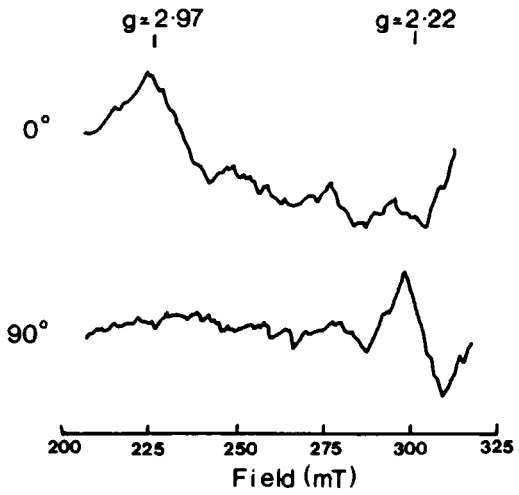

FIGURE 1 EPR spectra of cytochrome b_{559} in isolated PS II reaction centers when partially dried on mylar. Instrument settings were as follows: temperature, 15 K; microwave power, 15 dB down from 200 mW; modulation amplitude, 20 G. Spectra are shown which were obtained when the mylar sheets were parallel (0°) and perpendicular (90°) to the magnetic field.

cules that are perpendicular to each other (see reference 2). This is also the case in the analogous purple bacteria reaction center (4). Secondly, the orientation of the PS II primary donor chlorophyll in the plane of the membrane is different from the situation in purple bacteria where the primary donor bacteriochlorophyll is oriented almost perpendicular to the membrane plane. This represents a significant structural difference between the reaction centers of PS II and purple bacteria. Such differences are not unexpected, because the operating redox potential of P680, as an oxidant of water, is estimated to be at least 0.5 V more positive than that of purple bacteria.

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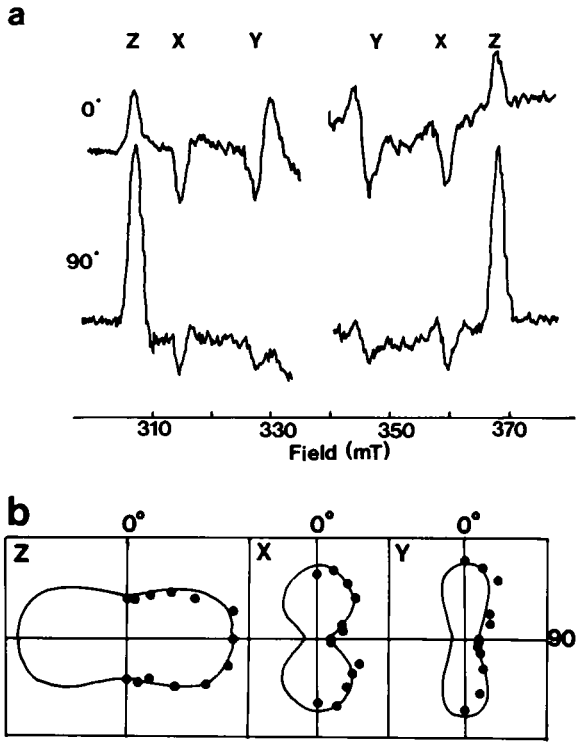

FIGURE 2 The orientation dependence of the P680 triplet in isolated PS II reaction centers. The sample, which was partially dried on mylar sheets, was submerged in sodium dithionite (20% in 200 mM glycine pH 9.0) under an 0_2 free argon atmosphere for 5 min in darkness at 22°C. Instrument settings were as follows: temperature, 4.2 K; microwave power 35 dB down from 200 mW, modulation amplitude 20 G. Spectra were recorded under illumination using white light from an 800 watt projector. (*a*) EPR spectra recorded when the mylar sheets were parallel (0°) and perpendicular (90°) to the magnetic field. (*b*) Polar plots of the *ZXY* features of the triplet signal.

REFERENCES

1. Picaud, A., S. Acker, and J. Duranton. 1982. A single step separation of PS I, PS II and chlorophyll antenna particles from spinach chloroplasts. *Photosynth. Res.* 3:203–213.
2. Rutherford, A. W. 1985. Orientation of EPR signals arising from components in Photosystem II membranes. *Biochim. Biophys. Acta.* 807:189–201.
3. Thurnauer, M. C., and J. R. Norris. 1977. The ordering of the zero field triplet spin sublevels in the chlorophylls. A magneto-photoselection study. *Chem. Phys. Lett.* 47:100–105.
4. Tiede, D. M. 1985. Redox center and secondary protein structure organization in bacterial reaction centers and the cytochrome b/c_1 complex. *In* Encyclopedia for Plant Physiology "Photosynthetic Membranes." A. Staehelin and C. J. Arntzen, editors. Springer Verlag. In press.

