JOURNAL OF BACTERIOLOGY, Nov., 1966
Vol. 92, No. 5

Characterization of a Low Molecular Weight Antigenic Protein from the Envelope of Influenza Virus

EDWARD A. ECKERT

Department of Epidemiology, School of Public Health, The University of Michigan, Ann Arbor, Michigan

Received for publication 27 July 1966

ABSTRACT

ECKERT, EDWARD A. (The University of Michigan, Ann Arbor). Characterization of a low molecular weight antigenic protein from the envelope of influenza virus. J. Bacteriol. **92**:1430–1434. 1966.—An antigenic protein from the lipid-extracted residue of influenza virus strain PR8 was solubilized with urea-dithiothreitol (DTT). The protein subunits had a sedimentation coefficient of 2*S* in urea-DTT and reassociated to a 4*S* state on dialysis. This form of the envelope protein did not agglutinate erythrocytes, but reacted with strain-specific antisera in the complement-fixation and blocking-antigen tests.

The general objectives of this series of studies are the isolation of the surface proteins of influenza virus and the elucidation of their role in the structure and antigenic character of the virus envelope.

Previously (4), it was demonstrated that removal of lipids from influenza virus strain PR8 by methanol-chloroform extraction gave a denatured protein residue which was essentially devoid of serological activity. Solubilization and disaggregation into protein subunits was accomplished with acetic acid, and, after removal of the acid, reassociation of subunits occurred spontaneously. A heterogeneous protein was recovered, which was capable of agglutinating erythrocytes, which induced antibodies reactive with the intact virus, and which reacted with antiserum prepared with whole virus.

In the present investigation, another protein-dissociating reagent, urea, in combination with a reducing agent, was employed to obtain active envelope protein preparations. The resulting product was found to differ in some of its biological and physical properties from the acetic acid-extracted protein.

MATERIALS AND METHODS

Virus. Influenza virus concentrates were prepared from infected embryonated egg fluids by adsorption-elution, first with chicken red blood cells and then with barium sulfate (3), followed by two cycles of low- and high-speed centrifugation (6,300 and 26,340 × *g*). The virus pellets were suspended in 0.15 M NaCl, and the resulting concentrates ranged from 48,000 to 120,000 hemagglutinating units per ml.

Virus antisera. Antisera were prepared in rabbits by use of virus concentrates with adjuvant (arlacel and mineral oil) as described in a previous report (4).

Solubilization of virus protein. Lipids were extracted from influenza virus strain PR 8 with methanol-chloroform by the procedure of Kates et al. (5). The precipitate from 4 ml of virus concentrate was suspended in 1 ml of freshly prepared 8 M urea, and organic solvents were removed by bubbling a stream of nitrogen through the suspension. The sample was diluted to 4 ml with 8 M urea, and 2 mg of the reducing agent dithiothreitol (DTT) was added (1). Stirring of the suspension in a sealed bottle with a nitrogen gas phase was continued overnight. A large sediment was removed by centrifugation at 12,800 × *g* for 15 min, and discarded. The supernatnat fluid was dialyzed against two changes of 2 liters of tris(hydroxymethyl)-aminomethane (Tris)-HCl buffer (0.02 M, *p*H 8). Preparations for the analytical ultracentrifuge were more concentrated, 4 ml of virus concentrate being used to prepare 1 ml of protein preparation.

Hemagglutination (HA), hemagglutination-inhibition (HI), and complement-fixation (CF) tests. These tests followed standard procedures as outlined in an earlier report (4).

Blocking-antigen (BA) test. Preliminary tests were performed to standardize the virus preparations at 4 HA units and the antisera at 4 HI units. To 0.2 ml each of successive twofold dilutions of the protein was added 0.2 ml of the antiserum containing 4 HI units. After 1 hr at room temperature, 4 HA units of virus in 0.2 ml was added to each reaction mixture. The virus was allowed to react with any unbound

antibodies for 1 hr, and then 0.2 ml of 1% chicken erythrocytes was added. Titers were read as the highest dilution of protein giving a positive HA reaction as determined by the pattern test.

