# IMPORTANCE OF INTERPEPTIDE DISULFIDE BOND IN A VIRAL GLYCOPROTEIN WITH HEMAGGLUTINATION AND NEURAMINIDASE ACTIVITIES

Masayuki OZAWA and Akira ASANO
*Institute for Protein Research, Osaka University, Osaka 565, Japan*

and

Yoshio OKADA
*Research Institute for Microbial Diseases, Osaka University, Japan*

Received 30 August 1976

## 1. Introduction

Cell fusion induced by a group of enveloped viruses such as HVJ (Sendai virus) [1] is known to proceed as follows: adsorption of the virus to cell membranes, agglutination of the cells, fusion of the virus envelope (membrane) to the cell membrane and fusion of cell membrane to cell membrane [2]. HVJ and the other paramyxoviruses which are the most powerful inducers of cell fusion are known to have two glycoproteins (spikes) projecting from the surface of the virion. One of them, abbreviated as HANA is responsible for its hemagglutination (i.e. adsorbing on cell surfaces) and neuraminidase activities [3,4] and another one (F) is required for its cell fusion activity [5]. Since not all of the spiked viruses are active for cell fusion, we are interested in the structure–function relationship of the viral glycoprotein spikes.

In this paper, we report that HANA glycoproteins of HVJ are present as oligomers connected with interpeptide disulfide bonds. This disulfide bond is easily split by several sulfhydryl compounds under non-denaturing conditions, and the reductive cleavage of HANA protein is accompanied by concomitant loss of the biological activities. Importance of this finding will be discussed in relationship to the other membrane-penetrating toxins.

*Abbreviations:* SDS, sodium dodecyl sulfate; HVJ, hemagglutinating virus of Japan; HAU, hemagglutination units; PAS, periodic acid-Schiff.

## 2. Materials and methods

HVJ, Z strain, was propagated in the allantoic sacs of chick embryos. The virus was purified by differential centrifugation as described previously [6]. Hemagglutination units were determined by Salk's pattern methods [7]. Treatment of the virus (1 mg/ml) with sulfhydryl compounds and the other reagents was performed in BSS, pH 8.0 (0.14 M NaCl, 54 mM KCl, 0.34 mM $Na_2HPO_4$, 0.44 mM $KH_2PO_4$ and 10 mM Tris–HCl buffer pH was adjusted to 8.0) at 37°C for 20 min. The treated virus was washed with Tricine-buffered saline (pH 7.6) [8] 3 ×. Human red blood cells were obtained from a blood bank (Midori-zyuzi) and used within 4 weeks after drawing. Hemolysis of human erythrocytes were determined as described previously [8]. SDS–polyacrylamide gel electrophoresis was performed as described by Fairbanks et al. [9], at 4 mA/tube for 2 h except that 2-mercaptoethanol was omitted when indicated. Samples were boiled for 3 min in the solubilizing buffer before application to the gel.

## 3. Results

### 3.1. *SDS-polyacrylamide gel electrophoresis under non-reducing conditions*

Usually viral protein components were identified by gel electrophoresis under reducing conditions. Five major bands are seen as in fig.1(a) and this is in good agreement with those reported earlier [3,5,10]. Partially different patterns were, however, obtained by performing electrophoresis under non-reducing conditions. As is shown in fig.1(b), protein bands marked P, NP and M did not appreciably change from those of fig.1(a). Bands marked HANA and $F_1$ had, however, disappeared and new bands marked B-1, B-2, B-3 and F appeared when electrophoresed without 2-mercaptoethanol. These differences can be more clearly seen when the gels were stained for carbohydrates. Under reducing conditions (fig.1(c)) bands HANA and $F_1$ with some minor band were seen, whereas in the absence of mercaptoethanol (fig.1(d)) all of these bands were replaced by bands which corresponded to B-1, B-3 and F of fig.1(b). Therefore, it seemed likely that major changes occurring with exclusion of the reducing reagent, 2-mercaptoethanol, from the medium were observed on glycoproteins except for band B-2 which was not stained with PAS-method. Electrophoretic profile similar to fig.1(a) (reduced conditions) was obtained when the virus was treated with 10 mM dithiothreitol, washed and electrophoresed under non-reducing conditions. Interestingly, hemagglutinin, neuraminidase and hemolytic activities of the virus were all lost by the dithiothreitol treatment described above.

