Hydroxylamine Treatment Differentially Inactivates Purified Rat Hepatic Asialoglycoprotein Receptors and Distinguishes Two Receptor Populations*

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Fu-Yue Zeng and Paul H. Weigel‡

From the Department of Biochemistry & Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190

We previously showed that two subpopulations of asialoglycoprotein receptors (ASGP-Rs), designated State 1 and State 2 ASGP-Rs, are present in intact cells and that State 2 ASGP-Rs can be inactivated in permeable rat hepatocytes in a temperature- and ATP-dependent manner. These inactivated ASGP-Rs can be quantitatively reactivated by the addition of palmitoyl-CoA and that State 2 ASGP-Rs can be inactivated in permeabilized cells, the State 2 ASGP-Rs can be inactivated in the absence of cytosol when fatty acyl-CoAs were added (9). These studies demonstrated the occurrence of a novel ASGP-R inactivation-reactivation cycle that could regulate receptor activity during endocytosis and receptor recycling (4). In digitonin-permeabilized cells, the State 2 ASGP-Rs can be inactivated in the absence of cytosol in a temperature- and ATP-dependent manner (8). Recent investigations demonstrated that the ATP-inactivated receptor population, which corresponds to the State 2 receptors, was rapidly and quantitatively reactivated in the absence of cytosol when fatty acyl-CoAs were added (9). These studies demonstrated the occurrence of a novel ASGP-R inactivation-reactivation cycle that could regulate receptor activity during endocytosis and receptor recycling (10). Although these above results support the existence of two functionally different populations of ASGP-Rs and the occurrence of an inactivation-reactivation cycle for the State 2 ASGP-R population, the structural basis for the two receptor states and the molecular mechanism(s) responsible for this cycle are unknown.

The molecular mass of the rat ASGP-R is approximately 264 kDa in nonionic detergent with a ligand-binding domain of 105–148 kDa (11, 12). Based on SDS-PAGE (13), the rat ASGP-R contains three subunits (RHL1, 2, and 3) with molecular masses of ~42, 49, and 54 kDa, respectively. These three subunits are the products of two different genes. RHL2 and RHL3 have the same core protein, but differ in the type and extent of post-translational carbohydrate modification (14). The stoichiometry and subunit composition of native rat ASGP-Rs are still unknown, although we have suggested that ASGP-Rs are hetero-hexamers composed of four RHL1 subunits and two subunits of either RHL2 and/or RHL3 (15). That previous studies also demonstrated that the surface and inter-

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‡ To whom correspondence should be sent. Tel.: 405-271-2227; Fax: 405-271-3092; E-mail: paul-weigel@uokhsc.edu.

1 The abbreviations used are: ASGP-R, asialoglycoprotein receptor; ASGP, asialoglycoprotein; ASOR, asialo-orosomucoid; CoA, coenzyme A; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride.
nal ASGP-Rs of the two functionally distinct State 1 and State 2 receptor populations have the same hetero-oligomeric subunit composition (16). The populations of ASGP-Rs may not be attributed to different subunit compositions.

The finding that fatty acyl-CoAs such as palmitoyl-CoA can regulate the activity of one receptor population (State 2 ASGP-Rs) led us to suggest that this receptor population may be fatty acylated in vivo (9, 10). A palmitoylation/depalmitoylation cycle might regulate the ligand binding activity of State 2 ASGP-Rs. A growing number of membrane proteins have been found to be modified by fatty acids. Fatty acids are often covalently attached to proteins through an amide bond to an N-terminal glycine residue or through an ester or thioester bond to internal serine, threonine, or cysteine (17, 18). The thioester linkages of fatty acids such as palmitic acid, are very labile to mild alkaline or NH₂OH treatment. NH₂OH, a chemical usually used to release thioester-linked fatty acids from acylated proteins (17, 18), has been used to study the role of palmitate in the function of rhodopsin and the influenza virus spike glycoprotein (19, 20).

Here we report that ~50% of the activity of affinity-purified ASGP-Rs is lost by treatment with NH₂OH under mild conditions and this NH₂OH-sensitive receptor population corresponds to the State 2 ASGP-Rs. A preliminary report of these results has been presented (21).