Ultracentrifugation. Analyses were performed with a Spinco model E analytical ultracentrifuge using schlieren optics and a 12-mm cell. Sedimentation rates were determined at 50,740 rev/min at 20 C. Sedimentation coefficients ($S_{20,w}$) were corrected to standard conditions, assuming a partial specific volume of the solute of 0.74 ml/g.

Rate zonal centrifugation. Preformed 5 to 20% sucrose gradients were formed with 0.15 M NaCl buffered with 0.05 M Tris-HCl (*p*H 8.0) as solvent. A 0.5-ml sample was layered on the gradient, and the tubes were centrifuged in an SW 39 head at 35,000 rev/min for 20 hr. Ten 0.4-ml fractions were collected from a pin hole in the bottom of the tube. A sample containing 10 mg/ml of crystalline bovine serum albumin (BSA) was centrifuged in parallel with the envelope protein as a control for estimating sedimentation coefficients. Its distribution was measured by absorption at 280 mμ.

Spectrophotometry. The Spectronic 505 recording spectrophotometer (Bausch & Lomb) was employed to obtain ultraviolet (UV)-absorption spectra.

RESULTS

UV-absorption spectrum of solubilized protein. An influenza virus strain PR8 concentrate, after removal of lipids, was extracted with urea-DTT as described in Materials and Methods, and was clarified by centrifugation at 100,000 × *g* for 60 min. After passage through a membrane filter (pore size, 50 mμ; Millipore Filter Corp., Bedford, Mass.), the UV-absorption spectrum was measured. The extract gave a peak at 281 mμ and a minimum at 252 mμ (Fig. 1). The ratio of absorbancy at 260 mμ to that at 280 mμ was 0.66. The spectrum was essentially that expected of a solution of a protein.

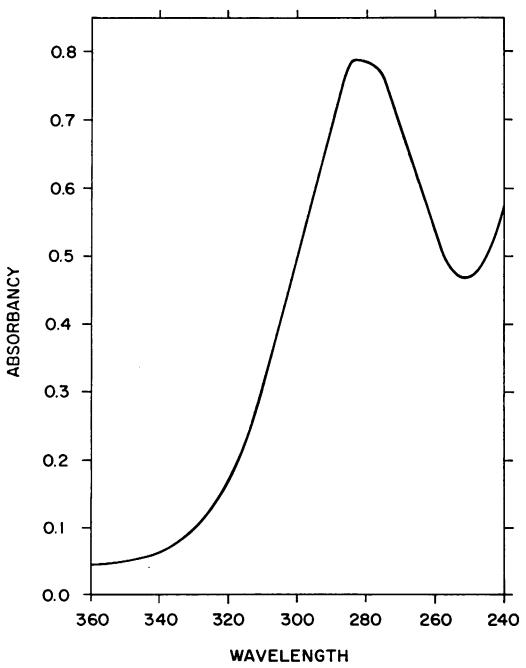

FIG. 1. *UV-absorption spectrum of the urea-DTT extract of envelope protein.*

Analytical ultracentrifugation. Analyses of the sedimentation patterns of the protein extract disclosed in all cases a single slow-moving peak. However, the sedimentation coefficients of this uniform material depended on the nature of the suspending medium, suggesting that the solvent determined the state of association of the protein. Centrifugation of the protein in 8 M urea-DTT (0.5 mg/ml) gave a corrected sedimentation coefficient, $S_{20,w}$, of 2.2*S*. After dialysis of the same sample against 0.15 M NaCl-0.05 M Tris-HCl (*p*H 8), the centrifugation was repeated, and again a single peak was observed, but the rate of sedimentation was more rapid, and the $S_{20,w}$ increased to 4.3*S*. The presence of only a single major component in each solvent system and the increase in sedimentation coefficient suggests that, on removal of the urea, a reassociation of subunits occurred. Although exact molecular weights cannot be calculated from the data, the order of increase in molecular size should be approximately threefold.

