Proc. Natl. Acad. Sci. USA
Vol. 77, No. 9, pp. 5087–5091, September 1980
Biochemistry

Reconstitution of the hepatic asialoglycoprotein receptor with phospholipid vesicles

(liposomes/circular dichroism/tryptophan fluorescence quenching)

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ABSTRACT A solubilized detergent-free preparation of the hepatic binding protein specific for asialoglycoproteins associates spontaneously with small unilamellar lipid vesicles. This process is independent of the phase transition of the lipid and effectively restores the specific binding activity of the receptor protein. The insensitivity of the resulting lipid–protein complex to ionic strength provides evidence for a hydrophobic interaction. There is a perturbation of the lipid phase transition concomitant with addition of the protein. Circular dichroism studies indicate that the protein undergoes a conformational change on association with lipid. Binding of specific ligand produces further physical changes in the receptor as indicated by alterations in the tryptophan fluorescence quenching pattern.

The study of membrane receptors is a burgeoning area of cell biology, and yet knowledge of conformation and conformational changes in these receptors is scant. With a purified receptor preparation, the molecular details of the structural consequences of ligand-binding and structure–function correlations could be examined. If such a preparation were free from detergent and lipid, the possibility of lipid-induced changes in receptor conformation could be explored. We have begun a series of biophysical studies using the purified asialoglycoprotein receptor of rabbit hepatocytes, which mediates the internalization of desialylated serum glycoproteins. This integral membrane receptor is uniquely suited to answer the questions posed above as a consequence of its availability in homogeneous form, in milligram amounts, and in an aqueous solution free from detectable amounts of lipid or detergent. Data are presented here to document the spontaneous insertion of this receptor into the lipid bilayer of unilamellar vesicles with concomitant physical changes associated with restoration of functional binding activity.

MATERIALS AND METHODS

Proteins and Lipids. The rabbit hepatic asialoglycoprotein receptor was isolated and purified as described (1). Dipalmitoyl phosphatidylcholine was obtained from Avanti Biochemicals. To make sonicated vesicles, the lipid in benzene was dried under argon and lyophilized overnight. It was rehydrated at 50°C for 15 min in buffer containing 139 mM NaCl, 6 mM KCl, 5 mM $CaCl_2$, and 10 mM Hepes, pH 7.4. For studies that included EDTA or ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), $CaCl_2$ was omitted from the buffer. Lipid, 25 mg in 5 ml of buffer, was sonicated with a probe sonicator (Heat Systems Ultrasonic, Plainview, NY) at 75–100 W for 1 hr and passed over a Sepharose 4B column, and the retained small unilamellar vesicles were collected. The result was a population of uniform vesicles 200–300 Å in diameter, each containing about 5000 lipid molecules. In some cases, ^{14}C-labeled dipalmitoyl phosphatidylcholine was used in the preparation of vesicles, which had a final specific activity of approximately 12,000 cpm per μmol of lipid.

Orosomucoid was treated with neuraminidase purified from *Streptococcus pneumoniae* to yield asialo-orosomucoid, which was in turn treated with β-galactosidase from the same source to yield agalacto-orosomucoid (2). Both proteins were labeled with ^{125}I by using the chloramine-T procedure (2); final specific activities ranged from 3000 to 6000 cpm per ng of protein.

Spectroscopy. All fluorescence measurements were made with an Aminco-Bowman spectrofluorometer (Aminco, Silver Spring, MD) with 2-mm slits. Tryptophan fluorescence was obtained by excitation at 290 nm. Potassium iodide quenching studies were performed by dilution of a 2 M stock solution into the cuvette with stirring. Sodium bisulfite was added to a final concentration of 10 μM to absorb any I_3^- formed. Diphenylhexatriene (Aldrich) was dissolved in tetrahydrofuran and diluted 1:1000 into the vesicle suspension to give a lipid-to-probe molar ratio of 800:1. Polarization was obtained with Glan polarizers with excitation wavelength = 376 nm and emission wavelength = 431 nm. Measurements were corrected for scatter and for transmission optics. Circular dichroism was determined by using a Cary model 60 spectrophotometer with a light path of 1 cm. Mean residue ellipticity (θ_m) was calculated as follows:

$$\theta_m = \frac{\text{ellipticity (millidegrees)} \times \text{mean residue weight (mg/mmol)}}{\text{path length (cm)} \times \text{concentration (mg/ml)} \times 10}.$$

