Acta virol. **35** : 135 - 143, 1991

AGGREGATION OF INFLUENZA VIRUS HAEMAGGLUTININ IN THE PRESENCE OF DETERGENTS

R. STAHN[1], H. SCHÄFER[1], J. MALUR[2], A. LADHOFF[3]

[1]Central Institute of Isotope and Radiation Research, Robert-Rössle-Str. 10, 1115 Berlin; [2]Central Institute of Hygiene, Microbiology, and Epidemiology, Berlin; and [3]Humboldt University Berlin, Department of Medicine, Charité, Berlin, Germany

Received March 15, 1990

Summary. - In the haemagglutinin (HA) detergent mixtures coexist various protein complexes. Using ^{125}I-HA tracer, gel chromatography, and centrifugation techniques we found protein aggregates comprising up to eight HA trimers. Formation of these structures seemed to be a function of the protein storage medium only. By contrast, special properties of the detergent as well as physicochemical conditions during the protein/detergent interaction had nearly no influence.

Key words: aggregation; detergents; haemagglutinin; influenza virus

Introduction

Detergents are frequently used for isolation, purification, characterization, and reconstitution of membrane proteins. The exact way of interaction between detergents and proteins is unknown. It is often assumed that the protein molecule gets inserted into a detergent micelle or, alternatively, that detergent molecules form a monolayer around the hydrophobic domains of protein molecule (Moeller *et al.*, 1986). But it is also possible that protein/detergent complexes with several protein molecules may be formed. Structures like these were extensively studied by Simons and coworkers using the spike proteins of the Semliki Forest virus (Simons *et al.*, 1973). The degree of protein aggregation and the nature of protein/detergent interaction may seriously affect the functional properties of the protein or its reactivity in the protein/lipid reconstitution process.

We were concerned with these problems when investigating the incorporation of functionally intact influenza virus HA into liposomes by removal of the detergent from the protein/lipid/detergent mixture. The HA exhibits a couple of functions: it bears antigenic properties, mediates the attachment of the virus to the cell surface and induces membrane fusion. Literature data as well as results of our own showed that the reproducibility of HA incorporation into

liposomes was rather poor with respect to the quantity and quality of protein binding (Huang *et al.*, 1979; Trudel *et al.*, 1981; Hosaka *et al.*, 1983; Kawasaki *et al.*, 1983; Nussbaum *et al.*, 1987; Stegmann *et al.*, 1987; Stahn *et al.*, unpublished). The reason for these observations may be due to differences in the protein association within the starting mixture (Helenius *et al.*, 1981; Mimms *et al.*, 1981). Therefore, we decided to analyse various HA/detergent mixtures for different HA species by gel filtration and centrifugation. A few data refer to a trimeric HA structure occurring in detergent solutions at neutral pH, e. g. in Triton X 100 (Doms *et al.*, 1986), in Lubrol (Ruigrok *et al.*, 1986) or in Lubrol PX and Brij-36 (Flanagan *et al.*, 1977). We investigated the aggregation state of HA in detergents of different chemical structure. The results revealed the coexistence of several HA aggregates differing in the number of HA trimers. The number of fractions, as well as their size, strongly depended on the storage medium of the protein. Even in solutions of the denaturing ionic detergent SDS different HA aggregates were present and only a partial dissociation of HA trimers could be observed. The antigenicity of HA in mixtures with some nondenaturing detergents, that were investigated with this respect, was not impaired.

Materials and Methods

Haemagglutinin (HA) and ^{125}I-haemagglutinin (^{125}I-HA). Influenza X73 (H3N2) viruses were grown in 11-day-old embryonated chicken eggs and purified by centrifugation at 102 000 x g in sucrose gradient (0 to 60 %). HA was split from the virions by continuous flow centrifugation of the virus suspension (100 mg/ml) in sucrose gradient 0 to 50 %, containing 1 to 1.5 % sodium deoxycholate (102 000 x g, for 4 hr). Sucrose and, if necessary, the detergent were removed by excessive dialysis. The HA was stored in phosphate buffered saline, pH 7.2 (PBS) at 4 ^{0}C in the presence or absence of the detergent.