Tests for hemagglutinating and serological activities. A series of tests was performed with urea-DTT protein extracts after dialysis against Tris-HCl buffer. The protein was examined for HA activity with chicken, pigeon, and guinea pig erythrocytes at 4, 20, and 35 C. Although the original virus concentrate had an HA titer of 100,000 units per ml, the derived protein caused no detectable hemagglutination in any of the systems. In contrast to this loss of HA activity, there was complete retention of complement-fixing antigenicity when virus and derived protein were tested with rabbit antiserum to intact PR8 virus (Table 1).

To determine whether the serological reactivity of the protein extract resulted from identity or similarity to the HA antigen of the viral envelope, tests for blocking antigen were carried out. These

TABLE 1. *HA and CF titers of the urea-DTT extracted protein and the PR8 virus sample*

Test	Protein	PR8 virus concentrate
HA	<2	20,000
CF	360	360

would determine whether the protein, although it lacked the capacity to hemagglutinate, would still bind antibodies directed against the HA antigens of the intact virus particle. The protein extract, even after considerable dilution, was found to bind 4 units of HI antibody. Thus, in a series of 15 preparations, in which PR8 virus concentrates with titers of 25,600 to 102,400 HA units were used as starting materials, the BA titers of the derived protein ranged from 160 to 1,280.

The strain specificity of the reaction was demonstrated by comparing the reactivity of the PR8 protein when tested in the BA test with homologous antiserum and virus and in two similar test systems with FM1 and Japan/305 influenza virus strains and their respective antisera. Whereas this PR8 protein preparation had a BA titer of 256 in the homologous sytem, it did not interfere with the HI reactions of FM1 or Japan/305 antisera even at a 1:2 dilution of the protein.

The demonstration that the urea-DTT extracted envelope protein would bind HI antibodies raised a question as to whether the lipid-extracted denatured protein residue was also reactive. Previously (4), it had been shown that only trace HA and CF activities could be detected. Now, when the BA test was used, preparations of denatured viral protein were never found to react beyond a 1:2 dilution. The results of the CF, HA, and BA tests all were in accord with the concept that the denatured viral protein was essentially in an inactive form and that activities were regained after protein dissociation and reassociation of the subunits.

Adsorption of envelope protein to erythrocytes. One possible explanation of the inability of the envelope protein preparations to agglutinate erythrocytes is the failure of the protein to bind to the cells. In this experiment, adsorption-elution of the envelope protein to red cells was attempted. A mixture of 0.6 ml of the protein with 0.2 ml of packed chicken red blood cells was held in an ice bath for 1 hr, after which the cells were removed by centrifugation and the supernatant fluid was tested for BA titer. The cells were washed once with iced BS; then 0.6 ml of BS was added and the mixture was held at 37 C for 1 hr. The eluate fluid was collected, and the cells were treated with an additional 0.6 ml of BS, this time containing receptor destroying enzyme (RDE). In parallel, a similar adsorption-elution procedure was performed with the original virus concentrate, concentrations being measured by the HA test. The results (Table 2) demonstrated that, although the centration of red cells was adequate for adsorption of virus which was subsequently eluted, the urea-DTT extracted envelope protein did not adsorb to chicken erythrocytes, nor was elution demonstrated.

TABLE 2. *Test of adsorption-elution of an envelope protein preparation, compared with a parallel test of PR8 virus concentrate*

Sample	Protein (BA titer)	Virus concentrate (HA titer)
Original	320	10,240
Adsorbed	320	<20
Eluate-BS	<10	5,120
Eluate-BS-RDE	<10	ND[a]

[a] Not determined.

