pooled. Virus preparations were stored at −80°C.

SFV was labeled biosynthetically with pyrene, as described previously [16]. This labeling procedure relies on the production of virus from cells cultured beforehand in the presence of pyrene-labeled hexadecanoic acid (1-hexadecylpyrene). Because this fatty acid is readily incorporated into the cellular membrane lipids, a virus preparation is obtained that contains a significant amount of pyrene-labeled phospholipids in the viral envelope. Labeling of SFV with the pyrene fluorophore does not affect the infectivity of the virus. Pyrene-labeled SFV was harvested and purified as described [16]. For all virus preparations, the phospholipid contents were determined by phosphate analysis [17] after extraction [18] and digestion [19] of the lipids.

Derivatization of Proteins With Succinic Acid and Aconitic Acid

Proteins were derivatized with succinic anhydride or *cis*-aconitic anhydride as follows: 10 mg of the protein was dissolved in 10 mL 0.20 M K_2HPO_4 (pH 8.0). Solid anhydride (10 mg) was added and the solution was stirred until all anhydride was dissolved. The solution was kept at pH 8–8.5 using 3.0 M NaOH. Free anhydride was removed by gel filtration on a Sephadex G25 column (Pharmacia, Uppsala, Sweden). The reaction products were washed with distilled water in a stirred cell concentrator (Amicon Inc, Beverly, MA, USA) equipped with a so-called omega membrane (Filtron Technology Co., MA, USA), and finally lyophilized. Partial derivatizations were performed by using variable amounts of the anhydrides.

Protein concentrations were determined according to the method of Lowry *et al.* [20]. The number of free primary amino groups present in the derivatized proteins were determined according to the method described by Habeeb [21]. Molecular mass estimations and determinations of the monomeric fractions of the proteins were performed by FPLC on a Superose-12 column (Pharmacia) as described previously [22]. Elution was performed with PBS (pH 7.4) at a flow rate of 0.4 mL/min. The relative net charge of derivatized proteins was determined by FPLC on a Mono-Q anion exchange column (Pharmacia) as described previously [23]. The column was developed with a linear gradient of 0–1.0 M NaCl in 20 mM Tris-HCl (pH 7.4), at a flow rate of 0.25 mL/min, for 30 min.

Virosomes

Reconstituted viral envelopes (virosomes) from influenza virus were prepared by a detergent solubilization and removal procedure based on $C_{12}E_8$ [24, 25]. Briefly, an amount of virus representing 1.5 μmol of phospholipid was sedimented by centrifugation, and subsequently solubilized with 0.70 mL 0.10 M $C_{12}E_8$ in HBS. The nucleocapsid fraction was removed by centrifugation, and the resulting supernatant (containing mixed micelles of $C_{12}E_8$, viral lipids, and the viral membrane proteins) was gently shaken with Bio-Beads SM-2 to remove the detergent. Thereafter, the virosomes were purified by sucrose density centrifugation. Incorporation of pyrPC in the virosomal membranes was achieved by adding $C_{12}E_8$ supernatants to 0.15 μmol of the dry probe prior to removal of the detergent with Bio-Beads SM-2. The phospholipid content of virosomes was determined by phosphate analysis [17] after lipid digestion [19].

Erythrocyte Ghosts

Ghosts were prepared from outdated red blood cell concentrates (blood type B, rhesus factor negative) by the method of Steck and Kant [26]. Erythrocytes were hemolysed at 4°C in 5.0 mM HEPES (pH 8.0), containing 5.0 mM EDTA. Sealing was performed in 5.0 mM HEPES (pH 7.4), containing 0.15 M NaCl and 5.0 mM $MgCl_2$, for 1 hr at 37°C. Sealed ghosts were collected by centrifugation and stored in HBS at −20°C. Ghost phospholipid content was determined by phosphate analysis [17] after extraction [18] and digestion [19] of the lipids.

Liposomes

Dry lipid films composed of phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, and cholesterol (molar ratio 1:1:0.35:1.5) were dispersed in HBS. The resulting dispersion was subjected to five cycles of freezing and thawing, and subsequently extruded 10 times [27] through a Nucleopore polycarbonate membrane with a pore size of 0.2 μM (Costar Co., Cambridge, MA, USA), using a miniextruder (Avanti Polar Lipids, Inc.). The phospholipid content was determined by phosphate analysis [17] after lipid digestion [19].

