Binding of α_1-Acid Glycoprotein to Membrane Results in a Unique Structural Change and Ligand Release

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ABSTRACT: α_1-Acid glycoprotein (AGP) consists of 183 amino acid residues and 5 carbohydrate chains and binds to basic and neutral drugs as well as steroid hormones. We investigated the structural properties and ligand-binding capacity of AGP under mild acidic conditions and its interactions with liposomes prepared from neutral or anionic lipids and the neutral drug, progesterone. Interestingly, AGP had a unique structure at pH 4.5, at which the tertiary structure changed, whereas the secondary structure remained intact. Furthermore, the binding capacity of AGP for progesterone did not significantly change under these conditions. It was also observed that AGP was strongly bound to the anionic membrane at pH 4.5, forming an α-helix-rich structure from the original β-sheet-rich structure, which significantly decreased the binding capacity of AGP for progesterone. The structural transitions as well as the membrane binding were suppressed by adding NaCl. These results indicate that AGP has a unique structure on the membrane surface under mild acidic conditions. The conformational change induces binding to the membrane aided by electrostatic interaction, and AGP subsequently takes on a predominantly α-helical conformation.

α_1-Acid glycoprotein (AGP),[1] a member of the lipocalin family, is a polypeptide with two disulfide bonds and five carbohydrate chains, which account for about 40% of its total mass of 36 kDa (*1*). It is a major binding protein for neutral and basic ligands (*2*−*5*). Although the three-dimensional structure and biological functions are still unknown, circular dichroism measurements (*6*) and molecular modeling (*7*) have revealed that this protein has a largely β-sheet structure in aqueous solution.

The hypothesis that membrane transport of a drug depends on the nonbound drug concentration is widely accepted. However, because this hypothesis does not fully explain the uptake mechanism of some AGP-binding drugs, a protein-mediated uptake system has been proposed (*8*−*11*). In such a system, structural changes in the protein due to interaction with the membrane surface decrease the drug-binding capacity. The recent ESR spectroscopic finding that the structure of HSA changes after interaction with the surface of hepatocytes supports this proposed system (*12*). It was recently reported that AGP binds to the vascular endothelial cell surface and then causes transcytosis across the cell without passing the intercellular junction (*13*). Andersen detected AGP on the surface of human monocytes, granulocytes, and lymphocytes using fluorescent electron microscopy (*14*, *15*). Other studies of AGP interacting with vesicles (*16*) and liposomes (*17*) also support the conclusion that AGP interacts with the membrane in circulation and may influence intracellular events. Furthermore, the oligosaccharide moiety of AGP is recognized by cell surface lectins (*18*).

We previously reported that the interaction between AGP and a biomembrane model (reverse micelles) resulted in structural change and a decrease in ligand-binding capacity. Moreover, this interaction resulted in a unique conformational transition: β-sheet to α-helix (*19*). Based on these results, it is important to investigate the structural properties and ligand-binding capacity of AGP under mild acidic conditions because local changes in pH on the biomembrane surface influence these parameters (*20*). Indeed, there are several reports that proteins undergo structural and functional changes under mild acidic conditions on the membrane surface and intracellularly (*21*−*23*), including other lipocalins (*24*).

In the present paper, we examined the relationship between structural properties under mild acidic conditions and the ability of AGP to interact with phospholipid interfaces containing neutral and anionic phospholipids. We demonstrate that AGP undergoes a unique conformational change under mild acidic conditions and that this conformational change promotes an interaction with the membrane. The ligand is then released due to either a change in affinity or closer membrane association. The process is a potentially useful model for studying the pharmacokinetics of both endogenous and exogenous substance binding to AGP and for elucidating further functions of AGP.

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[1] Abbreviations: AGP, α_1-acid glycoprotein; HSA, human serum albumin; PC, L-α-phosphatidylcholine; PG, L-α-phosphatidyl-DL-glycerol; PS, L-α-phosphatidyl-L-serine; PE, L-α-phosphatidyl ethanolamine; CD, circular dichroism; PAI-1, plasminogen activator inhibitor 1.