BILAYER PENETRATION BY MEMBRANE-ASSOCIATED PROTEINS

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The influenza virus consists of a core complex of protein and RNA that becomes coated with a lipid envelope as the maturing virus buds from the host cell (1). This envelope contains two major intrinsic proteins; one of which, hemagglutinin (HA), is known to form trimeric aggregates (2) appearing as characteristic spikes in electron micrographs

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of the viral envelope surface. The mature HA protein consists of two individual polypeptide chains, HA-1 (M_r 58,000) and HA-2 (M_r 25,000), covalently linked by a single disulfide bridge (2, 3). Sequence analysis of these chains has revealed two regions rich in hydrophobic amino acids, both found in the HA-2 peptide (4). It is generally thought that the COOH-terminal sequence of HA-2, the location of one of the hydrophobic domains, traverses the lipid bilayer and serves to anchor the entire HA complex in the membrane (2); whereas the second, highly conserved, hydrophobic segment is located at the NH_2-terminus of HA-2 and apparently does not penetrate the viral envelope. The HA-1 peptide is postulated to have no direct contact with the lipid envelope, being attached to the virus particle solely through a single covalent disulfide bridge to HA-2, which is strengthened by other noncovalent associations (5).

Until recently it has been extremely difficult to study disposition of membrane proteins within the hydrophobic domain of the lipid bilayer. The introduction of hydrophobic and amphiphilic photoreactive probes has, for the first time, allowed a detailed analysis of the integral proteins of complex membranes (6). Here we report the use of a photoreactive glycolipid analog, which shows a high level of selectivity for membrane-associated sequences (7–10), to identify components of the influenza virus particle that are intimately associated with its lipid envelope.

METHODS

Influenza virus strain A/PR/8/34 was propagated in the allantoic cavity of 10-d old embryonated hens' eggs (11). Virus was grown at a temperature of 35°C and harvested 48 h after inoculation. Viral particles were subjected to differential centrifugation and then further purified on consecutive sucrose and renografin-76 (Squibb, Princeton, NJ) gradients (7). The purified virus was stored at −70°C in 10 mM Tris buffer (pH 7.4) containing 100 mM NaCl and 1 mM EDTA (TSE buffer) at a protein concentration of 5 mg/ml. Purity of viral preparations was determined by protein fractionation by SDS-polyacrylamide gel electrophoresis (see below). The integrity of the viral envelope was demonstrated by lactoperoxidase-catalyzed surface iodination performed at 4°C according to the method of Sefton et al. (12).

Viral particles were solubilized by treating the virus with 0.1% (vol/vol) Triton X-100 in TSE buffer at 20°C for 15 min. The nucleocapsid was separated from solubilized proteins by centrifugation, as described below under proteolysis. Samples were analyzed by SDS-polyacrylamide gel electrophoresis.

Removal of exposed viral polypeptides was achieved by using the method of Brand and Skehel (13). Briefly, virus particles were suspended at a protein concentration of 2 mg/ml in TSE buffer containing 50 mM 2-mercaptoethanol and 1 mg/ml bromelain (E.C. 3.4.22.4; Sigma Chemical Co., St. Louis, MO) and incubated for 14 h at 37°C. Following digestion, the mixture was centrifuged at 135,000 × *g* for 30 min to separate intact enveloped capsids from peptide fragments released by proteolysis. Both the supernatant fluid and the sedimented material were analyzed by SDS-polyacrylamide gel electrophoresis.

The photoreactive probe 12-(4-azido-2-nitrophenoxy)stearoyl-[1-^{14}C] glucosamine (12APS-GA) was synthesized as previously described (7) and stored in 95% ethanol in the dark at 5°C. The specific radioactivity was 50 *m*Ci/*m*Mol. Twenty μl (100 μg protein) of viral suspension were added to 600 μl of TSE buffer at 37°C. Under safelight conditions 6 μl 12APS-GA (1.2 ± 10^5 cpm) were added; the mixture was incubated for a further 5 min before being irradiated for 15 s at 366 nm with a high intensity mercury-xenon arc lamp. The labeled virus was harvested by centrifugation at 200,000*g* for 30 min.