Fluorescence Measurement of Membrane Fusion

Fusion of pyrPC-labeled influenza virosomes with ghosts or of pyrene-labeled SFV with extruded liposomes was investigated as follows: ghosts and virosomes, or liposomes and SFV, were added to a quartz microcuvette containing HBS, to a final volume of 665 μL. After a 2-min incubation at neutral pH, the medium was acidified by the addition of 35 μL of a solution of 0.10 M acetic acid and 0.10 M 2-[*N*-morpholino]ethanesulfonic acid. By using aliquots of this solution, which were pretitrated with NaOH or HCl, the solutions in the cuvette became adjusted to final pH values between pH 4.5 and pH 6.0. The final concentrations were 60 μM ghosts and 2.5 μM virosomes, or 200 μM liposomes and 0.50 μM SFV (all based on phospholipid contents). Polyanionic proteins were admixed with the ghosts and virosomes, or with the liposomes and SFV, prior to the 2-min incubation at neutral pH and the subsequent acidification.

The experiments were carried out in an Aminco-Bowman Series 2 spectrometer (SLM-Aminco, Urbana, IL, USA), equipped with a cuvette holder that was thermostated at 37°C. The time course of pyrene excimer fluorescence was measured, with continuous stirring, at excitation and emission wavelengths of 345 nm (bandpass 1 nm) and 490 nm (bandpass 16 nm), respectively, in the presence of a 475 nm cutoff filter in the emission beam. Background fluorescence was assessed at infinite dilution of the probe, which was obtained by adding 35 μL of 0.20 M $C_{12}E_8$. The changes in fluorescence were converted to extents of fusion (f) by calculating $f = 100 \times (E_0 - E)/(E_0 - E_\infty)$, where E represents the excimer fluorescence intensity at 490 nm, and E_0 and E_∞ represent, respectively, the intensities at 490 nm at time zero and after the addition of $C_{12}E_8$, both corrected for dilution effects.

Binding of Virosomes to Ghosts

Binding of virosomes to ghosts was measured by mixing ghosts and virosomes in the absence or presence of modified proteins in 700 μL HBS at 37°C. The proteins were present at a concentration of 250 μg/mL in the presence of 60 μM ghosts and 2.5 μM virosomes. Aliquots were taken to assess the total amount of virosomes (by measuring pyrene monomer fluorescence). After a 2-min incubation at neutral pH, which allowed for binding to take place, the suspension was centrifuged at 2,800 × g for 1 min at room temperature. Levels of pyrene monomer fluorescence were assessed in the supernatants and resuspended pellets, and expressed relative to the total amount of fluorescence initially measured. Pyrene monomer fluorescence was measured at infinite dilution of the pyrPC by adding $C_{12}E_8$ to a final concentration of 10 mM. Excitation of the pyrene fluorophore was performed at 345 nm (bandpass 1 nm) and the monomer fluorescence was recorded at an emission wavelength of 378 nm (bandpass 4 nm).

RESULTS

Effect of Charge-Modified Proteins on HA-Mediated Membrane Fusion of pyrPC-Labeled Virosomes With Erythrocyte Membranes

Influenza virus membrane fusion was studied in a model system involving the use of pyrPC-labeled virosomes and erythrocyte ghosts as fusion targets. Erythrocyte ghosts contain sialylated proteins and lipids, which serve as receptors for HA. Thus, virosomes were allowed to bind to the ghosts during an incubation at neutral pH, and fusion was subsequently induced by acidification of the medium. Upon fu-

sion, the virosomal pyrPC mixed with the target membrane lipids, which resulted in probe dilution and a concomitant decrease in pyrene excimer fluorescence. The rate-limiting step in this process was the fusion step itself (and not the subsequent lipid mixing) [28], and upon fusion the excimer fluorescence of the particular virosome involved disappeared completely. The relative decreases in fluorescence were converted to fusion curves, as described in Materials and Methods, which thus continuously reflect the fraction of virosomes fused. A typical fusion curve is shown in Fig. 1 (curve a).