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MATERIALS AND METHODS

Materials. AGP (Cohn fraction VI), progesterone, L-α-phosphatidylcholine (PC) and ethanolamine (PE) from egg yolks, L-α-phosphatidyl-L-serine (PS) from bovine brain, and L-α-phosphatidyl-DL-glycerol sodium salt (PG) were all from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were of analytical grade.

Liposome Preparation. Liposomes were prepared as described previously (*25*). Briefly, phospholipids were dissolved in chloroform, and the solvent was evaporated under a stream of nitrogen and then under vacuum for at least 1 h. The phospholipid film was dispersed in the following 20 mM buffers: sodium phosphate (pH 6.0−7.4) and sodium acetate (pH 4.5−5.5), each containing 0−150 mM NaCl. Small unilamellar vesicles were prepared by sonication with a probe sonicator to near optical clarity, and residual multilamellar vesicles and titanium particles released from the probe were removed by centrifugation at 14000*g* for 20 min. Vesicles were mixed with AGP in the buffer at a final concentration of 10 mM AGP and 50−600 μM phospholipids (1:5−1:60 molar ratio of protein to phospholipids), and samples were incubated at room temperature for at least 1 h.

Measurement of Circular Dichroism Spectra. Circular dichroism spectra were recorded with a Jasco J-720 spectropolarimeter, using 10 μM AGP in 20 mM buffer (described above) at each pH. UV spectra were recorded in 10 mm and in 1 mm path length cells for near- and far-UV spectra, respectively.

Measurement of Fluorescence Spectra. Fluorescence was measured using a Jasco FP-770 fluorometer (Tokyo). AGP was dissolved at 10 μM in appropriate buffers. For Trp fluorescence, the excitation wavelength was 280 nm, and emission was monitored from 300 to 400 nm. ANS was added to a final concentration of 20 μM (2:1 molar ratio of ANS to AGP), which was enough to form the complex of AGP−ANS as much as possible and prevent nonspecific binding because it was reported that AGP had a high-affinity site for ANS (*25*). Spectra were recorded immediately after mixing. The excitation wavelength was 380 nm, and emission was monitored from 450 to 550 nm.

Membrane-Binding Experiment. Sucrose-loaded large unilamellar vesicles were prepared as previously described (*26*). The buffers (20 mM) used at each pH were as described above. Repeating the experiment with various buffers showed that the results depended on pH and not the buffer used. After incubation for 10 min at room temperature, solutions were centrifuged (20000*g*, 30 min) to separate vesicles and associated protein (pellet) from soluble protein (supernatant). Control experiments at all pH values showed that, in the absence of phospholipid vesicles, no AGP was present in the pellet after centrifugation. Samples were analyzed by the Bradford assay.

Progesterone−AGP-Binding Experiment. Binding of progesterone to AGP was determined by the ultrafiltration method. Progesterone was dissolved as 1 mg/mL in acetonitrile, and the stock solution was diluted with 20 mM sodium phosphate buffer (pH 6.0−7.4) and sodium acetate buffer (pH 4.5−5.5). The final concentration of acetonitrile did not exceed 1%. AGP solution (1 mL) containing progesterone was incubated for 10 min on ice and then centrifuged at 2000*g* for 40 min at 4 °C. After centrifugation, the filtrate (10 μL) was analyzed by HPLC to determine the free progesterone concentration. The HPLC system consisted of a Hitachi 655A-11 pump (Hitachi, Tokyo) and a Hitachi L-4000 UV detector set at 244 nm. The analytical column used was an AM312 ODS column (150 × 6.0 mm i.d., S-5 mm, 120 Å) (YMC, Kyoto) and was maintained at room temperature. The mobile phase was acetonitrile−20 mM sodium phosphate buffer (pH 7.4) (50:50 v/v) at a flow rate of 1 mL/min. AGP and PG concentrations were 10 and 400 μM, respectively. AGP−progesterone-binding experiments in the presence of liposomes had a final progesterone concentration of 10 μM (1:1 molar ratio of progesterone to AGP) after the liposomes had been saturated in progesterone to limit its nonspecific adsorption by the liposomes.