**EXPERIMENTAL PROCEDURES**

**Materials**—Human promasocid, CNBr-activated Sepharose 4B, neumaminidase (type X), chloroquine, monensin, Triton X-100, Tween 20, phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin A, soybean trypsin inhibitor, Tris, and DTE were from Sigma. NaCl (10–20 mCi/μg of iodine) was from Amersham Corp. 1,3,4,6-Tetrachloro-3,6-diaphenylglycuroid (TDO-GEN), BSA, and neuraminidase reagents were from Pierce Chemical Co. 125I-ASOR was prepared by deacylation of ASOR with neumaminidase and subsequent purification as described previously (22). Diginon X-100 was from Eastman Kodak Co. Hydroxylamine was from Aldrich (catalog number 25558-0), Sigma (catalog number H-2391), or Fluka (catalog number 55460). Heps was from Research Organics Corp. Collagenase (type D) was from Boehringer Mannheim. Neurulcollase (0.45 μm) was from Schleicher & Schuell. Acrylamide (twice recrystallized) was from U. S. Biochemical Corp. SDS, ammonium persulfate, N,N'-methylenebisacrylamide, and SDS-PAGE molecular weight markers were from Bio-Rad. All other chemicals were reagent grade.

**Buffers and Media**—Medium 1 contains modified Eagle's medium supplemented with 0.22 g of NaHCO₃/liter and 2.4 g of Hepes/liter, pH 7.4. Medium 1/BSA is medium 1 containing 1% (w/v) BSA. Buffer 1 contains 142 mM NaCl, 6.7 mM KCl, 10 mM Heps, pH 7.4. BIC20 is buffer 1 containing 20 mM CaCl₂. Permeabilization buffer is buffer 1 containing 0.2% (w/v) digitonin, 1 mM PMSF, 1 μM leupeptin, and 10 μM soybean trypsin inhibitor. Extraction buffer is buffer 1 containing 1.5% (w/v) Triton X-100, 10 mM CaCl₂, 1 mM PMSF, 1 μM leupeptin, and 10 μM soybean trypsin inhibitor. Elution buffer contains 10 mM sodium acetate, pH 5.0, 150 mM NaCl, and 10 mM EGTA.

**Preparation of Hepatocytes**—Male Sprague-Dawley rats (200–300 g) were from Harlan Breeding Laboratories (Houston, TX) and from SasCo (Oklahoma City, OK). Rat hepatocytes, isolated by a modification (23) of the collagenase perfusion procedure of Seglen (24), were suspended in Medium 1/BSA (2–3 × 10⁷ cells/ml) and incubated at 37°C for 1 h in a gyratory water bath to increase and stabilize the cell surface ASGP-R concentration (25). To inactivate the State 2 ASGP-Rs, in some experiments following equilibration at 37°C, hepatocytes in medium 1/BSA (2 × 10⁷ cells/ml) were incubated at 37°C for 60 min either with 1 mM chloroquine or 50 μM monensin (26). Untreated and treated cells were rapidly chilled on ice and then centrifuged at 37°C for 1 h in a gyratory water bath to increase and stabilize the cell surface ASGP-R concentration (25).

**Purification of Active ASGP-Rs**—All steps were carried out at 4°C, unless otherwise stated. ASOR was coupled to CNBr-activated Sepharose 4B to a density of 2–3 mg of ASOR/ml resin according to the manufacturer’s guidelines. Active ASGP-Rs were purified from isolated rat hepatocytes (to prepare State 1 ASGP-Rs) by a modification of a procedure described previously (27). Briefly, rat hepatocytes, prepared as described above, were chilled with 3 volumes of ice-cold buffer 1, centrifuged, and resuspended at 1 × 10⁷ cells/ml in permeabilization buffer for 20 min. Permeabilized cells were washed twice with 10 mM EGTA in buffer 1 containing 1 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A to remove cytosol and any endogenous ligands. The cells were then resuspended and incubated in extraction buffer (2 × 10⁷ cells/ml) for 1–2 h on a rotator at 4°C. After centrifugation for 10 min, the supernatant was filtered sequentially through 1.2-, 0.8-, 0.45-, and 0.2-μm filters, and incubated with ASOR-Sepharose for at least 2 h on a rotator. The nonspecifically bound components were removed by extensive sequential washes with BIC10 containing 0.5% Triton X-100, and then 0.05% Triton X-100. Active ASGP-Rs were removed with elution buffer (10 mM sodium acetate, pH 5.0, 150 mM NaCl, and 10 mM CaCl₂), and supplemented with CaCl₂ to a final concentration of 10 mM. Individual eluted fractions were subjected to a ligand-binding competition assay to determine the presence of active ASGP-Rs (27). The pooled fractions were concentrated using a Centricon-10 device (Amicon), and stored at 4°C before using.