The effect of a series of (aconitylated or succinylated) proteins on HA-mediated membrane fusion was investigated by mixing the proteins with ghosts, prior to the addition of the virosomes. Fusion was measured as outlined above by acidification after a 2-min incubation period. Figure 1 shows fusion of virosomes with erythrocyte ghosts in the absence of any protein (curve a), and in the presence of 250 μg/mL of unmodified HSA (curve b), Suc_{60}-HSA (curve c), Aco_{51}-HSA (curve d), Aco-β-lactoglobulin A (curve e), and Aco_{60}-HSA (curve f). The data show that HSA by itself had no effect on HA-mediated membrane fusion, and that these polyanionic proteins were able to inhibit the rates and extents of HA-mediated membrane fusion. In addition, the inhibitory effects were not accompanied by the induction of lag-times of fusion. Notably, significant lag-times are routinely observed at suboptimal

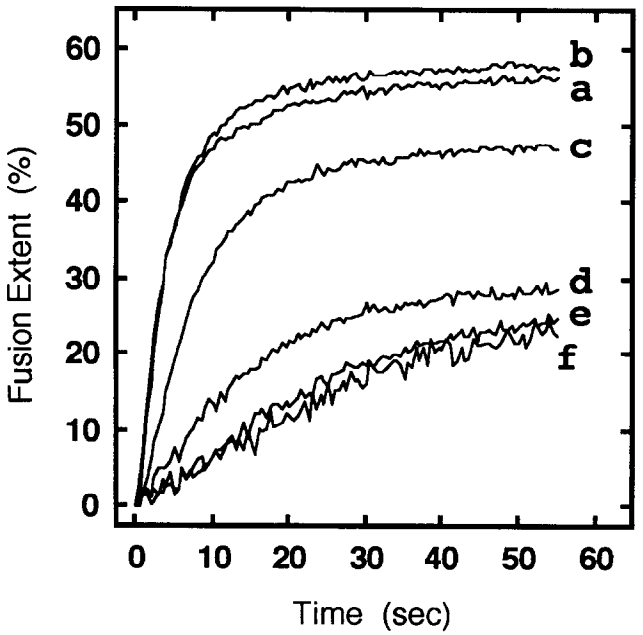

FIG. 1. Fusion of virosomes with erythrocyte ghosts. PyrPC-labeled virosomes (2.5 μM phospholipid) were incubated with ghosts (60 μM phospholipid) either in the absence of any exogenous protein (a), or in the presence of 250 μg/mL HSA (b), Suc_{60}-HSA (c), Aco_{51}-HSA (d), Aco-β-lactoglobulin A (e) or Aco_{60}-HSA (f). After a 2-min incubation at neutral pH, the medium was acidified to pH 5.0, and fusion was continuously monitored at 37°C by the decrease in pyrene excimer fluorescence.

pH values and/or lower reaction temperatures, conditions where the rates of fusion are comparable to those observed here in the presence of the polyanionic proteins. Table 1 summarizes the effects of all of the (modified) proteins on the rates and extents of fusion. Succinylated lactoferrin, lysozyme, and transferrin were not able to significantly inhibit HA-mediated membrane fusion, although the extents of modification of lactoferrin and transferrin were high (Δcharge > 100). Succinylated lactalbumin, at a charge density slightly above that of Suc_{60}-HSA, was less effective in inhibiting influenza virus fusion. Together, these data suggest that negative charge is not the only factor important for influenza virus fusion–inhibitory activity. This notion is further supported by the observation that Suc_{60}-BSA was without effect, whereas Suc_{60}-HSA was a potent inhibitor.

The data in Table 1 further show that for modified HSA the anionic-charge density plays an important role in the relative fusion–inhibitory activity. HSA, in which the (positive) charges of 18 of the ε-lysine residues (Aco_{18}-HSA) had been changed into 36 negative charges, had no significant effect on either the rate or extent of HA-mediated membrane fusion. With increasing negative charge, the antifusion activity increased. Furthermore, derivatization of 51 or 60 residues with *cis*-aconitic anhydride (which replaced the positive charge of each modified residue with two negative charges) resulted in a higher antifusion activity than derivatization of 60 residues with succinic anhydride (which replaced the positive charge of each modified residue with one negative charge), suggesting that the number of introduced negative charges played a more important role than the number of positively charged residues that had been modified. Moreover, Aco-β-lactoglobulin A, which had a small charge shift (48), but a relatively high shift in charge density, due to its small molecular weight, was as active as Aco_{60}-HSA, which had almost the same shift in charge density. In conclusion, the influenza virus fusion–inhibitory activity of aconitylated or succinylated proteins depended on the protein used, in combination with the introduced anionic-charge density.