### 3.2. *Identification of B-1 and B-3 as HANA glycoprotein*

For further identification of bands appeared under nonreducing conditions, glycoprotein which bears hemagglutinin and neuraminidase activities (HANA) [3,4] was purified by an affinity column containing fetuin-Sepharose as described by Scheid and Choppin [11]. A glycoprotein fraction which may be released by digestion of sialic acid residues of fetuin by its own neuraminidase was obtained. As shown in fig.1(e), the protein thus obtained is almost pure HANA glycoprotein when tested under reducing conditions, whereas under non-reducing conditions it exhibited two peaks corresponding to B-1 and B-3 (fig.1(f)). Thus, B-1 and B-3 seem to be oligomers of HANA glycoprotein. The same conclusion was obtained from the

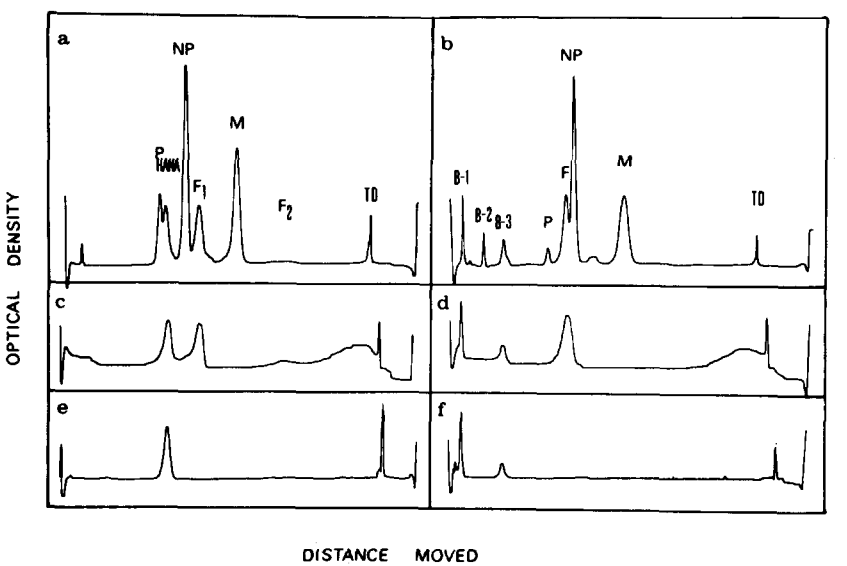


Fig.1. SDS-gel electrophoresis of HVJ and its component. (a,c,e) Reducing conditions, 1.0% of 2-mercaptoethanol was included in the solubilizing medium. (b,d,f) Non-reducing conditions, 2-mercaptoethanol was omitted from the medium. (a,b,e,f) Stained with Coomassie Brilliant Blue. (c,d) Stained by PAS-method as described by Kapitany and Zebrowski [21]. (e,f) Purified HANA glycoprotein fraction was applied.

experiments in which B-1 and B-3 extracted from the gels were re-electrophoresed under reducing conditions. Both bands show the same HANA band in the presence of 2-mercaptoethanol. Band F may be composed of $F_1$ and $F_2$ connected by disulfide bond as reported for NDV [12].

### 3.3. *Importance of disulfide bond in HANA glycoprotein*

Treatment of the virus under mildly reducing conditions ($\geq$6 mM reduced glutathione) resulted in disappearance of bands B-1 and B-3, but not of bands B-2 and F (fig.2). Reduced glutathione at and below 4 mM was, however, apparently without effect on the electrophoretic profile. As shown in fig.3, the loss of the biological activities of HANA glycoprotein, namely hemagglutination and neuraminidase, and hemolysis which is the activity of F glycoprotein but also