Bromelain cleaved HA (B-HA) was prepared according to Brand and Skehel (1972). B-HA was stored in PBS at 4 ^{0}C. Radioiodination of HA and B-HA was carried out applying the chloramine T method, as described (Stahn *et al.*, 1987). The specific activities of the ^{125}-I-tracers ranged from 0.03 to 0.3 MBq/μg. In every single experiment, protein and the ^{125}I-tracer used were coming from the same batch.

Detergents. All detergents were products of SERVA (analytical grade) and were used without further purification.

Protein/detergent mixtures. About 0.26 to 0.43 μmol HA were mixed with the following detergents: octyl-glucoside, sodium cholate (Na-cholate), sodium deoxycholate (NaDoc), cetyltrimethylammonium bromide (CTAB), lauroylsarcosinate, Triton X 100 (TX 100), TWEEN-80, CHAPS, Lubrol, sodium dodecylsulphate (SDS). Mixtures with variation of the detergent were prepared by exchanging the detergents. The ratio of detergent to protein was always $>$ 6000 (mol/mol). After at least 16 hr (4 ^{0}C and room temperature, respectively) the mixtures were separated by gel chromatography or density gradient centrifugation without change of the detergent concentration. With some detergents the conditions of incubation were changed: ionic strength (from 0.001 to 0.15), pH (from 7.2 to 8.5), temperature (-200 ^{0}C, -20 ^{0}C, 4 ^{0}C, 22 ^{0}C, 55 ^{0}C), time (from 1 hour to 21 days), application of ultrasound (for 0.5 to 15 minutes).

Gel chromatography. ^{125}I-HA/HA- or ^{125}I-B-HA/B-HA mixtures were separated on Sephadex G-150 (0.8 x 50 cm) or on Sepharose 4-B (0.8 x 50 cm), presaturated with 0.2 % bovine serum albumin, respectively. The elution buffer was 0.0066 mol/l PBS, containing 0.02 % NaN_3 and the same detergent in a concentration used in the starting mixture. The elution peaks were characterized by $K_{av} = \frac{v_e - v_o}{v_t - v_o}$, v_e being the elution volume of the peak, v_o the void volume and v_t the total volume of the column (Determan, 1967).

Density gradient centrifugation. Protein/detergent mixtures (containing the ^{125}I-protein tracer) were applied on the top of a 5 to 25 % or 10 to 50 % continuous sucrose gradient, containing the same type and concentration of detergent used in the mixture and throughout centrifuged for a period of 16 hr at 150 000 x g. The gradients were fractionated and measured in a gamma-counter. The sedimentation coefficient of each protein peak was calculated by the method of McEwan (1967).

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). HA, various HA/detergent mixtures and isolated HA/detergent fractions, containing the appropriate ^{125}I-HA tracer, were electrophoresed according to Laemmli (1970) using 10 % or 15 % resolving gels, at pH 8.7, and 3 % stacking gels, pH 6.8. The samples were heated for 3 min in boiling water bath with the incubation buffer (0.1 mol/l Tris-HCl, pH 6.8, 4.6 % SDS and 17 % glycerol). Under reducing conditions the buffer contained 11 % ß-mercaptoethanol. About 10 to 20 μl of the cooled samples were applied on the stacking gel. The electrophoresis was performed at 2 mA for 2 hr at room temperature (electrode buffer: 0.05 mol/l Tris-HCl, 0.38 mol/l glycine, 1 % SDS, pH 8.3). The gel was cut into 5 mm pieces immediately after the run, and radioactivity was measured in a gamma-counter.

Electron microscopy. Preparations of HA and HA/octyl glucoside mixtures were made by adsorbing the samples to carbon films and negative staining with 2 % phosphortungstic acid, pH 6.5, for 3 min.

Single radial immunodiffusion (SRD). Various HA/detergent mixtures were analysed by the method of Schild *et al.* (1975). The anti-HA serum Anti/A/Leningrad/385/80 (sheep) was obtained from Staatliches Kontrollinstitut für Immunbiologische Arzneimittel (Berlin, Germany).