Virosome Binding to Ghosts in the Presence of Charge-Modified Proteins

The first step in the investigation of the fusion–inhibitory activity involved the mixing of the (modified) proteins with erythrocyte ghosts and virosomes. After this, fusion was induced by lowering of the pH. If binding of virosomes (to erythrocyte ghosts) is inhibited before fusion is induced, the rates and extents of fusion are substantially lower (see [29]). Therefore, we assessed whether the influenza virus fusion–inhibitory activity of Aco_{60}-HSA, Aco_{51}-HSA, Suc_{60}-HSA, and Aco-β-lactoglobulin A might be related to an effect on the virosome–ghost binding interaction at neutral pH.

PyrPC-labeled virosomes and ghosts were mixed in the

TABLE 1. Effect of polyanionic proteins on HA-mediated membrane fusion

Protein*	ΔCharge†	ΔCharge/AA‡	Fusion rate§ (%/sec)	Fusion rate§ (% Inhibition)¶	Fusion extent§ (%)
–			9.2		59
HSA	0	0	9.5	0	59
Aco_{60}-HSA	180	0.308	0.70	92	24
Aco_{51}-HSA	153	0.262	1.5	84	30
Aco_{28}-HSA	84	0.144	7.2	22	54
Aco_{18}-HSA	54	0.092	8.0	n.s.	60
Aco-β-lactoglobulin A	48	0.296	0.85	91	29
Suc_{60}-HSA	120	0.205	4.5	51	48
Suc_{60}-BSA	120	0.206	9.2	0	56
Suc-lactalbumin	28	0.228	6.5	29	54
Suc-lactoferrin	110	0.160	8.0	n.s.	56
Suc-lysozyme	14	0.109	8.0	n.s.	60
Suc-transferrin	116	0.171	8.0	n.s.	57

Fusion of virosomes with erythrocyte ghosts was studied as in Fig. 1, either in the absence or presence of 250 μg/mL of the modified proteins. Rates of fusion were obtained from the fusion curves by determining the slope of the tangent to the initial parts of the curves. The extents of fusion were read from the fusion curves after fusion had proceeded for 5 min.

* Except for HSA and lysozyme, which was from chicken egg, all proteins were of bovine origin. The digits in subscript indicate the number of positively charged residues that were modified.

† The data represent the number of positively charged residues that were modified, to which the number of negative charges that were introduced is added. Succinylation introduces 1, and aconitylation introduces 2 net negative charges.

‡ The anionic charge density is the ratio of the charge shift introduced (previous column) and the number of amino acids present in the protein primary structure.

§ Listed data are representative values from typical experiments. The rates of fusion vary between ±1/10th of the indicated values. The variations between the extents of fusion is 2–3%.

¶ Decrease in the rate of fusion, relative to the rate of fusion in the absence of any protein (n.s., nonsignificant inhibition of the rate of fusion).

absence or presence of modified proteins, at the same concentrations as used in Table 1. The ghosts were pelleted by a brief centrifugation, and the relative association of the virosomes to the ghosts was determined by measuring total pyrene fluorescence in the supernatants and in the resuspended pellets (Table 2). In the absence of ghosts, 90% of the fluorescence was recovered in the supernatant. After an incubation with ghosts and subsequent centrifugation, 65% of the fluorescence became associated with the ghosts in the pellets. Aco_{60}-HSA, Aco_{51}-HSA, Suc_{60}-HSA, or Aco-β-lactoglobulin A had no significant effect on the amounts of fluorescence associated with the ghosts in the pellets, nor on the amounts of fluorescence remaining in the supernatants. Thus, the influenza virus fusion–inhibitory activity of these preparations could not be accounted for by an effect on HA-mediated virosome-ghost interaction at neutral pH.