The fractional contents of α-helical, β-, and random structure were determined by a computer curve-fitting procedure, using the reference curves of Chen *et al.* (3). The best fit was determined from the root mean square error (4).

Isolation of the Vesicle–Receptor Complex. Potassium bromide density gradients (densities of 1.01–1.25 g/ml) were run at 4°C for 20 hr at 105,000 × *g* in a swinging-bucket rotor. Molecular sieve chromatography on Sephacryl S-200 was used to separate free ligand from ligand bound to reconstituted vesicle–receptor complexes as described in *Results*.

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RESULTS

Receptor Association with Unilamellar Vesicles. In order to determine whether the solubilized receptor would associate spontaneously with the phospholipid bilayers, it was mixed with ^{14}C-labeled vesicles. As shown in Fig. 1, vesicles alone were insufficiently dense to enter the potassium bromide gradient. Upon addition of the binding protein, at a protein-to-lipid molar ratio of 1:130, all of the vesicle lipid was recovered near the bottom of the gradient, a finding indicative of an association stable both to the centrifugal field and to the high ionic strength of the gradient.

A clearer demonstration of the stable association of the receptor protein with the lipid vesicles was achieved by modifying the protein-to-lipid ratio to obtain a complex of intermediate buoyant density. The complex formed at a protein-to-lipid molar ratio of 1:520 enters only partially into the gradient and is readily separable from the empty vesicles and the protein-enriched complex. The protein–lipid association was unaffected by the presence of 2 M NaCl and remained invariant when the receptor was mixed with the vesicles at, above, or below the lipid phase transition temperature. Similarly, the association took place equally well in the presence or absence of 5 mM EDTA.

Effect of Receptor on Vesicle Structure. To determine whether insertion of the receptor disrupted the lipid bilayer, vesicles were prepared with 100 mM carboxyfluorescein entrapped within the internal space (5). The addition of 10–20 molecules of receptor per vesicle failed to cause leakage of the dye even at the lipid phase transition temperature. Leakage was determined by an increased fluorescence due to loss of self-quenching, which occurs when entrapped dye escapes from the vesicles. Further evidence attesting to the vesicular nature of the complex was obtained by isolation of the complexes from potassium bromide density gradients or after chromatography on Sephacryl S-200. In both cases, the recovered vesicles contained entrapped dye that was readily liberated by the addition of Triton X-100 (0.1%) and monitored by measurement of fluorescence (5).

Depolarization of diphenylhexatriene fluorescence was used to ascertain the effect of protein addition on the lipid phase transition of the vesicles. As shown in Fig. 2, association of the receptor with the vesicles effectively lowered the transition