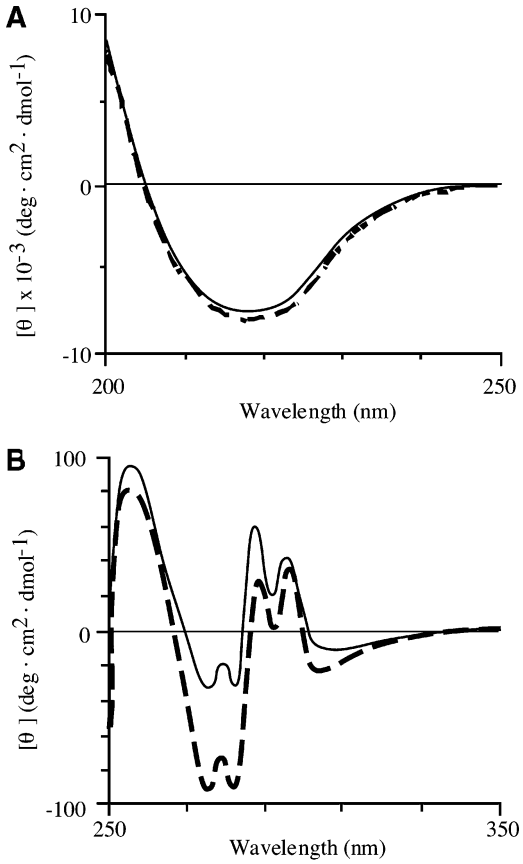

FIGURE 1: Effects of pH on the far-UV (A) and near-UV (B) CD spectra of AGP at pH 7.4 and 4.5, AGP spectra are shown as a continuous line (pH 7.4) and a dashed line (pH 4.5). Circular dichroism spectra were recorded using 10 μM AGP in a solution containing 20 mM buffer: sodium phosphate (pH 7.4) or sodium acetate (pH 4.5).

RESULTS

Effects of pH on the Conformational Structure of AGP. It has been reported that the pH at the membrane surface is lowered due to the membrane potential (*20*). This pH decrease may mediate interactions between proteins and the membrane itself (*24*). We investigated how the tertiary and secondary structures of AGP were affected under mild acidic conditions (pH 4.5−6.5) using circular dichroism spectroscopy (Figure 1). The tertiary structure changed slightly compared with that at pH 7.4, but the secondary structure was unaffected, even at pH 4.5. To obtain more information about the conformational changes in AGP under mild acidic

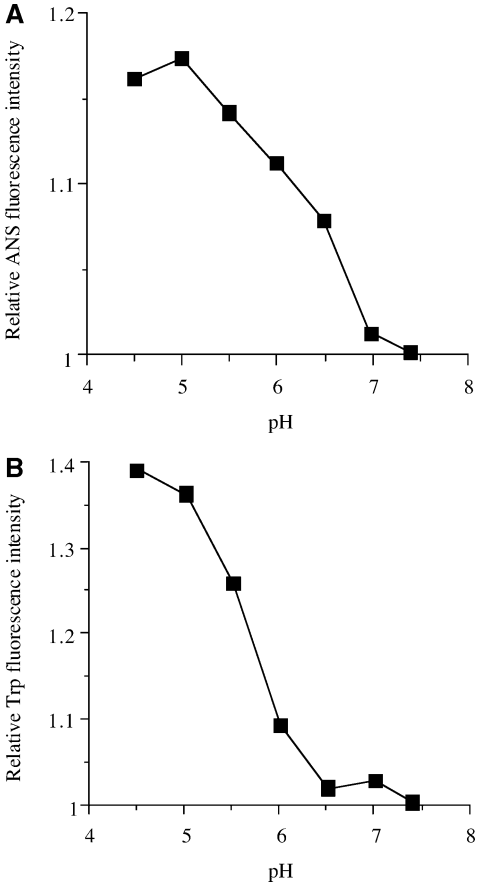

FIGURE 2: Fluorescence spectra of ANS and Trp residues at various pHs. AGP was dissolved at 10 μM in the appropriate buffers, and ANS was added to a final concentration of 20 μM (2:1 molar ratio of ANS to AGP). ANS: excitation wavelength, 380 nm; Trp: excitation wavelength, 280 nm.