TABLE 2. Binding of virosomes to erythrocyte ghosts in the presence of proteins with influenza virus fusion–inhibitory activity

Protein	Fusion rate (%/sec)	Fusion rate (% Inhibition*)	Fusion extent (%)	Binding† S(%)	Binding† P(%)
–	11.5		82	21	65
Aco_{60}-HSA	0.70	94	24	23	62
Aco_{51}-HSA	1.2	90	27	25	61
Suc_{60}-HSA	5.6	51	62	22	68
Aco-β-lactoglobulin A	0.70	94	21	22	60

Fusion of virosomes with erythrocyte ghosts was studied as in Fig. 1, either in the absence or presence of 250 μg/mL of the modified proteins. The rates and extents of fusion were obtained as in Table 1. Binding measurements were performed by incubating virosomes, ghosts, and modified proteins under the same conditions. Aliquots were taken to assess the total amount of virosomes (by measuring pyrene monomer fluorescence; see Materials and Methods for further details). After the 2-min binding period at neutral pH, ghosts were pelleted by centrifugation. Levels of pyrene monomer fluorescence were assessed in both the supernatant and in the resuspended pellets and expressed relatively to the total amount of fluorescence initially determined. Measurements were performed in triplicate and averaged.

* Decrease in the rate of fusion, relative to the rate of fusion in the absence of any protein.

† The relative recoveries of virosomes in the supernatant (S) and pellet (P) fractions are indicated.

Effect of the Concentration of Influenza Virus Fusion-Inhibiting Protein on the Inhibition of Membrane Fusion

The specific fusion-inhibitory activities of Aco_{60}-HSA and Aco-β-lactoglobulin A were further characterized by investigating dose–effect relationships (Fig. 2). Membrane fusion was measured as outlined above, in the presence of Aco_{60}-HSA or Aco-β-lactoglobulin A up to concentrations of 0.50 mg/mL. The inhibitory effects were concentration dependent. The rates of fusion were very sensitive to the presence of Aco_{60}-HSA (Fig. 2A) and Aco-β-lactoglobulin A (Fig. 2C). Their IC_{50} values were about 4 and 10 μg/mL, respectively. The extents of fusion were also sensitive to low amounts of Aco_{60}-HSA (Fig. 2B) and Aco-β-lactoglobulin A (Fig. 2D). However, the data do suggest that a complete block of HA-mediated membrane fusion was difficult to achieve.

Effect of Charge-Modified Proteins on the Fusion of Other Viruses

Aco_{60}-HSA, Aco_{51}-HSA, Suc_{60}-HSA, and Aco-β-lactoglobulin A appeared to inhibit low pH-dependent HA-mediated membrane fusion in a specific manner. This was further documented by investigating fusion of virosomes prepared from another strain of influenza virus, A/Shangdong/9/93. The HA from this virus has a higher optimum pH for fusion than the HA from the X47 strain of the virus (pH 5.5 vs. pH 5.0). Aco_{60}-HSA, Aco_{51}-HSA, Suc_{60}-HSA, and Aco-β-lactoglobulin clearly inhibited membrane fusion at pH 5.5, from the A/Shangdong strain, although the inhibitory effects were less pronounced than those obtained at pH 5.0 (with the X47 strain). The polyanionic proteins had the same order of activity noted above: Aco_{60}-HSA was the most potent, followed by Aco-β-lactoglobulin, Aco_{51}-HSA, and Suc_{60}-HSA. As the inhibition of membrane fusion was smaller at pH 5.5 than at pH 5.0, the question that subsequently arose was whether these proteins inhibited HA-mediated membrane fusion over the whole specific pH range of a certain strain of virus.

To this end, the effects of the protein with the most potent fusion–inhibitory activity (Aco_{60}-HSA) on the pH dependence of membrane fusion mediated by HA from the influenza strains X47 (Fig. 3) or A/Shangdong (Fig. 4) were studied. Aco_{60}-HSA inhibited the rate of membrane fusion (Figs. 3A and 4A) at each specific pH value, for both strains of virus. The effects on the extents of fusion (Figs. 3B and 4B) were smaller, especially for the A/Shangdong strain of virus at pH values above pH 5.2. Under these conditions, fusion mediated by A/Shangdong HA is very rapid and highly efficient, so that the activity of Aco_{60}-HSA is probably not high enough to efficiently interfere with the fusion reaction. At pH values above pH 5.2, fusion mediated by X47 HA is slow, and then inhibition of fusion by Aco_{60}-HSA is more readily seen. At any rate, the data supported the conclusion that these polyanionic proteins did not merely induce a shift in the pH dependence, but inhibited influenza virus membrane fusion in a specific manner.