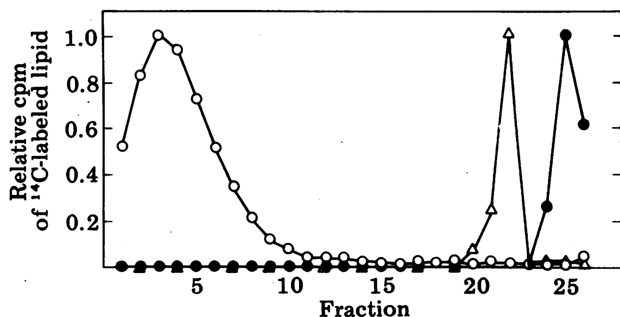

FIG. 1. KBr density gradient centrifugation of ^{14}C-labeled vesicles and ^{14}C-labeled vesicle–receptor complex. A 100-μl sample of ^{14}C-labeled vesicles (2.5 mM lipid) in 139 mM NaCl/6 mM KCl/5 mM $CaCl_2$/10 mM Hepes, pH 7.4, was layered on a 5-ml KBr gradient, 1.01–1.25 g/ml, in 1/2-inch × 2-inch tubes (1 inch = 2.54 cm) and centrifuged at 105,000 × *g* for 24 hr (●). Fractions were 0.2 ml. ^{14}C-Labeled vesicle–receptor complexes of different densities were prepared by combining either 5 μl of receptor preparation (3.3 mg/ml) and 20 μl of ^{14}C-labeled vesicles (protein-to-lipid molar ratio = 1:130) (○) or 10 μl of receptor preparation and 160 μl of ^{14}C-labeled vesicles (protein-to-lipid molar ratio = 1:520) (△). The samples were incubated at room temperature for 15 min prior to application to a KBr gradient and centrifugation, as above.

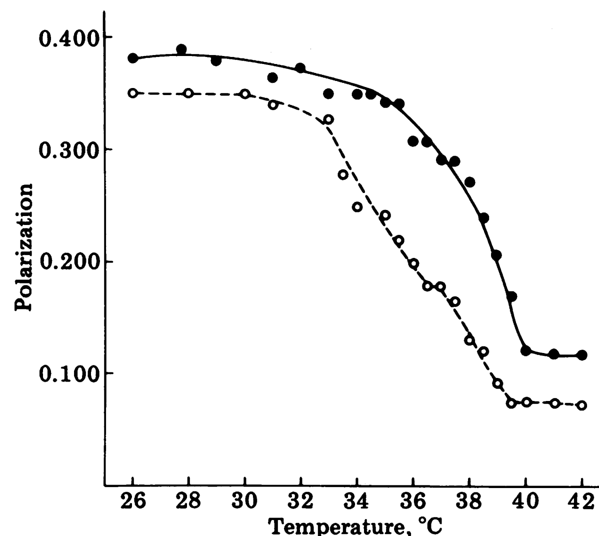

FIG. 2. Fluorescence polarization of 0.5 μM diphenylhexatriene in vesicles in the presence (○) or absence (●) of receptor. The receptor–vesicle complex was prepared as in Fig. 1 with a protein-to-lipid molar ratio of 1:500. The temperature was controlled with a circulating water bath and monitored constantly in the cuvette with a probe (Bailey, Saddle Brook, NJ). The temperature was held at each point for about 2 min.

temperature in accordance with the hypothesis that the protein inserts in a manner that disturbs the orderly packing of the lipid molecules. This observation is in good agreement with the density gradient data, which also suggest a strong lipid–protein interaction.

Ligand Binding to Receptor–Vesicle Complex. The isolated detergent-solubilized hepatic binding protein (receptor) binds to asialoglycoproteins by recognition of the terminal nonreducing galactose or *N*-acetylgalactosamine residues of their carbohydrate chains. However, at detergent levels below the critical micellar concentration, no significant binding occurs. To determine whether ligand-specific binding was restored when the solubilized detergent-free receptor associated with vesicles, studies employing Sephacryl S-200 were undertaken.

After a brief incubation of lipid vesicles with ^{125}I-labeled asialo-orosomucoid, the mixture was applied to a column of Sephacryl S-200. A single radioactive peak was recovered in fractions 30–35 (Fig. 3*A*); it cochromatographed with free asialo-orosomucoid and was clearly resolved from the vesicles that emerged close to the void volume of the column, as determined in earlier trials utilizing ^{14}C-labeled lipid vesicles. The inability of the naked vesicles to bind ligand was contrasted by the binding capacity of the receptor–vesicle complex, as shown in Fig. 3*A*. In this case, the radioactive ligand comigrated with the protein–lipid vesicles and was recovered in fractions 20–25.