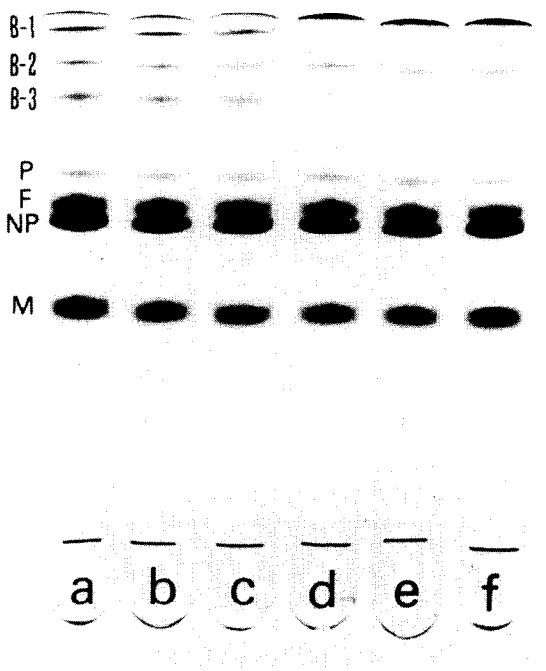


Fig.2. SDS–gel electrophoresis of HVJ treated with different concentrations of reduced glutathione. HVJ was preincubated with indicated concentrations of reduced glutathione as described in the text. Electrophoresis was performed under non-reducing conditions, and stained with Coomassie Brilliant Blue R-250. Concentrations of reduced glutathione used were as follows: a, 0 mM; b, 2 mM; c, 4 mM; d, 6 mM; e, 8 mM; f, 10 mM.

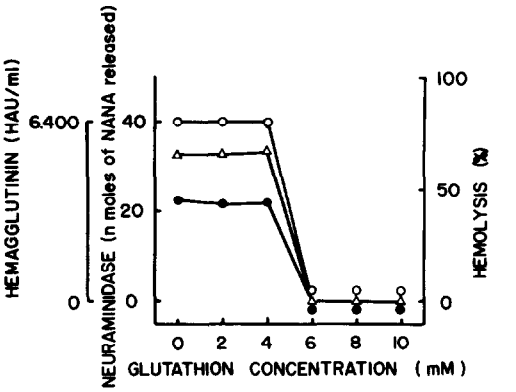


Fig.3. Effect of reduced glutathione-treatment on the biological activities of HVJ. HVJ treated with indicated concentrations of reduced glutathione was washed and its neuraminidase activity was determined after incubation at 37°C for 5 min with fetuin as a substrate. Released sialic acid was estimated by the method of Aminoff [22]. Hemolysis was measured as described in the text. Hemagglutination activity of the treated virus was expressed as HAU/ml. (–○–) hemagglutinating activity. (–△–) hemolysis. (–●–) neuraminidase activity.

requires intact HANA glycoprotein were all well paralleled with splitting of the interpeptide disulfide bond. Thus, importance of this easily split disulfide bond of HANA in its biological activities has to be considered. A discrepancy that the disappearance of bands B-1 and B-3 is not accompanied by the appearance of HANA band after reduced glutathione treatment may be explained such that the mild reduction did not split intrapeptide disulfide bond and this resulted in anomalous behavior of the peptide under nonreducing conditions [13], since complete reduction of the partially reduced samples exhibited the appearance of HANA band.

### 3.4. *Disulfide bonds in the other proteins*

Re-electrophoresis of extracted B-2 under reducing conditions exhibited band P, therefore, B-2 may be composed of a dimer of P peptide. Biological importance of this is not clear at present.

Although a glycoprotein of influenza virus with hemagglutinin activity (HA) was known to be consisted of two peptide $HA_1$ and $HA_2$ connected with a disulfide bond [14], the disulfide bond was not split by the addition of 20 mM dithiothreitol to the virus suspension (data not shown). At the same time,

no decrease in hemagglutinin activity was observed after the dithiothreitol treatment. Similar resistance of interpeptide disulfide bond and the enzyme activity to the reducing reagent was also found in the case of influenza neuraminidase. On the other hand, similar treatment of NDV a fusogenic virus with the sulfhydryl reagent resulted in complete inactivation of hemagglutinin activity.