This notion was further supported by investigating fusion of SFV, another virus that infects cells in a low pH-dependent manner. Fusion of pyrene-labeled SFV with lipid vesicles has been well characterized (see [30]). Here, we investigated the effects of the polyanionic proteins on the fusion of pyrene-labeled SFV with extruded liposomes, composed of phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, and cholesterol at pH 5.5. Experi-

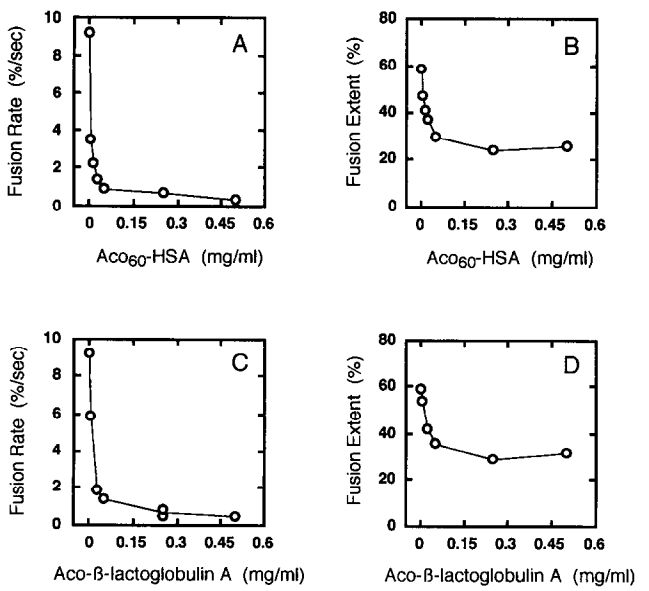

FIG. 2. Inhibition of the rates and extents of HA-mediated fusion by Aco_{60}-HSA and Aco-β-lactoglobulin. Fusion of virosomes with erythrocyte ghosts was studied as in Fig. 1, in the presence of increasing concentrations of Aco_{60}-HSA (A and B) and Aco-β-lactoglobulin (C and D). Rates of fusion (A and C) were obtained from the fusion curves by determining the slope of the tangent to the initial parts of the curves. The extents of fusion (B and D) were read from the fusion curves after fusion had proceeded for 5 min.

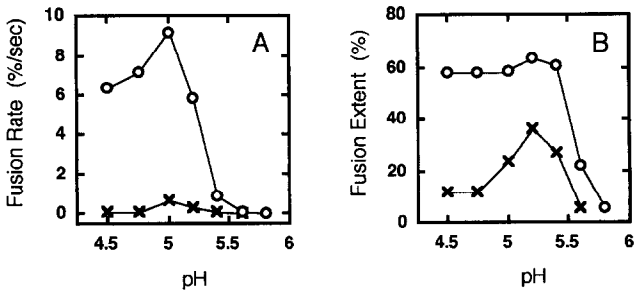

FIG. 3. Fusion of virosomes, carrying the HA from A/Victoria/3/75 (X47), with erythrocyte ghosts, as function of pH. Virosomes were induced to fuse with ghosts as in Fig. 1, employing the addition of aliquots of different solutions of pretitrated 0.10 M acetic acid and 0.10 M MES, to obtain the indicated pH values. Fusion was measured either in the absence of exogenous protein (○), or in the presence of 250 μg/mL of Aco_{60}-HSA (✖). The rates (A) and extents of fusion (B) were obtained as in Fig. 2.

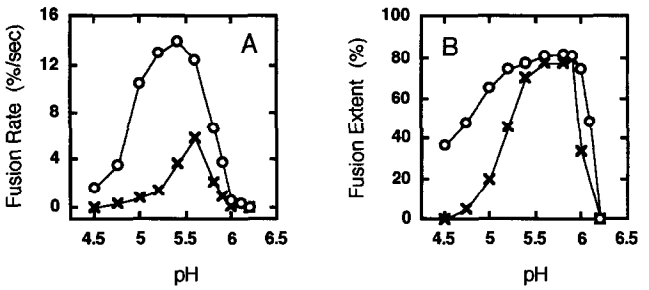

FIG. 4. Fusion of virosomes, carrying the HA from A/Shangdong, with erythrocyte ghosts, as function of pH. Virosomes were induced to fuse with ghosts as in Fig. 3, either in the absence of exogenous protein (○), or in the presence of 250 μg/mL of Aco_{60}-HSA (✱). The rates (A) and extents of fusion (B) were obtained as in Fig. 2.