conditions, we measured the fluorescence spectra of ANS and Trp residues on AGP at pH 4.5−7.4 (Figure 2). ANS is often used to evaluate hydrophobic regions, which are generally in the protein interior (*25*, *27*, *28*). The fluorescence intensity of ANS and Trp residues increased with lowering of pH. These results indicate that AGP has a unique conformational structure under the mild acidic conditions of the membrane surface, even in the absence of direct interactions between AGP and the membrane.

Effects of pH and NaCl on the AGP−Membrane Binding. The membrane environment has been shown to have a negative charge, in addition to being mildly acidic (*20*). We therefore examined the binding of AGP to PG- and PC-based membranes at each pH (pH 4.5−7.4) (Figure 3A). AGP bound strongly to the PG−membrane with lower pH, whereas significant binding was not observed with the PC−membrane at any pH. To confirm the presence of electrostatic forces in this interaction, the effects of NaCl on this binding were examined with the PG−membrane at pH 4.5 (Figure 3B). NaCl suppressed binding in a concentration-dependent manner (0 mM, 96.0 ± 0.58%; 75 mM, 37.5 ± 2.64%; and 150 mM, 10.0 ± 16.3%). The finding that other cations, Ca^{2+} and K^{+}, also inhibited the interaction between AGP and membrane suggests the presence of electrostatic force (data not shown). The above interaction was also observed even in physiological buffer, but it was small, as expected from the NaCl effect. To test the validity of this model and

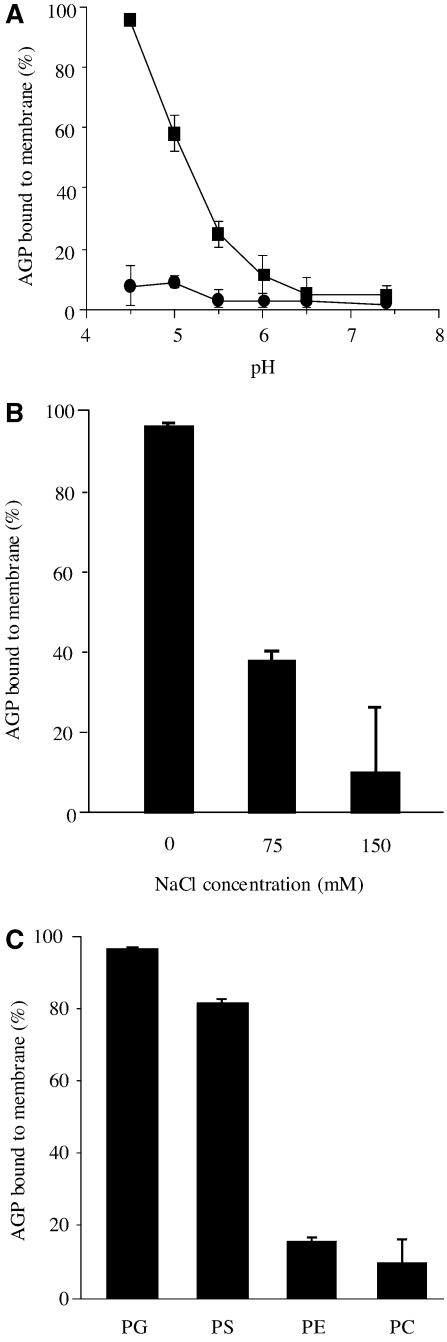

FIGURE 3: Interaction mode of AGP−membrane binding. (A) pH dependence of AGP binding to the membrane [PC (●), PG (■)]. (B) Effect of NaCl on the binding of AGP to the membrane. (C) Binding of AGP to the membrane made from other phospholipids. Experiments were performed using 10 μM AGP in solutions containing 20 mM buffers as described in Materials and Methods. Phospholipid vesicles were prepared at 400 μM.