## 4. Discussion

Inhibition of hemagglutinin, neuraminidase and hemolytic activities by dithiothreitol treatment has been reported previously by Neurath et al. [15]. Importance of interpeptide disulfide bond was, however, not recognized at that time. From the studies reported above, it is reasonable to assume the importance of such disulfide bond in the biological activities. The other possibility that sulfhydryl compounds reacted with metal ions which may be required for the biological activities was checked by the use of the other metal chelating reagents. Incubation of the virus with metal chelators, such as 6 mM EDTA, 8 mM allylthiourea and 8 mM *o*-phenanthroline did not influence the biological activities described above, whereas treatment of the virus with 10 mM reduced glutathione, 0.5% 2-mercaptoethanol, 1 mM dithiothreitol and 20 mM cysteine all resulted in complete loss of the biological activities, and at the same time disappearance of the bands B-1 and B-3. Thus, splitting of interpeptide disulfide bond by the latter may be relevant to the inactivation of the activities. Formation of the disulfide bond by autooxidation during solubilization as reported in the case of H-2 antigen [16] can be excluded, since pretreatment of the virus with 10 mM monoiodoacetate before solubilization did not change the electrophoretic profile of the virus. At the same time, the biological activities was not inhibited, thus, free sulfhydryl group seems to be not essential for the biological activities.

Importance and/or presence of intrapeptide disulfide bonds in HANA glycoprotein of the virus are not known at present, but higher sensitivity of the interpeptide disulfide bonds of the protein to sulfhydryl compounds than those of F-glycoprotein of the same virus, HA- and NA-glycoproteins of influenza virus (non-fusogenic) and intrapeptide disulfide bonds of many other biologically active proteins is evident. The other examples whcih are known to have similar sensitive-interpeptide disulfide bond required for their biological activities are bacterial toxins (diphteria [17], Botulinus [18] and others) and plant toxins (ricin [19] and abrin [19]). It is very interesting that all of these proteins including HANA glycoprotein of HVJ react with cell membranes from the outside. Further, the disulfide bond is required for these toxins only when they are reacting with the cell surface but not with cell free extracts [17,19,20]. Importance of this similarity will be discussed in detail in a future publication.

## Acknowledgments

We would like to thank Professor Ryo Sato of our laboratory for his helpful discussions and Professor Y. Kawade of Kyoto University for kindly providing NDV.

## References

[1] Okada, Y. (1958) Biken's J. 1, 103–110.
[2] Poste, G. and Allison, A. G. (1973) Biochim. Biophys. Acta 300, 421–465.
[3] Shimizu, K., Shimizu, Y. K., Tohara, T. and Ishida, N. (1974) Virology 62, 90–101.
[4] Porter, A., Scroggs, R. A., Marx, P. A. and Kingsbury, D. W. (1975) Virology 67, 179–187.
[5] Homma, M. and Ohuchi, M. (1973) J. Virol. 12, 1457–1465.
[6] Maeda, T., Asano, A., Ohki, K., Okada, Y. and Ohnishi, S. (1975) Biochemistry 14, 3736–3741.
[7] Salk, J. E. (1944) J. Immunol. 49, 87–97.
[8] Sekiguchi, K. and Asano, A. (1976) Life Sci. 18, 1383–1389.
[9] Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606–2617.
[10] Scheid, A. and Choppin, P. W. (1974) Virology 57, 475–490.
[11] Scheid, A. and Choppin, P. W. (1974) Virology 62, 125–133.
[12] Nagai, Y., Ogura, H. and Klenk, H.-D. (1976) Virology 69, 523–538.
[13] Dunker, A. K. and Kenyon, A. J. (1976) Biochem. J. 153, 191–197.

[14] Laver, W. G. (1971) Virology 45, 275–288.
[15] Neurath, A. R., Vernon, S. K., Hartzell, R. W. and Rubin, B. A. (1973) J. Gen. Virol. 19, 9–20.
[16] Henning, R., Milner, R. J., Reske, K., Cunningham, B. A. and Edelman, G. M. (1976) Proc. Natl. Acad. Sci. USA 73, 118–122.
[17] Gill, D. M. and Pappenheimer, A. M. Jr. (1971) J. Biol. Chem. 246, 1492–1495.
[18] Sugiyama, H., DasGupta, B. R. and Yang, K. H. (1973) Proc. Soc. Exp. Biol. Med. 143, 589–591.
[19] Olsnes, S. and Pihl, A. (1972) FEBS Lett. 28, 48–50.
[20] Collier, R. J. and Kendel, J. (1971) J. Biol. Chem. 246, 1496–1503.
[21] Kapitany, R. A. and Zebrowski, E. J. (1973) Anal. Biochem.56 361–369.
[22] Aminoff, D. (1961) Biochem. J. 81, 384–392.