Viral proteins were separated in gels of 9% acrylamide/0.27% N,N′-methylene bisacrylamide using the discontinuous buffer system of Laemmli (14) under a constant voltage of 200 V. Before electrophoresis, samples were boiled for 2 min in buffer containing 67 mM Tris, 1% (wt/vol) SDS, 8 M urea, with or without 1% (vol/vol) 2-mercaptoethanol. Proteins were visualized by staining with Coomassie Blue dye R-250 and radiolabeled proteins were subsequently identified by treating gels with 1 M sodium salicylate (15) before exposure to preflashed X-Omat AR film (Eastman Kodak Co., Rochester, NY). Fluorograms were developed after 4 to 6 wks exposure at −70°C.

RESULTS AND DISCUSSION

The chemistry of photocatalyzed reactions is complex (6, 10) and, although they may not be completely predictable, we have no evidence that suggests that the reactive species of 12APS-GA shows any functional specificity for particular peptide sequences. Previous studies with other membrane systems show that it labels only integral membrane components (9, 16). Our objective in this study was to identify those components of the influenza virus that are in close association with the viral lipid envelope. Before we

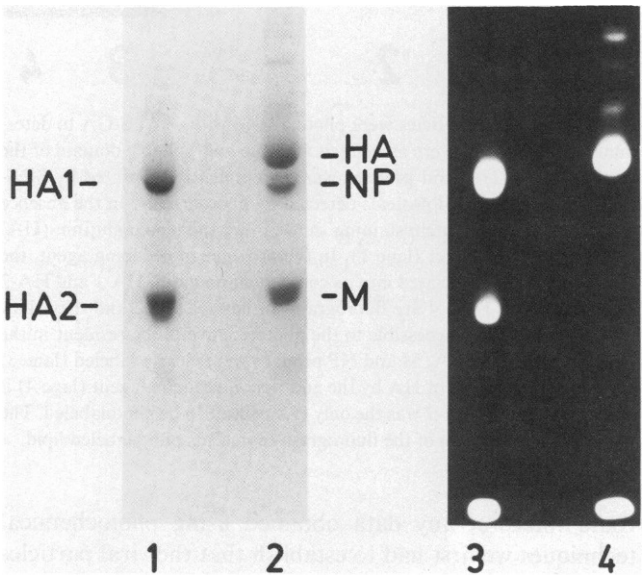

FIGURE 1 Lactoperoxidase catalyzed surface iodination of the virion surface proteins was carried out to establish that the virus particles were intact. When proteins of the iodinated virus were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in the absence of reducing agent, the hemagglutinin (HA) protein (M_r 75,000) remained intact (lane 2). The corresponding autoradiogram (lane 4) showed that this complex was efficiently labeled. However, no labeling was detected in either the nucleoprotein (NP) or the matrix protein (M). After addition of reducing agent the HA protein dissociated into the peptides HA-1 (M_r 53,000) and HA-2 (M_r 25,000), (lane 1). The autoradiogram showed that both these peptides were efficiently iodinated (lane 3), but again no labeling of the NP or M proteins was detected, confirming that the viral particles used in these studies were intact. Sensitive protein staining, combined with the results of surface iodination, indicated that the viral neuraminidase protein (NM) was present in very small copy numbers in the viral samples used for this study.