ments were performed and analysed as described in Materials and Methods. Figure 5 shows the time course of fusion in the presence of 1.0 mg/mL HSA (curve a), which had no influenza virus fusion–inhibitory activity, and in the presence of 1.0 mg/mL Aco_{60}-HSA (curve b), the most potent compound. Aco_{60}-HSA had no specific inhibitory effect on SFV fusion. Similar results were obtained with the other polyanionic proteins with influenza virus fusion–inhibitory activity (results not shown). When the protein concentrations were increased to 5.0 mg/mL, limited a-specific effects on the rates of fusion were noted, which were related to effects on the bulk pH. Thus, the influenza virus and HIV-1 membrane fusion–inhibitory activities of polyanionic proteins were not paralleled by inhibition of SFV membrane fusion.

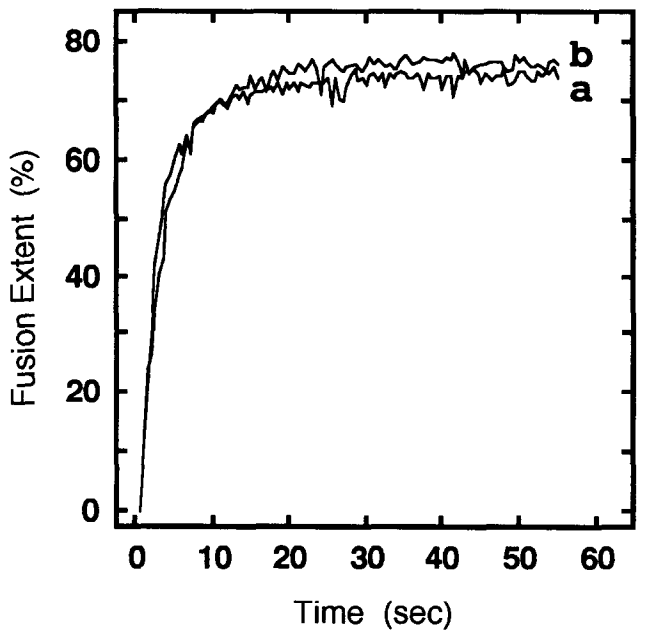

FIG. 5. Lack of effect of Aco_{60}-HSA on SFV fusion with liposomes. Fusion of pyrene-labeled SFV with liposomes was studied in the presence of 1 mg/mL of unmodified HSA (a) or Aco_{60}-HSA (b) as outlined in the text.

DISCUSSION

In this article, we describe that polyanionic HSA and β-lactoglobulin A strongly inhibit influenza HA-mediated membrane fusion. We used a well-defined model system based on reconstituted viral envelopes (virosomes) fusing with erythrocyte ghosts. These ghosts contain sialic acid-bearing receptors for HA. The virosome-ghost system allows a detailed investigation of the effects of drugs on the membrane fusion characteristics of influenza virus. Such systems are not yet available for HIV-1 membrane fusion.

Suc-HSA, Aco-HSA, and Aco-β-lactoglobulin A inhibited the rate and extent of HA-mediated membrane fusion in a concentration-dependent fashion. The relative fusion–inhibitory activity of derivatized HSA depended on the overall negative-charge density. However, this is not the only important factor for the fusion–inhibitory activity of polyanionic proteins, because there was a certain degree of specificity with respect to the protein: derivatized HSA and β-lactoglobulin A inhibited HA-mediated fusion, whereas other derivatized plasma (i.e. BSA, lysozyme, and transferrin) or milk proteins (i.e. lactalbumin and lactoferrin) were inactive (Table 1). Thus, a purely electrostatic effect of the derivatized proteins on influenza virus membrane fusion does not suffice for fusion inhibition; apparently a more specific interaction must also be involved.

Rapid and extensive fusion of virosomes with ghosts depends on their binding interaction at neutral pH [29]. However, the fusion–inhibitory activity reported here could not be accounted for by an effect on the initial binding of virosomes to ghosts (Table 2). Thus, the inhibition of fusion occurs at some step after binding and acidification. An alternative explanation for the observed inhibition of fusion might have been a trivial effect on the pH dependence of membrane fusion. This, however, was not the case either (Figs. 3 and 4). The pH thresholds and optima of fusion of influenza A/Victoria/3/75 (X47) and A/Shangdong/9/93 were unaffected. Therefore, we conclude that the active proteins affect the fusion step itself.