Antibody induction. The ability of an envelope protein preparation to evoke virus-specific antibodies was tested in rabbits. Two rabbits were each inoculated with 0.5 ml of a preparation with a BA titer of 360 in an adjuvant emulsion and 6 weeks later with similar protein intravenously. After 1 week, the rabbits were bled and the trypsin-periodate treated sera were tested. Both had HI titers with PR8 virus of 512, whereas the preimmunization sera titered 8.

Infectivity. Volumes of 0.1 ml of dilutions of protein preparations (10^{-1} to 10^{-3}) were inoculated into groups of four fertile eggs. No hemagglutinin was detected in any of the egg fluids. Two successive blind passages with the egg fluids were also negative.

Rate zonal centrifugation. Although analytical ultracentrifugation of envelope protein in Tris-HCl buffered saline gave a single component with an $S_{20,w}$ of about 4*S*, it remained to be demonstrated whether serological reactivity was associated with this component. For this purpose, the envelope protein extract was centrifuged in a sucrose gradient. The sedimentation profile was determined with the use of CF and BA tests to measure titers; a single, slow-moving peak of activity was found (Fig. 2). The data disclosed no essential difference in sedimentation rate or shape of the profile when measured by either the CF or the BA test. A parallel run of BSA permitted calculation of a sedimentation coefficient, a value of about 4*S* being determined. Comparison of the shape of the peak of antigenic reactivity with that of BSA also discloses a high degree of homogeneity of the antigen, an observation confirming that found with the analytical ultracentrifuge.

DISCUSSION

The term envelope proteins refers to those proteins associated with the lipid envelope of influenza virus, active in viral hemagglutination, and contributing a major share of the im-

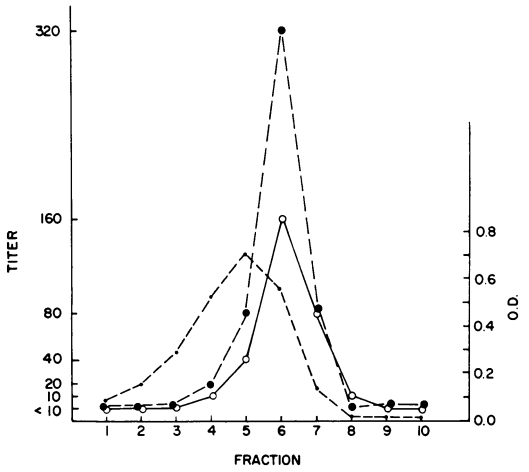

FIG. 2. *Rate zonal centrifugation of urea-DTT extracted envelope protein through a sucrose gradient. Activities of the protein were measured by the BA (●) and CF (○) tests. A control run of BSA (•) was included for comparison. Both titers and the UV absorption of BSA were plotted on a linear scale. Fraction 1 refers to the lowest part of the tube.*

munological attributes of the virus surface. It is not clear whether these antigens are limited to projections from the exposed surface or, in addition, form a matrix with viral lipids and are responsible for the structure of the envelope.

The disruption of the viral envelope with methanol-chloroform and subsequent isolation of protein by the urea-DTT procedure led to a series of changes of state which is informative with regard to the structure and properties of the viral envelope protein. Extraction of virus concentrates with the organic solvent mixture resulted in the removal of viral lipids and, at the same time, denaturation of viral protein. The resultant disorientation of the protein structure eliminated all serological attributes except for trace activities. Solubilization of the protein in urea with a reducing agent led to the formation of protein subunits, with a sedimentation coefficient of 2*S*, suggesting a unit consisting of, at most, one or two polypeptides. The serological activity of the protein in this state could not be measured because of the presence of urea; removal of the urea led to spontaneous association, resulting in a stable 4*S* subunit. Serological tests disclosed that in this state, where a molecular weight of the order of magnitude of 50,000 would be expected, the essential strain-specific antigenic character of the envelope protein was already determined, i.e., the 4*S* component reacted specifically in the blocking antigen test with antibody to the homologous strain of influenza virus, and also induced in rabbits antibodies which would block the hemagglutinating activity of intact virus. In an earlier study, Tyrrell and Horsfall (6) described an influenza virus derivative with similar biological properties, prepared by repeated freezing and thawing.