In order to test the specificity of binding, the terminal galactose residues of asialo-orosomucoid were removed by enzymatic hydrolysis with β-galactosidase. The resulting product, agalacto-orosomucoid, was iodinated and incubated as above. In accordance with the known specificity of the binding protein (6), the receptor–vesicle complex failed to bind the modified ligand and all of the radioactivity appeared in the included peak (Fig. 3*B*). Similarly, use was made of the monosaccharide ligand *N*-acetylgalactosamine, which is a competitive inhibitor of binding. Inclusion of this material in the assay system completely blocked the binding of ^{125}I-labeled asialo-orosomucoid to the vesicles as seen by the lack of radioactivity emerging at, or near, the void volume of the column (Fig. 3*B*).

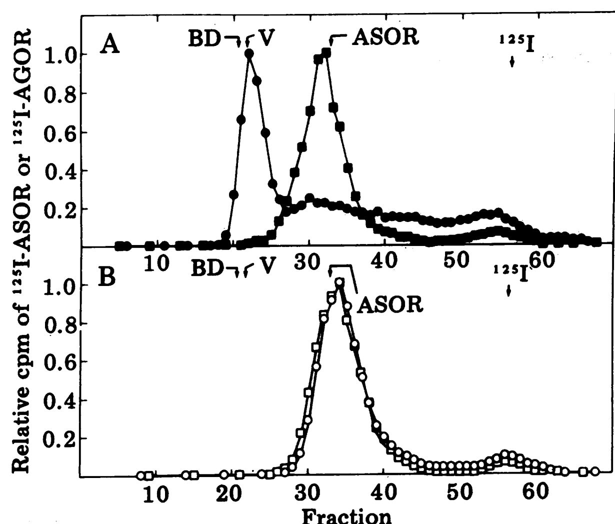

FIG. 3. Ligand binding of receptor–vesicle complex. Arrows indicate the elution points from the 0.6 × 15 cm Sephacryl S-200 column of blue dextran (BD), ^{14}C-labeled vesicles (V), ^{125}I-labeled asialo-orosomucoid (ASOR), and $^{125}I^-$ as determined in preliminary experiments. All studies were conducted at room temperature in buffer containing 10 mM Tris·HCl at pH 7.8, 400 mM KCl, 5 mM $CaCl_2$, and 1% bovine serum albumin. (*A*) ^{125}I-Labeled asialo-orosomucoid (20 μl of 10 μg/ml) was added to 60 μl of vesicles (3 mM lipid) and 100 μl of buffer and incubated for 20 min prior to chromatography (■). Next, receptor–vesicle complex was formed by adding 15 μl of receptor preparation (3.3 mg/ml) to 300 μl of ^{14}C-labeled vesicles with a 5-min incubation. This mixture was applied to a 0.6 × 15 cm Sephadex G-25 column and the receptor–vesicle complex eluted in the void volume; excess unincorporated aqueous receptor adhered to the resin. Two hundred microliters of the filtered complex was then incubated with 20 μl of the ^{125}I-labeled asialo-orosomucoid and 100 μl of buffer for 20 min and applied to the above Sephacryl S-200 column with monitoring of ^{125}I (●). (*B*) The receptor–vesicle complex was formed as in *A* and was made 100 mM in *N*-acetylgalactosamine and incubated for 15 min. Twenty microliters of ^{125}I-labeled asialo-orosomucoid and 100 μl of buffer were then added with a 20-min incubation prior to chromatography on the S-200 column (○). The receptor–vesicle complex was incubated with 20 μl of 20 μg/ml ^{125}I-labeled agalacto-orosomucoid (AGOR) and 100 μl of buffer followed by Sephacryl S-200 chromatography (□).