experiment, the binding experiment was repeated using other phospholipids: PS and PE (Figure 3C). PG, PS, and PE (the degree of the negative charge: PG > PS > PE) are anionic lipids, PC is neutral, and the pattern of interaction of AGP with the lipids also supported the existence of an electrostatic interaction between AGP and the membrane. Moreover, at each pH, binding of AGP to PG−membrane had a significant correlation with the fluorescence intensity of Trp residues ($r = 0.9901$, $p < 0.01$) (Figure 4) but not ANS (data not shown). These results strongly suggest that the slight changes in AGP conformation observed under mild acidic conditions

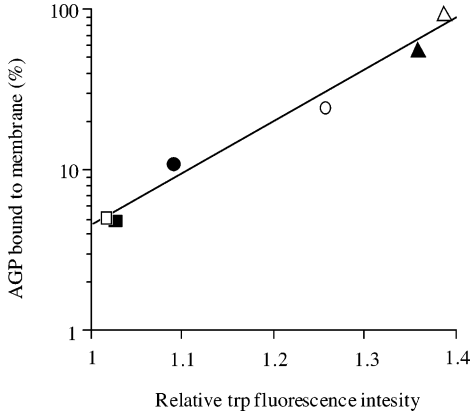

FIGURE 4: Correlation between AGP−membrane interaction and Trp residues of the AGP environment under mild acidic conditions. Each symbol represents the following pH: (■) 7.0, (□) 6.5, (●) 6.0, (○) 5.5, (▲) 5.0, and (△) 4.5.

lead to the binding of AGP to the membrane. This interaction may involve an electrostatic component.

Effects of pH and NaCl on the Conformation of AGP in Membrane Interactions. To evaluate the structural properties of AGP in membrane interactions, we examined the effects of pH and NaCl on the conformation of AGP in the presence of a PG−membrane (Figure 5). Panels B−D of Figure 5 show a $-[\theta]$ value of 222 nm as an index of the α-helix content. The secondary structure of AGP shifted from being β-sheet-rich to an α-helix-rich structure at lower pH (Figure 5A,B). In addition, the degree of this conformational transition depended on the PG concentration (Figure 5C) and was inhibited by higher NaCl concentrations (Figure 5D). These results indicated that this conformational transition was initiated following, or during, binding of AGP to the membrane.

Effects of Ligand-Binding Capacity of AGP in Membrane Interaction. It is important to understand how the ligand-binding capacity of AGP changes when the structural transition (β-sheet to α-helix) occurs in the presence of the PG−membrane. We used a representative AGP-binding ligand, progesterone, because it is uncharged and therefore unaffected by pH. The binding of progesterone to AGP had a good correlation with the α-helix content of AGP interacting with the PG−membrane at pH 4.5−7.4 ($r = 0.9545$, $p < 0.01$) (Figure 6). No changes in binding capacity were observed for pH 4.5−7.4 in the absence of PG−membrane. These results show that the binding of progesterone to AGP was strongly affected by its interaction with the PG−membrane but was not affected by mild acidic conditions.

DISCUSSION

The hypothesis that uptake of a drug depends on the free drug concentration (not bound by protein) is widely accepted. However, a pharmacokinetic study using albumin- and AGP-binding ligands found that uptake is more efficient than predicted by this model (*8*). One explanation for this phenomenon is that structural changes in the carrier protein may be induced by interaction with the target cell surface. Consequently, the ligand is released concomitantly with conformational changes in the protein (*8*−*11*). This idea is supported by ESR spectroscopic findings showing that albumin undergoes structural changes when interacting with hepatocytes (*12*). It should also be noted that AGP binds to