It is of interest to compare the derivatives obtained by solubilization of the denatured protein with the two dissociating reagents: acetic acid (4) and urea-DTT. Concentrated acetic acid extraction (Federation Proc. **25**:250, 1966) produced a 2*S* subunit, as did urea-DTT. In like fashion, dialysis of the acetic acid extract against acid buffer gave a component with a sedimentation coefficient of 4*S*, comparable to the urea-DTT protein in neutral buffered saline. However, the 4*S* components produced by the two procedures were not identical in their overall properties; the acetic acid derivative underwent further spontaneous association to form a heterogeneous, hemagglutinating protein as the *p*H of the solvent approached neutrality. In contrast, the protein extracted with urea-DTT differed in its chemical nature, in that association beyond the 4*S* state did not occur in neutral saline, and, functionally, in that it neither agglutinated nor was bound to erythrocytes.

Two explanations appear possible for the failure of the urea-DTT derived 4*S* component to attach to the red cell surface. Either some groups essential for attachment are blocked or lacking, or the binding energy between cell surface and antigen is very low, and only a larger associated antigen, such as the acetic acid-derived protein, has a sufficient number of sites to overcome the disruptive kinetic forces. The latter explanation would be in agreement with the proposal of Choppin and Stockenius (2), that the hemagglutinating potential of ether-split virus fragments decreases as the size of the units decreases. On the other hand, the inability of urea-DTT subunits to associate beyond the 4*S* stage suggests a basic modification of its structure, so that these subunits will combine neither with red cells nor with one another. It is noteworthy that, despite these differences, a common strain-specific antigenicity is shared by the low molecular weight urea-DTT derived blocking antigen, the heterogeneous hemagglutinating protein produced by the acetic acid extraction procedure, and the intact PR8 influenza particle.

The characterization of the envelope protein derived from influenza virus by both the acetic acid and the urea-DTT procedures provides a framework for a possible model of a self-assembly process for viral envelope antigen in the infected cell. The results suggest that structural associations arise by combinations of individual poly-

peptides or dimers, first to form a serologically specific low molecular weight intermediate, and then finally a larger hemagglutinating protein. In vitro, such associations occur as spontaneous reactions, determined by the chemical character of the components, and there appears to be no requirement for a directing agent or enzyme.

ACKNOWLEDGMENTS

This investigation was conducted under the auspices of the Commission on Influenza, Armed Forces Epidemiological Board, and was supported by the Office of the Surgeon General, U.S. Army, Washington, D.C.

LITERATURE CITED

1. CLELAND, W. W. 1964. Dithiothreitol, a new protective reagent for SH groups. Biochemistry **3**: 480–482.
2. CHOPPIN, P. W., AND W. STOCKENIUS. 1964. Interactions of ether-disrupted influenza A_2 virus with erythrocytes, inhibitors and antibodies. Virology **22**:482–492.
3. DRESHER, J., A. V. HENNESSY, AND F. W. DAVENPORT. 1962. Photometric methods for the measurement of hemagglutinating virus and antibody. I. Further experiences with a novel photometric method for measuring hemagglutinins. J. Immunol. **89**:794–804.
4. ECKERT, E. A. 1966. Envelope protein(s) derived from influenza virus. J. Bacteriol. **91**:1907–1910.
5. KATES, M., A. C. ALLISON, D. A. J. TYRRELL, AND A. T. JAMES. 1961. Lipids of influenza virus and their relation to those of the host cell. Biochim. Biophys. Acta **52**:455–466.
6. TYRRELL, D. A. J., AND F. L. HORSFALL, JR. 1954. Disruption of influenza virus. Properties of degradation products of the virus particle. J. Exptl. Med. **99**:321–342.