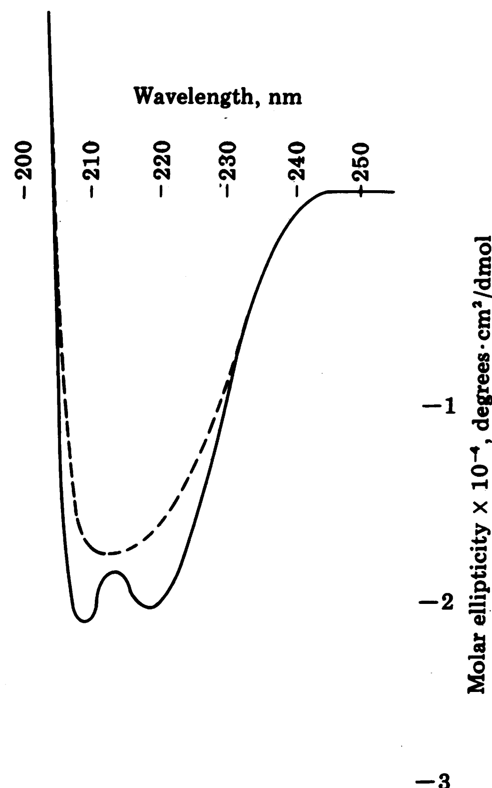

FIG. 4. Circular dichroism of the receptor. Receptor–vesicle complex was prepared as in Fig. 1, with a protein-to-lipid molar ratio of 1:1000. —, Receptor; - - -, receptor–vesicle complex in phosphate-buffered saline. The concentrations of receptor in the samples were identical.

Changes in Circular Dichroism upon Receptor–Vesicle Interaction. The circular dichroism pattern of the isolated receptor in phosphate-buffered saline containing 5 mM $CaCl_2$ is shown in Fig. 4. Under conditions in which vesicles alone showed no ellipticity, even at wavelengths as short as 200 nm, the addition of these vesicles to the soluble receptor produced a marked change in pattern. This observation suggests that the receptor undergoes a conformational change upon association with the lipid. The intrinsic nature of this change is supported by observing identical changes (*i*) over a 10-fold change in lipid-to-protein ratio or protein concentration, (*ii*) as a function of path length, and (*iii*) as a function of the distance between the sample and the photomultiplier. Analysis of the secondary structure of the protein, made from the circular dichroism data and presented in Table 1, revealed a gain in β structure upon membrane insertion. Unfortunately, studies of the effects of ligand binding on the circular dichroism pattern of the receptor were not possible because the protein ligand, asialo-orosomucoid, would itself contribute to the pattern and the monosaccharide ligand, *N*-acetylgalactosamine, exhibited a high absorbance in the ultraviolet range.

Intrinsic Fluorescence Changes upon Receptor–Vesicle Interaction. An alternative aspect of the physical changes resulting from association of the receptor with lipid vesicles was revealed by studies on the accessibility of the intrinsic tryptophan fluorescence to collisional quenching by potassium iodide (7). As shown in Fig. 5*A*, significant quenching of tryptophan fluorescence was observed when potassium iodide was added to the free receptor (curve 1). However, upon association of the receptor with the lipid bilayer of added vesicles, the extent of quenching was markedly diminished as a result of reduced availability to the quenching agent (curve 2).

The diminished quenching observed upon insertion of the receptor into the lipid bilayer of the vesicles was even more markedly pronounced by the addition of the monosaccharide ligand, *N*-acetylgalactosamine, to the receptor–vesicle complex (curve 3 in Fig. 5*A*). The effect of *N*-acetylgalactosamine was concentration dependent and reached apparent saturation at approximately 100 mM ligand. Assuming that saturation of the quenching effect reflects saturation of ligand binding, an affinity binding constant of 10^{-2}–10^{-3} M could be calculated for this monovalent ligand. In the absence of added vesicles, *N*-acetylgalactosamine was without effect on the quenching seen with the free receptor, as might be predicted from the known inability of the solubilized, detergent-free receptor to bind ligand (1).

Table 1. Secondary structure of receptor

Protein	% α-Helix	% β-Structure	% Random structure	RMS*
Free	39	17	44	450
In vesicles	26	29	45	500

Percentages of α-, β-, and random structure from circular dichroism are given. Protein concentration was 20 μg/ml and protein-to-lipid molar ratio was 1:500.