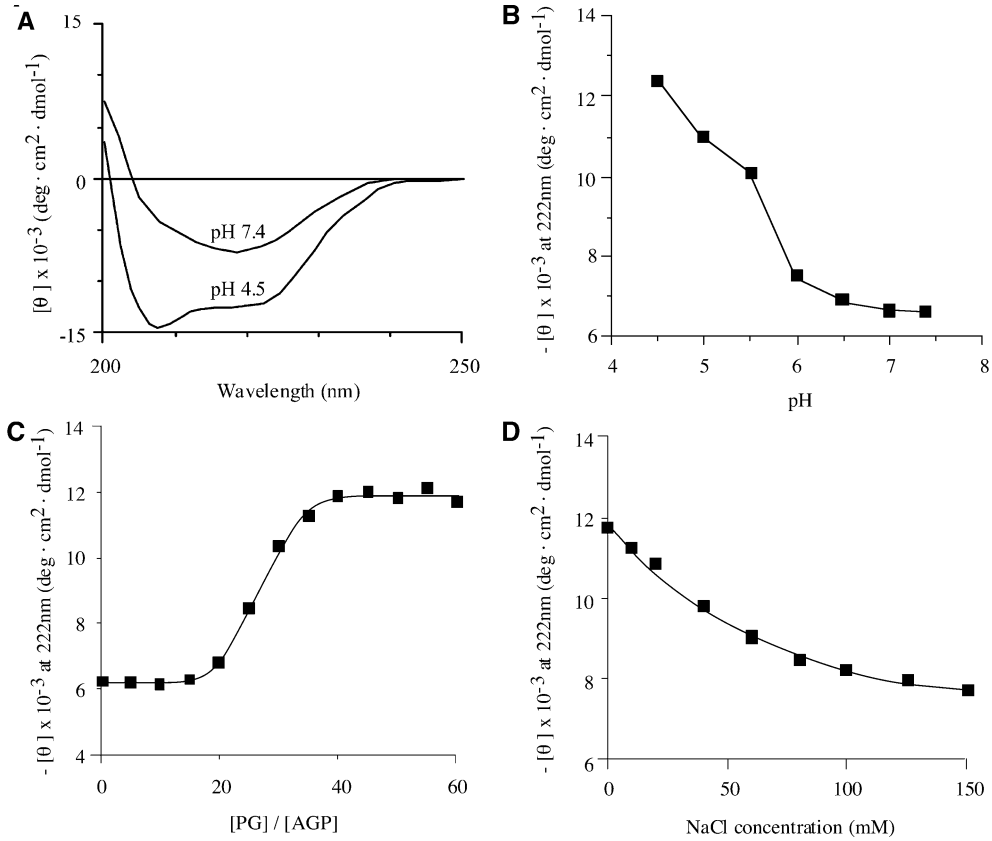

FIGURE 5: Effect of pH (A, B), PG concentration (C), and NaCl (D) on the conformational transition of AGP interacting with the membrane. Experiments were performed using 10 μM AGP in solution containing 20 mM buffer as described in Materials and Methods. The pH was 4.5 or 7.4 (A), was varied from 4.5 to 7.4 (B), or was 4.5 (C, D). Phospholipid vesicles were prepared at 400 μM (A, B, and D).

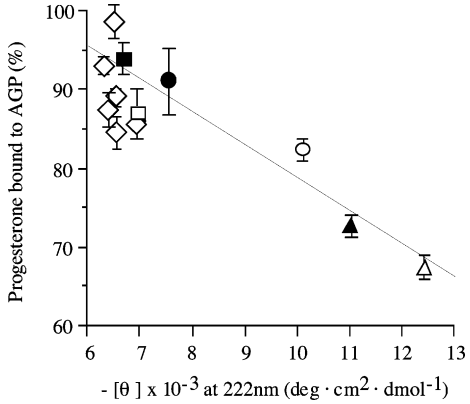

FIGURE 6: Binding of progesterone to AGP interacting with the membrane. AGP and PG concentrations were set at 10 and 400 μM, respectively. AGP−progesterone-binding experiments in the presence of liposomes were performed at a final progesterone concentration of 10 μM (1:1 molar ratio of progesterone to AGP) after liposomes were saturated in progesterone. The experiment was performed in a solution containing 20 mM appropriate buffer. Each symbol represents the following pH: (■) 7.0, (□) 6.5, (●) 6.0, (○) 5.5, (▲) 5.0, (△) 4.5, and (◇) 7.0−4.5 in the absence of PG−membrane.