During HA-mediated membrane fusion, the HA2 N-terminal fusion peptides rapidly insert into the target membrane [31, 32]. Before both bilayers actually merge, rearrangements at the site of contact of both membranes take place. The time required for these changes to occur (lag time) depends on the reaction conditions, the pH, and the temperature in particular. Our current results suggest that negative charge-modified proteins do not inhibit the low pH-dependent conformational change in HA itself. Furthermore, the proteins do not appear to inhibit the rearrangements at the contact site, because they did not induce lag times of fusion (Fig. 1).

Another highly negatively charged agent, dextran sulfate, besides having anti-HIV-1 activity, also inhibits influenza virus membrane fusion in both erythrocyte and liposome model systems [33–36]. Interestingly, the fusion–inhibitory activities of dextran sulfate show similarities with the activities of the proteins reported here. Like Aco-

HSA, Suc-HSA and Aco-β-lactoglobulin A, dextran sulfate has no effect on HA-mediated binding at neutral pH [34] and does not affect the low pH-induced conformational change of HA [34, 35]. On the other hand, attachment of virus particles to erythrocyte membranes at low pH, which not only involves HA-mediated receptor binding, but also hydrophobic attachment mediated by the exposed fusion peptides, is inhibited by dextran sulfate [35]. Moreover, dextran sulfate binds with high affinity to the virus [35], but not to the erythrocytes. Therefore, it was suggested that dextran sulfate interacts with the fusion peptide of the viral envelope glycoprotein [36]. Although in the present study, the membrane fusion intermediate sensitive to the polyanionic proteins has not been identified, our results are compatible with an interaction of the fusion peptides of HA2 with these compounds.

It is interesting to note that both dextran sulfate and polyanionic HSA inhibit influenza virus membrane fusion and HIV-1 cell entry. Entry of HIV-1 into cells is mediated by the viral gp120/gp41 complex (see [37]). After gp120-mediated attachment to cell surface CD4, conformational changes in the transmembrane gp41 subunit occur, which result in gp41-mediated merging of the viral envelope with the cell plasma membrane. There are several similarities between influenza HA and HIV-1 gp120/gp41 [8, 37–41]. Both proteins are composed of two subunits, HA1 and HA2 in influenza virus, and gp120 and gp41 in HIV-1, which originate from an uncleaved precursor. The surface subunits (HA1 and gp120) bind to the host–cell receptor molecules. The transmembrane subunits (HA2 and gp41) carry the N-terminal fusion peptides, which are generated through cleavage of the precursor molecules. For fusion to occur the transmembrane subunits have to undergo a conformational change, and the fusion peptides have to insert into the target membrane. Finally, it seems likely that in this conformation an oligomeric state has to be formed [41, 42].

In fact, there appear to be more similarities between HIV and influenza virus, on the one hand, than between SFV and influenza virus on the other. Although membrane fusion of both SFV and influenza is low pH dependent, key differences between their membrane fusion reactions can be discerned. Prominent distinctions include a specific lipid requirement for SFV membrane fusion and the absence of a well-recognizable fusion peptide in SFV. Thus, while influenza HA2 and HIV gp41 have N-terminal fusion peptides, SFV E1 does not, although an internal E1 sequence has been suggested to function as a fusion sequence [43]. This has led to the conclusion that the membrane insertion and oligomerization of the fusion proteins of influenza and Semliki Forest virus are likely to be different [44]. Thus, it is possible that the lack of fusion inhibition by polyanionic proteins in the case of SFV (Fig. 5) is related to the absence of an N-terminal fusion peptide in the viral spike protein.

In conclusion, this work shows that highly negatively charged Aco-HSA, Suc-HSA, and Aco-β-lactoglobulin A inhibit influenza virus membrane fusion in a specific manner. Viral membrane fusion is a prerequisite for infection, and, indeed, the antifusion effects of dextran sulfate show a remarkable correlation with inhibition of influenza virus replication [33, 36]. This might point to a more general application of aconitylated and/or succinylated proteins than their potential use as anti-HIV-1 agents only. In this respect, it must be recognized that the biocompatibility of modified proteins might be larger than that of dextran sulfate, which is known for its anticoagulant properties and which also affects the functioning of macrophages [2, 45, 46].

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