* Root mean square error (degrees·cm^2/dmol).

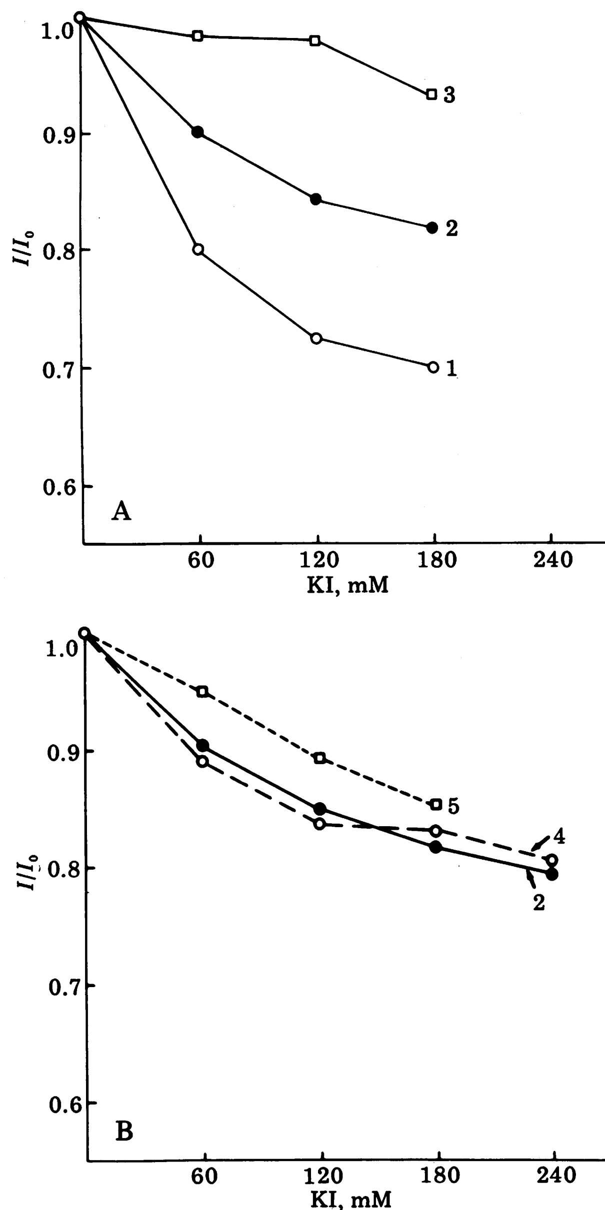

FIG. 5. KI quenching of receptor tryptophan fluorescence. Receptor–vesicle complex was prepared as in Fig. 1, and the KI concentrations shown were obtained by adding 5-μl portions of a 2 M KI solution to the 150-μl experimental solutions. Measurements were made immediately after stirring, but no time dependence of quenching was observed. Excitation wavelength = 290 nm. Fluorescence quenching is expressed as I/I_0, in which I is the fluorescence intensity at a given KI concentration and I_0 is the fluorescence intensity in the absence of KI. *N*-Acetylgalactosamine has no effect on the tryptophan fluorescence of either free or vesicle-bound protein. (*A*) KI quenching of tryptophan fluorescence of free receptor (curve 1), receptor–vesicle complex (protein-to-lipid molar ratio = 1:500) (curve 2), and receptor–vesicle complex plus 100 mM *N*-acetylgalactosamine (curve 3) in phosphate-buffered saline with 5 mM $CaCl_2$. (*B*) KI quenching of tryptophan fluorescence of receptor–vesicle complex (curve 2), complex plus 100 mM *N*-acetylglucosamine (curve 4), and complex plus 100 mM *N*-acetylgalactosamine in phosphate-buffered saline containing 5 mM $MgCl_2$ rather than $CaCl_2$ (curve 5).