vascular endothelial cell surfaces (*13*). pH-linked conformational transitions frequently reflect interactions of proteins with membranes. This is due to the fact that the membrane potential decreases the local pH as compared with the bulk of the solution (*29*). The difference in pH has been experimentally determined to be 1.6 pH units, and the calculated value reaches 2.7 pH units (*20*). We observed that the tertiary structure of AGP changed slightly but the secondary structure was conserved under acidic conditions. At pH 4.5, the ligand-binding capacity of AGP was almost equal to that at pH 7.4. These results show that the structure of the AGP-binding area for progesterone is retained, even at pH 4.5.

An increasing number of proteins, including transferrin (*21*), influenza hemagglutinin (*22*), and the lipocalins retinol-binding protein (*23*) and tear lipocalin (*24*), undergo pH-linked structural changes. Such conformational transitions may be physiologically significant, because they may mediate ligand release or membrane fusion. In the case of transferrin, translocation across membranes from the neutral pH of the bloodstream to the acidic intracellular environment leads to opening of the protein structure and the release of iron, apparently with active participation of the receptor (*21*), and tear lipocalin is postulated to deliver lipids at the tear−film interface by a pH-linked structural change (*24*). The unique structural changes in AGP, which were observed in this experiment, may be the first event in membrane interaction.

We examined the structural properties of AGP and its ligand-binding capacity in the membrane−water phase using liposomes, which are frequently used as a biomembrane model. It was observed that the α-helix content of AGP tended to increase in the presence of a PG−membrane under mild acidic conditions. This result supports the idea that the unique structural changes in AGP under mild acidic conditions induce interaction with the membrane. The results obtained from the ANS and Trp fluorescence experiment suggest that the interaction of AGP with the membrane under mild acidic conditions may be promoted by a slight change of conformation of AGP but not a greater exposure of the

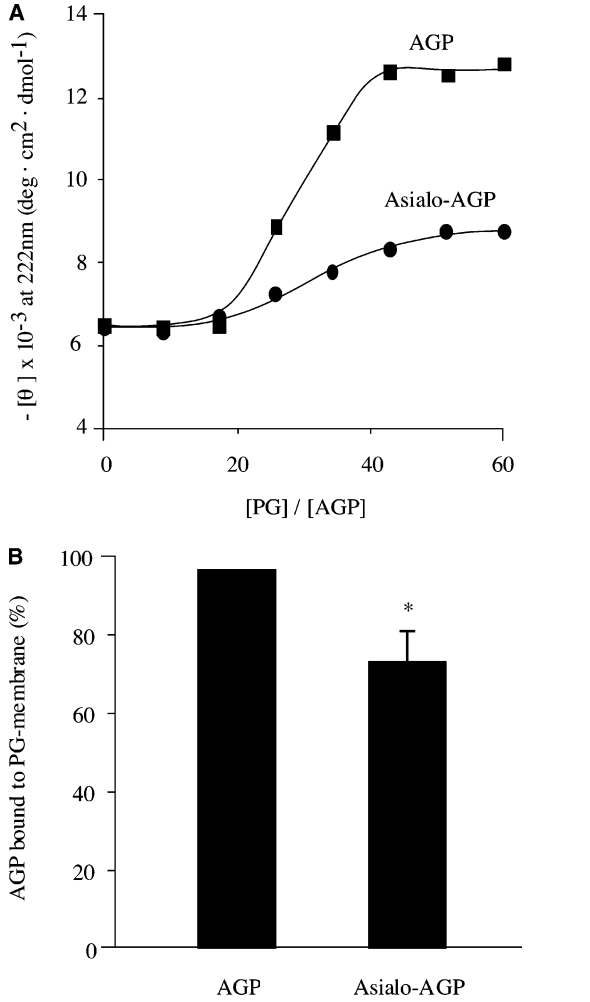

FIGURE 7: Effect of sialic acid on the interaction between AGP and PG−membrane. The degree of conformational transition of AGP (■) and asialo-AGP (●) interacting with PG−membrane (A) and the binding of AGP and asialo-AGP to PG−membrane (B) were monitored as in Figures 3 and 5.