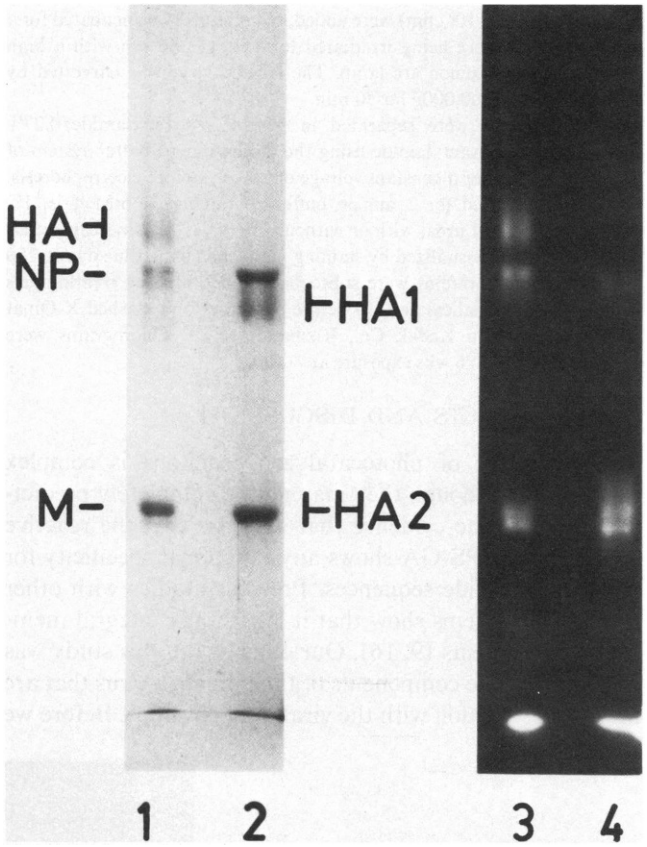

FIGURE 2 Virus particles were photolabeled with 12APS-GA to determine which proteins were associated with the hydrophobic domain of the viral envelope. The viral proteins were subsequently separated by SDS-PAGE and the labeled proteins detected by fluorography. In the absence of reducing agent, protein staining showed that the hemagglutinin (HA) protein remained intact (lane 1). In the presence of reducing agent, the HA protein was dissociated into its constituent peptides, HA-1 and HA-2 (lane 2). Lanes 3 and 4 are fluorograms of lanes 1 and 2 and they show which proteins were accessible to the photoreactive probe resident in the viral envelope. The HA, M and NP proteins were clearly labeled (lane 3) and after dissociation of HA by the addition of reducing agent (lane 4) it was apparent that HA-2 was the only HA peptide to be photolabeled. The material at the bottom of the fluorogram represents photolabeled lipid.

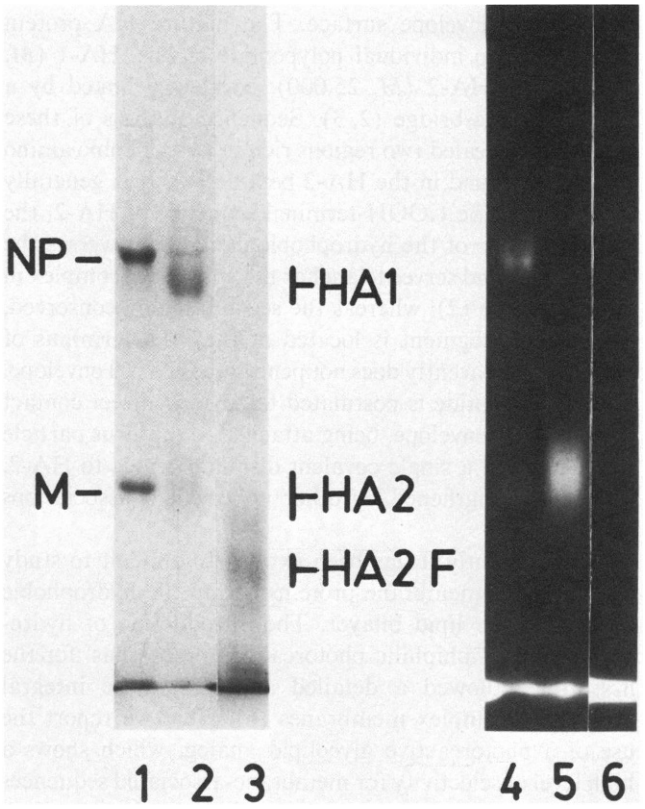

FIGURE 3 Because of similarities between the molecular weights of the component peptides of HA and other viral proteins such as NP and M, membrane proteins were isolated by treating 12APS-GA-labeled virus with Triton X-100, followed by centrifugation. After separation of viral proteins by SDS-PAGE it was clear that the pellet contained proteins M and NP (lane 1) and the supernatent fluid contained HA-1 and HA-2 (lane 2). The corresponding fluorogram indicated that NP, M, and HA-2 were photolabeled, whereas no labeling of HA-1 was detected (lanes 4 and 5). Fig. 3 also shows the result of treating the photolabeled virus with the proteolytic enzyme bromelain, which releases a fragment of HA-2, HA-2F (M_r 20,000). This N-NH_2-terminal HA-2 fragment (lane 3), lacking the proposed membrane-associated moiety, showed no evidence of being photolabeled even after extended exposure of the gel to film (lane 6). The lack of radioactivity associated with the bromelain-released NH_2-terminal fragment, in conjunction with the high efficiency labeling of intact HA-2 indicated that this peptide was anchored in the membrane by the COOH-terminal fragment.