Results and Discussion

Gel chromatography of HA/detergent mixtures using Sephadex G-150 or Sepharose-4B columns, and centrifugation applying continuous sucrose gradients of 5 to 25 % or 10 to 50 %, respectively, furnished separation profiles with several peak maxima. Typical profiles shown in Figs. 1 and 2 represent the separation results of HA/Triton X 100 and HA/SDS mixtures. Though differing slightly in size, the peaks appeared at the same positions irrespective of the special detergent applied. This was true also with respect to differences in the incubation time, temperature, pH, and ionic strength of the mixtures (see *Materials and Methods*).

The presence or absence of the detergent during the protein storage proved to be of critical importance generally for the formation of different protein fractions (Fig. 3). Fraction A, however, could be detected only if the HA had been stored in solutions free of detergent and fraction D arose only from the HA/SDS mixtures (Figs. 1 and 2). Rechromatography of the fractions obtained by centrifugation and vice versa has proved that the protein fractions A, B, C, D

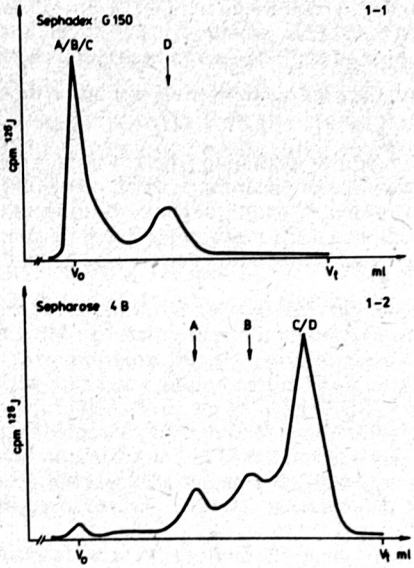

Fig. 1
Gelchromatography of ^{125}I-HA/HA/detergent mixtures in the presence of detergent
1-1 Elution profile of the separation of a HA/SDS mixture on Sephadex G-150
1-2 Elution profile of the separation of a HA/TX 100 mixture on Sepharose-4B

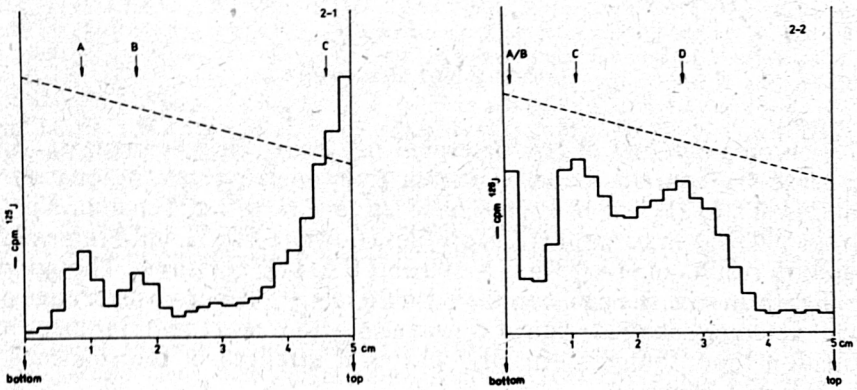

Fig. 2
Density gradient cetrifugation of ^{125}I-HA/HA/detergent mixtures in the presence of detergent
2-1 Centrifugation profile of the separation of a HA/TX 100 mixture on a 10 - 50 % sucrose gradient
2-2 Centrifugation profile of the separation of a HA/SDS mixture on a 5 - 25 % sucrose gradient

from gel chromatography and from density gradient centrifugation were identical. The ratio of different protein aggregates in the starting mixture, as well as the isolated fractions were stable over a period of at least 10 days at 4 °C. The elution constants K_{av} and the sedimentation coefficients were calculated for each individual peak. The results are summarized in Table 1. There were no significant differences between the values determined from mixtures with various detergents. On account of missing information about the proportion of detergent in the HA/detergent complexes, the calculation of the number of HA molecules per complex was impossible. Average values could be estimated, however, by combining electron microscopy and SDS-PAGE of HA fractions and including bromelain cleaved HA (B-HA) into the investigations.