hydrophobic side chains. When we examined the interactions of asialo-AGP with the anionic PG−membrane, the membrane-binding capacity and extent of the conformational transition both decreased at pH 4.5 (Figure 7). The finding that the conformational transition was decreased more than membrane binding may be due to an increase in the conformational stability of asialo-AGP. In accordance with this proposal, Friedman et al. have reported, by using fluorescence spectroscopy, that sequential removal of the sialic acids from AGP had no significant impact of the conformation of the protein core. However, the removal of sialic acids resulted in an increased stability of the core probably caused by interaction between the core and the asialoglycan chains (30). Aubert and Loucheux-Lefebvre also found by additive circular dichroism that the glycan chains do not perturb the protein core conformation (*6*). Villalobos et al. reported that the distribution of glycans on the AGP surface is asymmetric as found with monoclonal antibody probes (*31*), and Rojo-Dominguez and Hernandez-Arana and Schlueter predicted by molecular modeling that most of the glycans were distal to the proposed ligand-binding site (*7*, *32*). Together, these results strongly suggest that the influence of the terminal glycan sialic acid residues in the present work may be to orient the AGP at the PG−membrane surface and

present the relatively unprotected ligand-binding site to the membrane. Thus, it is likely that the initial conformational events that we observe in AGP involve residues that are present or closely coupled to those in the surface presented to the membrane.

In numerous proteins, insertion into the membrane seems to induce transition to an α-helix-rich structure (*33*−*38*). In the case of AGP, the ligand-binding area of AGP may involve regions that form an α-helix-rich structure and/or are inserted into the membrane since the λ_{max} of Trp fluorescence was blue shifted due to interaction with the PG−membrane (pH 4.5, 340 nm; pH 4.5 + PG−membrane, 335.5 nm). Our preliminary experiment using the Trp mutants (W25A, W122A, and W160A) showed that Trp25 and Trp160 were inserted into the membrane.

Structural transitions similar to those found in AGP (α to *β* or *β* to α) have been observed in other proteins including *β*-lactoglobulin and plasminogen activator inhibitor (PAI-1) (*38*−*40*). For example, *β*-lactoglobulin readily changes to an α-helical structure from a *β*-sheet one, when exposed to more hydrophobic alcohol (e.g., trifluoroethanol) (*39*). We obtained similar results for AGP, containing for a series of alcohols an α-helix induction effect in AGP of propanol > ethanol > methanol (data not shown). These results suggest that hydrophobic interactions contribute to the formation of an α-helix on AGP observed in the presence of the membrane. The folding intermediate of PAI-1 (α-helix structure) is reportedly a functional state, while the native state is inert (*40*−*42*). Furthermore, a conformational transition from α-helix to *β*-sheet seems to induce propagation of prion proteins, resulting in prion diseases, such as bovine spongiform encephalopathy (*43*). It has also been reported that slight structural change of prion due to interaction with membrane might induce such a conformational transition (α-helix to *β*-sheet) (*44*).

The biological functions of AGP are currently unknown, although numerous activities have been described of which the most common is binding ligands, particularly therapeutic drugs. For example, AGP binds thalidomide and thereby affects the drug's immunomodulatory activity against tumor necrosis factor α (*45*). AGP is reportedly involved in intracellular events, such as controlling thrombocytic agglutinability, controlling bacterial englobement, extension during engrafting, and inhibiting lymphocyte growth through various mechanisms (*2*, *5*, *46*). AGP has been detected on the surface of normal human lymphocytes, granulocytes, and monocytes by fluorescent electron microscopy (*13*, *14*).

The limited data obtained here suggest that interaction between AGP and biomembranes may be important for other aspects of the physiological functions of this protein in addition to the observed changes in ligand-binding capacity.

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