could interpret any data obtained using photochemical techniques we first had to establish that the viral particles used in this study were intact. Fig. 1 shows the results of lactoperoxidase-catalyzed surface iodination of the viral particles that confirmed the integrity of the virus. On the basis of much indirect evidence regarding influenza virus structure we anticipated that a membrane-restricted probe such as 12APS-GA would become associated exclusively with the HA-2 peptide of the HA complex. Fig. 2 indicates that this is indeed the case, with the probe showing complete specificity for HA-2, even though this peptide is in intimate contact with the much larger and superficially more accessible HA-1 peptide. Furthermore, cleavage of the labeled HA-2 peptide with bromelain (Fig. 3), an enzyme that cleaves at a single site close to the COOH-terminal hydrophobic domain of HA-2 (13), showed that the photoreactive probe reacts exclusively with the COOH-terminal hydrophobic sequence. This observation provides direct confirmation of the intramembrane location of this particular hydrophobic sequence, supporting the concept of a COOH-terminal membrane-anchoring domain. In contrast, the highly conserved hydrophobic region at the NH_2-terminus of HA-2 was completely unlabeled, a result consistent with its presumed extramembranous location.

HA is not the only protein that has a close association with the viral envelope. The M (matrix or membrane) protein is believed to form a layer in close contact with the inner surface of the lipid envelope (17, 18). Evidence for such a location was first obtained from electron microscopic studies of the influenza virus (19, 20). A subsequent

study showed that glutaraldehyde fixed then lipid-extracted virus particles have a smooth-surfaced coating surrounding the nucleocapsid that was thought to correspond to the M protein (3). Inside this smooth coat lie ribonucleoprotein structures containing eight segments of RNA coiled into helices. The ribonucleoprotein structures consist of single-stranded RNA and four proteins, of which NP is predominant. Because it appears that the nucleocapsid may be embedded in a matrix of M protein (21), NP could be expected to have a close association with the M protein. Fig. 2 shows that 12APS-GA–labeled matrix protein with high efficiency and surprisingly it also labeled NP. If the probe is truly membrane-restricted, then this result is incompatible with a model of viral structure in which the nucleocapsid is discrete from the overlying lipid envelope.

Surface-restricted iodination had established that the virus particles used for our studies were intact (Fig. 1) and therefore 12APS-GA labeling of the M and NP proteins could not be attributed to the presence of defects in the viral envelope. Furthermore, the surface-restricted iodination pattern was unchanged after photolabeling (data not shown), showing that the presence of the amphiphilic probe, at the concentration used in this study, caused no disruption of the viral envelope. It is unlikely that 12APS-GA in its photoactivated state will exhibit partition selectivity favoring the nucleocapsid aqueous space over the external buffer. The probe clearly shows a selectivity for HA subunits that can only be rationalized on the basis of membrane association and it is reasonable to assume that the same selectivity mechanisms operate on the inner side of the viral envelope.

A strong association between the M protein and the nucleocapsid of Sendai virus was shown by Caldwell and Lyles (22), who were unable to dissociate either protein from the inner surface of erythrocyte membranes after viral fusion, even with harsh conditions. Reaggregation studies with Sendai virus proteins also indicate a close association between the M and NP proteins (21). A tight association between M and NP would be an advantage during budding of enveloped viruses from a host cell membrane, where it would enable the NP protein and its associated RNA to bind to a part of the membrane rich in viral envelope proteins. Although two recent reports have alluded to the accessibility of M protein to membrane-restricted photoreactive reagents (23, 24), here we provide documentary evidence suggesting that both the M and NP proteins both have domains that are intercalated with the envelope lipids.

In summary, the photoreactive lipid analog, 12-(4-azido-2-nitrophenoxy)stearoyl-[1^{14}C] glucosamine provides direct evidence that the hemagglutinin protein of influenza is anchored to the viral lipid envelope solely through an hydrophobic segment at the carboxyl terminal of the HA-2 peptide. The highly conserved, hydrophobic segment at the amino terminal of HA-2, which is believed not to penetrate the lipid envelope bilayer, was unlabeled. The results of our study are consistent with a model for influenza virus in which both M and NP have regions that are integrated in the membrane; these proteins are presumably also in close association with each other, or with RNA, because they withstand detergent disruption of the envelope bilayer. We are currently using similar chemical techniques to monitor the direct insertion of the NH_2-terminus of HA-2 into target membranes during pH-dependent viral fusion with phospholipid vesicles.