Electron microscopic studies of octyl glucoside solutions of HA, which had previously been stored detergent free for several weeks, revealed three different types of protein structures: single rods and rods associated to rosettes or parts of rosettes. According to their thickness and length the rods represent HA trimers (Ruigrok *et al.*, 1986). The separated protein fractions A, B, C, D, were analysed by SDS-PAGE. As estimated by comparison with marker molecules, fraction A and B yielded bands corresponding to HA trimer aggregates, HA

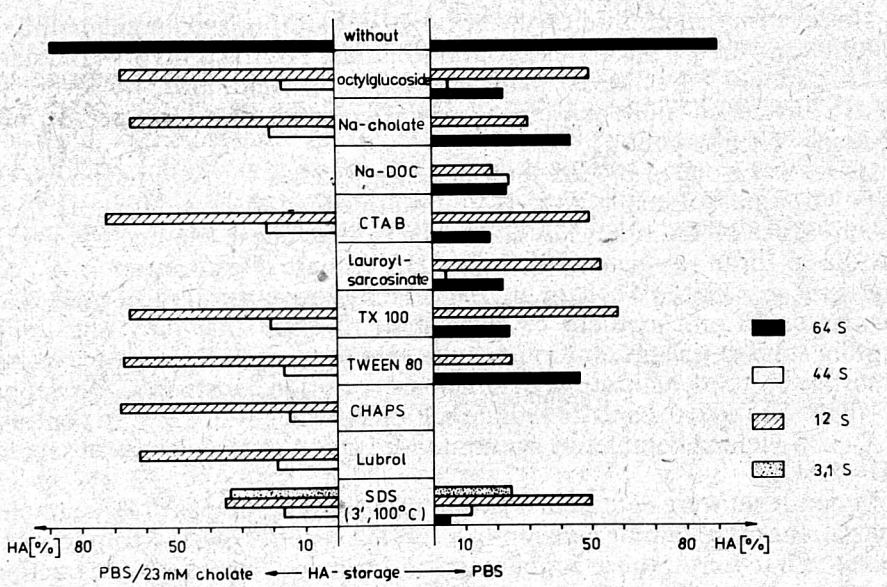

Fig. 3
Dependence of the HA aggregation in the presence of detergents on the protein storage medium
Data obtained by density gradient centrifugation techniques.

Table 1. Elution constants and sedimentation coefficients of separated HA fractions in the presence of detergent

Protein fraction	Gel chromatography matrix	K_{av}	Centrifugation Sed. coeff. (S)
A	Sepharose-4B	0.3	64 ± 6
B	Sepharose-4B	0.5	44 ± 9
C	Sepharose-4B	0.7	12 ± 1
D	Sephadex G-150	0.4	3.1 ± 1.3

trimers and HA monomers. Fraction C furnished HA trimer- and HA monomer bands, but in fraction D the HA monomers were predominant. Throughout, HA dimers were only slightly measurable. Quantitative evaluation of the SDS-PAGE bands prepared from differently stored HA preparations pointed to a more extensive aggregation of HA, that had been stored free of detergent. Table 2 lists the quantitation of multimer and monomer bands, as determined by γ-measurement.

Not even by admixture of the strongly denaturing agents guanidinhydrochloride and urea, a further dissociation of these HA fractions or of the starting mixture could be achieved. Applying reducing conditions, however, SDS-PAGE furnished monomeric HAl and HA2 bands only, irrespective of the protein storage medium.