Two additional controls were examined in an effort to test the specificity of the quenching reaction. In the first, *N*-acetylglucosamine, a monosaccharide that is structurally similar to *N*-acetylgalactosamine but is not a ligand for the receptor, did not diminish the quenching effect of potassium iodide on the receptor–vesicle complex (Fig. 5*B*, curve 4). In the second, $MgCl_2$ was substituted for $CaCl_2$. Comparison of curves 3 (Fig. 5*A*) and 5 (Fig. 5*B*) reveals that magnesium was unable to replace calcium in facilitating the *N*-acetylgalactosamine-induced reduction of quenching. These results have all been reproduced with the uncharged aqueous quencher acrylamide (results not shown) (8).

DISCUSSION

Currently, little information is available as to how the physical conformation of membrane receptor proteins is influenced by their special environment in the lipid bilayer or on the molecular sequelae accompanying their binding to specific ligands. The present report on the interaction of a purified mammalian binding protein with lipid vesicles represents a direct experimental approach to these questions in a clearly defined model system.

The data reported here provide evidence that the soluble detergent-free asialoglycoprotein binding protein associates spontaneously with lipid vesicles either below or above the lipid phase-transition temperature. That the association is not primarily ionic in nature was indicated by its stability in high salt and by the marked effect on the lipid phase transition revealed by diphenylhexatriene polarization studies (Fig. 2). Both cytochrome *c* and basic myelin protein A-1 produce similar phase-transition changes when exposed to lipid bilayers, and it has been suggested that this is due in part to strong interactions between the protein and the lipid bilayer (9). Significantly, the functional binding of the hepatic receptor, which was undetectable in the absence of detergent, was restored upon insertion into the lipid bilayer of the vesicles. The recovered binding activity retained the original parameters of specificity for galactose and *N*-acetylgalactosamine and an absolute requirement for calcium.

Concomitant conformational changes accompanying the association of protein and vesicles are shown in the circular dichroism studies, which reveal a change in the overall secondary structure, predominantly an increase in β-pleated sheet (Table 1). These conformational changes are strikingly different from those found with free apolipoproteins, wherein lipid association produces a marked gain in secondary structure with a large increase in α-helicity (10, 11). As with all such studies, exact predictions of secondary structure based on computer curve fittings must be made with caution because the original curves were generated from data of soluble proteins in aqueous solution and may not accurately represent the situation in which the protein exists in a lipid environment.

Similarly, the potassium iodide quenching of tryptophan fluorescence is another physical characteristic of the protein that is altered upon association with lipid (Fig. 5). Although no firm correlation between potassium iodide quenching and protein conformation exists, the decreased quenching is most simply interpreted as a burying of tryptophan within either the protein or the lipid bilayer, the result being a decrease in the accessibility to potassium iodide. In conjunction with the other studies, the diminution of tryptophan quenching occurring when the receptor associates with lipid is consistent with the view that the protein penetrates the hydrophobic interior of the bilayer.

It is noteworthy that the observed quenching of the receptor's tryptophans was further decreased by the binding of the monosaccharide ligand, *N*-acetylgalactosamine (Fig. 5). Because the size of the ligand is similar to that of tryptophan, it is unlikely that this effect is due to molecular shielding, as this would require that all exposed tryptophans be located at the binding site. These ligand-induced changes are more likely due to a

deeper penetration of the protein into the bilayer or to receptor aggregation. However, evidence has been obtained that ligand binding can enhance penetration of the receptor into the bilayer of black lipid membranes (12). It can be concluded from the above that ligand binding does change the physical state of the receptor embedded in the membrane. Corroborative studies, utilizing circular dichroism changes, were not feasible because the ligands examined absorb strongly in the region of the spectrum used for such measurements.

The immediate relevance of these studies to an understanding of the sequence of events taking place *in vivo* is not yet clear. However, it would appear reasonable to propose that the ligand-binding-induced changes seen here could serve as the first step of a signal leading to receptor-mediated endocytosis. Such a change would allow the cell to distinguish between occupied and unoccupied receptors, permitting selective endocytosis of the former.

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