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REFERENCES

1. Laver, W. G., and E. D. Kilbourne. 1966. Identification in a recombinant influenza virus of the structural proteins derived from both parents. *Virology.* 30:493–501.
2. Wilson, I. A., J. J. Skehel, and D. C. Wiley. 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. *Nature (Lond.).* 289:366–373.
3. Schulze, I. T. 1972. The structure of the influenza virus II. A model based on the morphology, and composition of subviral particles. *Virology.* 47:181–196.
4. Verhoeyen, M., R. Fang, W. M. Jou, R. Devos, D. Huylebroeck, E. Saman, and W. Fiers. 1980. Antigenic drift between the haemagglutinin of the Hong Kong influenza strains A/Aichi/2/68 and A/Victoria/3/75. *Nature (Lond.).* 286:771–776.
5. Dopheide, T. A., and C. W. Ward. 1980. Structural studies on the Hong Kong influenza hemagglutinin. The structure of the light chain and the arrangement of the disulfide bonds. *In* Structure and Variation in Influenza Virus. W. G. Laver and G. M. Air, editors. Elsevier Verlag, Amsterdam. 21–26.
6. Bayley, H. A. 1982. Photoactivated hydrophobic reagents for integral membrane proteins. *In* Membranes and Transport. A. N. Martonosi, editor. Plenum Publishing Corp., New York. 1:185–194.
7. Bramhall, J. S., M. A. Shifflett, and B. J. Wisnieski. 1979. Mapping the membrane proteins of Newcastle disease virus with a photoreactive glycolipid probe. *Biochem. J.* 177:765–768.
8. Bramhall, J., B. Ishida, and B. Wisnieski. 1978. Photolabile and paramagnetic reagents for the investigation of transmembrane signalling events. *J. Supramol. Struct.* 9:399–406.
9. Hu, V. W., A. F. Esser, E. R. Podack, and B. J. Wisnieski. 1981. The membrane attack mechanism of complement: photolabeling reveals insertion of terminal proteins into target membrane. *J. Immunol.* 127:380–386.
10. Hu, V. W., and B. J. Wisnieski. 1979. Photoreactive labeling of M13 coat protein in model membranes by the use of a glycolipid probe. *Proc. Natl. Acad. Sci. USA.* 76:5460–5464.
11. Maeda, T., A. Asano, K. Ohki, Y. Okada, and S. Ohnishi. 1975. A spin-label study on fusion of red blood cells induced by hemagglutinating virus of Japan. *Biochemistry.* 14:3736–3741.
12. Sefton, B. M., G. G. Wickus, and B. W. Burge. 1973. Enzymatic iodination of *Sinbis* virus proteins. *J. Virol.* 11:730–735.
13. Brand, C. M., and J. J. Skehel. 1972. Crystalline antigen of

influenza virus envelope. *Nature New Biol.* (*Lond.*). 238:145–147.

14. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (*Lond.*). 227:680–685.
15. Chamberlain, J. P. 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. *Anal. Biochem.* 98:132–135.
16. Wisnieski, B. J., and J. S. Bramhall. 1981. Photolabeling of cholera toxin during membrane penetration reveals kinetics of specific subunit entry. *Nature* (*Lond.*). 289:319–321.
17. Bucher, D. J., L. G. Kharitonenkow, J. A. Zakomiridin, V. B. Grigoriev, S. M. Klimenko, and J. F. Davies. 1980. Incorporation of influenza M protein into liposomes. *J. Virol.* 36:586–590.
18. Schulze, I. T. 1970. The structure of influenza virus I. The polypeptides of the virion. *Virology.* 42:890–904.
19. Bachi, T., W. Gerhard, J. Lindenmann, and K. Muhlenthaler. 1969. Morphogenesis of influenza A virus in Ehrlich ascites tumor cells as revealed by thin sectioning and freeze etching. *J. Virol.* 4:769–776.
20. Compans, R. W., K. V. Holmes, S. Dales, and P. W. Choppin. 1966. An electron microscopic study of single cycle infection of chick embryo fibroblasts by influenza virus. *Virology.* 30:411–426.
21. Yoshida, T., Y. Nagai, S. Yoshii, K. Maeno, T. Matsumoto, and M. Hoshino. 1976. Membrane (M) protein of HVL (Sendai virus): its role in virus assembly. *Virology.* 71:143–161.
22. Caldwell, S. E., and D. S. Lyles. 1981. Interaction off Sendai virus proteins with the cytoplasmic surface of the erythrocyte membrane following viral envelope fusion. *J. Biol. Chem.* 256:4838–4842.
23. Goldman, D. W., J. S. Pober, H. Bayley, and J. White. 1979. Selective labeling of the hydrophobic segments of intrinsic membrane proteins with a lipophilic photogenerated carbene. *Nature* (*Lond.*). 280:841–843.
24. Gregoriades, A., and B. Frangione. 1981. Insertion of influenza virus M protein into the viral lipid bilayer, and localization of the site of insertion. *J. Virol.* 40:323–328.