B-HA was isolated by digestion of virus particles with bromelain, cleaving the hydrophobic domain of the HA2 unit. In aqueous solutions at pH 7 and in the presence of TX 100 B-HA exists as a trimer. These trimers, however, are unable to form aggregates like intact HA trimers (Nestorowicz *et al.*, 1985; Doms *et al.*, 1986). Accordingly, gel chromatographic studies of B-HA on Sepharose-4B and gradient centrifugation revealed that only one uniform protein species was present in aqueous solutions, as in the absence as in the presence of nondenaturating detergents. Its elution constant K_{av} on Sepharose-4B amounts to 0.7 and its sedimentation coefficient to 8.9 S. In contrary to HA, SDS yielded completely monomerised B-HA molecules, as observed by SDS-PAGE.

In nondenaturing detergent solution three different types of HA complexes coexist: those containing apparently intact HA rosettes of 6 to 8 trimers – correspond to fraction A, those containing 3 to 4 trimers – correspond to fraction B, and those containing one trimer rod only – correspond to fraction C. HA monomers (fraction D) may only be obtained under denaturing conditions, for example in SDS solution. Reequilibration of any separate HA fraction did not occur within a period of two weeks. However, it seems to be very improbable that this behaviour is due to a high kinetic stability of the complexes exclusi-

vely. Considering, additionally, the different behaviour of HA and B-HA rather suggests, that some type of irreversible association between HA trimers took place in the hydrophobic domain of the HA2 subunit. Thus, not even the application of denaturing agents could dissociate the HA into one single uniform species as it was possible with B-HA.

It is not clear which type of interaction takes place between the hydrophobic domains of the HA molecules. A similar resistance to dissociation towards SDS has already been described for other hydrophobically anchored membrane proteins, for example for the coat protein of bacteriophage f 1 (Makino *et al.*, 1975) or for glycophorin (Silverberg *et al.*, 1978). This behaviour was discussed to be the result of a reduced attachment of SDS to α-helical structures. But some facts point to intermolecular S-S bridges between HA trimers and/or between HA molecules within the trimers: (i) the resistance to efficient denaturing conditions, (ii) the lack of reequilibration of single type associates, and (iii) the influence of the storage medium on the quantity of different protein aggregates. Moreover, the reaction yield of the SH-group specific reagent m-maleinimidobenzoyl-NHS-ester with HA was doubled, if the protein had been stored in the presence of detergent. Apparently, the incorporation of the detergent molecules into the protein rosette hinders HA trimers from intermolecular reactions.

Finally, we examined the reactivity of HA towards specific antibodies in different solutions by radial immunodiffusion. As a result, the antigenicity remained unaffected, irrespective of the detergent (lauroylsarcosinate, TX 100, Na-cholate, Lubrol, and octylglucoside) and of the storage medium. On the other hand, in the presence of SDS, the precipitation areas, if observed at all, were diffuse, indicating a strongly decreased antigenicity. Apparently, the presence of nondenaturing detergents and the protein aggregation in such solution are of minor importance for this property of HA. Investigations of the

Table 2. Dependence of the HA aggregation on the storage medium of the protein, determined by SDS PAGE

HA preparation	Storage medium	HA multimers (%)	HA monomers (%)
1	PBS	80	11
2	PBS	84	11
3	PBS	82	9
4	PBS	83	17
4	PBS/cholate*	67	24
5	PBS/CTAB*	71	22

*Na-cholate: 23 mM; CTAB: 10 mM

reconstitution of HA into liposomes shall clarify the influence of HA aggregation and of HA/detergent interaction on the efficiency of protein incorporation into liposomes and on the functional properties of HA. This could be of interest for isolation, purification, and storage not only of HA, but also of other proteins of comparable shape and structure.

References

Brand, C. M., and Skehel, J. J. (1972): Crystalline antigens from the influenza virus envelope. *Nature (New Biology)* **238**, 145 - 147.

Determann, H. (1967): *Gelchromatographie*, Springer Verlag Berlin, Heidelberg.

Doms, R. W., and Helenius, A. (1986): Quarternary structure of influenza virus hemagglutinin after acid treatment. *J. Virol.* **60**, 833 - 839.

Flanagan, M. T., and Skehel, J. J. (1977): The conformation of influenza virus hemagglutinin. *FEBS Lett.* **80**, 57 - 60.