USE OF POLYCLONAL ANTIBODIES TO DETERMINE WHICH PEPTIDES OF A TRANSMEMBRANE COMPLEX HAVE AQUEOUS EXPOSURES

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The proteins that catalyze photosynthetic electron transport in the thylakoid membranes of chloroplasts exist as transmembrane protein complexes. One of these protein complexes is photosystem II (PS II). PS II can be isolated from thylakoids by detergent extraction such that its O_2-evolving activity remains (1), but little is known about the protein topography of this complex. In this report we describe the isolation of antibodies specific for antigenic sites on both the lumen and stroma sides of the thylakoid membranes. These two groups of antibodies are then used to identify those proteins exposed on the stroma and the lumen sides of the membrane.

MATERIAL AND METHODS

PS II complexes were isolated, purified, and injected into rabbits as described elsewhere (2). The polyclonal antisera were challenged with either right-side–out (RSO) or inside-out (ISO) thylakoid preparations. RSO thylakoids were prepared by washing control thylakoids in 200 mM sucrose containing 1 mM EDTA (pH 7.5) at 20°C three times. ISO thylakoids were prepared and assayed as described in (3). One ml aliquots of antiserum previously dialyzed against 200 mM sucrose containing 10 mM TES (pH 7.5) and 10 mM KCl were challenged with either RSO or ISO thylakoids equivalent to 30 mg chlorophyll. Nonspecifically bound antibodies were removed by washing, and specifically bound antibodies were removed by suspending the antibody-precipitated thylakoids in 100 mM glycine/HCl (pH 2.8) containing 200 mM sucrose. Membranes were removed by centrifugation and the supernatants were adjusted to pH 7.5 with 1 M Tris base (pH 9.2). These solutions contained antibodies specific for determinants exposed on either the stroma or lumen side of the thylakoid membrane. After electrophoretic transfer to nitrocellulose paper (NCP), LDS-PAGE–separated proteins with determinants specific for the stroma side or lumen side antibodies were identified by Western Blotting as described in (4).

RESULTS AND DISCUSSION

Comparison of lanes 1 and 8 of Fig. 1 shows that we have antibodies to virtually every PS II peptide. Peptides with M_r of 59, 57, 44, 40, 33, 32, 29, 28, 27, 26, 25, 23, 18, 17, and 12×10^3 d are visible. Lanes 3 and 4 show the proteins that have been labeled by antibodies that bound to the stroma surface of the RSO thylakoids. Major bands include those at 59, 28, 27, and 26×10^3 d. Faint bands are seen at 57, 33, 23, and 18 (lane 3) as well as a very faint band at 33×10^3 d (lane 4). Missing from the major bands are the Tris-removable peptides (33, 25, and 18×10^3 d) (5, 6) which reside on the lumen surface of the membrane (7). Lanes 5 and 6 show the proteins that have been labeled by antibodies which bound to the lumen surface of ISO thylakoids. Major bands of lane 5 include those at 59, 32 (diffuse), 28, 27, 26, 23, and 12×10^3 d. The 32 and 12×10^3 d peptides are included because the relative band strengths appear at least as strong as the bands visible with whole serum (lane 1). Faint bands are visible at 57, and 18×10^3 d. Bands that have been identified by stroma side antibodies as well as lumen side antibodies include those at 59, 57, 28, 27, 26, and 23×10^3 d. We conclude that these proteins are transmembranous. The 12 and 32×10^3 d proteins (lane 5) are clearly recognized by antibodies that

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