Helenius, A., Sarvas, M., and Simons, K. (1981): Asymmetric and symmetric membrane reconstitution by detergent elimination. *Europ. J. Biochem.* **116**, 27 - 35.

Hosaka, Y., Yasuda, Y., and Fukai, K. (1983): Hemolysis by liposomes containing influenza virus hemagglutinin. *J. Virol.* **46**, 1014 - 1017.

Huang, R. T. C., Wahn, K., Klenk, H. D., and Rott, R. (1979): Association of envelope glycoproteins of influenza virus with liposomes - a model study on viral envelope assembly. *Virology* **97**, 212 - 217.

Kawasaki, K., Sato, S. B., and Ohnishi, S. I. (1983): Membrane fusion activity of reconstituted vesicles of influenza virus hemagglutinin glycoproteins. *Biochem. Biophys. Acta* **733**, 286 - 290.

Laemmli, U. K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680 - 685.

Makino, S., Woolford Jr., J. W., Tanford, Ch., and Webster, R. E. (1975): Interaction of deoxycholate and of detergens with the coat protein of bacteriophage fl. *J. Biol. Chem.* **250**, 4327 - 4332.

McEwen, C. R. (1967): Tables of estimating sedimentation through linear concentration gradients of sucrose solution. *Anal. Biochem.* **20**, 114 - 149.

Mimms, L. T., Zampighi, G., Nozaki, Y., Tanford, Ch., and Reynolds, J. A. (1981): Phospholipid vesicle formation and transmembrane protein incorporation using octyl glucoside. *Biochemistry* **20**, 833 - 840.

Moeller, J. V., le Maire, M., and Andersen, J. P. (1986): Uses of nonionic and bile salt detergents in the study of membrane proteins, pp. 147 - 196. In D. Watts, M. de Pont (Eds): *Progress in Protein-Lipid Interactions 2,* Elsevier Science Publishers BV.

Nestorowicz, A., Laver, G., and Jackson, D. C. (1985): Antigenic determinants of influenza virus hemagglutinin. X. A comparison of the physical and antigenic properties of monomeric and trimeric forms. *J. gen. Virol.* **66**, 1687 - 1695.

Nussbaum, O., Lapidot, M., and Loyter, A. (1987): Reconstitution of functional influenza virus envelopes and fusion with membranes and liposomes lacking virus receptors. *J. Virol.* **61**, 2245 - 2252.

Ruigrok, R. W. H., Wrigley, N. G., Calder, L. J., Cusack, S., Wharton, S. A., Brown, E. B., and Skehel, J. J. (1986): Electron microscopy of the low pH structure of influenza virus hemagglutinin. *EMBO J.* **5**, 41 - 49.

Schild, G. C., Wood, J. M., and Newman, R. W. (1975): A single radial immunodiffusion technique for the assay of influenza hemagglutinin antigen. *Bull. Wrld. Hlth. Org.* **52**, 223 - 231.

Stahn, R., Schäfer, H., Malur, J., and Petzold, G. (1987): Preparation and properties of ^{125}I-hemagglutinin from influenza viruses. Iodination of a membrane glycoprotein. *Isotopenpraxis* **23**, 41 - 46.

Stegmann, T., Morselt, H. W. M., Booy, F. P., van Breemen, J. F. L., Scherphof, G., and Wilschut, J. (1987): Functional reconstitution of influenza virus envelopes. *EMBO J.* **6**, 2651 - 2659.

Silverberg, M., Furthmayr, H., and Marchesi, V. T. (1976): The effect of carboxymethylating a single methionine residue on the subunit interactions of glycophorin A. *Biochemistry* **15**, 1448 - 1454.

Simons, K., Helenius, A., and Garoff, H. (1973): Solubilization of the membrane proteins from Semliki forest virus with Triton X 100. *J. mol. Biol.* **80**, 119 - 133.

Trudel, M., and Nadon, F. (1981): Virosome preparations: differences between influenza and rubella hemagglutinin adsorption. *Can. J. Microbiol.* **27**, 958 - 962.