**Incubation with NH₂OH**—The freshly purified ASGP-Rs were incubated in BIC10 containing 0.025% Triton X-100, and various concentrations of NH₂OH, pH 7.4, on ice for the indicated times. The reaction was stopped by dilution with BIC10 (to give ~0.005% Triton X-100) and loaded onto a nitrocellulose membrane. Other studies have shown that ASGP-R adsorption to the nitrocellulose is decreased if the Triton X-100 concentration is above 0.005%.

**Ligand-binding Assay**—Untreated and NH₂OH-treated ASGP-Rs were diluted with BIC20 to a concentration of Triton X-100 <0.005%, then loaded onto a 0.45-μm pore size nitrocellulose membrane (0.5 μg of ASGP-R well) using a dot-blot manifold (Schleicher & Schuell). The wells were rinsed twice with 500 μl of BIC20, the manifold was disassembled, and the nitrocellulose membrane was blocked with buffer 1 containing 0.1% Tween 20 at 4°C for 90 min. After washing with BIC20 containing 0.02% Tween 20, the sheet was then incubated with 1.0 μg/ml 125I-ASOR (100–200 cpm/fmol) in BIC20 containing 0.02% Tween 20 at 4°C for 2 h. After extensive washes with BIC20 containing 0.02% Tween 20, individual dots containing immobilized ASGP-Rs were cut out using a cork borer and transferred into γ-tubes to measure radioactivity. Non-specific binding, determined by the bound radioactivity remaining in the presence of a 50-fold excess of non-labeled ASOR, was less than 5% of the total in all cases. All binding assays were done in duplicate.

**Dissociation of Preformed Receptor-Ligand Complexes by NH₂OH**—Active ASGP-Rs, freshly purified as described above, were rebound to ASOR-Sepharose by incubating 10 μg of ASGP-Rs with 10 μl of ASOR-Sepharose in 50 μl of BIC20 containing 0.05% Triton X-100 at 4°C for 2 h on a rotator. After a brief centrifugation, the supernatant fluid was removed using a T-200R flat end tip (RPI, Inc.), and the Sepharose pellet containing ASGP-R-ASOR complexes was incubated in BIC20 containing 0.05% Triton X-100, and various concentrations of either NH₂OH or Tris at 4°C for a time course as indicated in Table I. After centrifugation, the supernatant fluids were transferred into a new tube using a T-200R flat end tip, and the pellet was incubated with 100 μl of EGTA-containing elution buffer to remove the bound ASGP-Rs. The protein content in the supernatant and pellet was then determined.

**Metabolic Labeling—Hepatocytes** were cultured with 1Hlalpinic acid and ASGP-Rs were affinity-purified as described in the accompanying paper (28).

**General—Protein content was measured by the method of Bradford** (29) using BSA as a standard. SDS-PAGE was carried out by the method of Laemmli (30); the samples were boiled for 1 min in the SDS sample buffer with 5% β-mercaptoethanol. Protein bands were visualized by silver staining (31). 125I-radioactivity was measured using a Packard multipurpose γ spectrometer. Statistical analyses were carried out using the Student’s t test.

**RESULTS**

One subpopulation of ASGP-Rs is more readily inactivated by NH₂OH—In order to assess quantitatively the effect of NH₂OH on the ligand binding activity of affinity-purified rat ASGP-Rs, we used a recently developed dot-blot assay2 to measure the 125I-ASOR binding activity of purified ASGP-Rs. This assay permits rapid and reproducible quantitation of changes in the ligand binding activity of both immobilized 2 P. A. Haynes, F-Y. Zeng, and P. H. Weigel, manuscript in preparation.
TABLE I

| Treatment | Total ASGP-Rs from untreated hepatocytes | State 1 ASGP-Rs | State 2 ASGP-Rs |
|-----------|----------------------------------------|----------------|----------------|
|           | dissociated ASGP-R (% of initial protein bound) | From choloroquine-treated hepatocytes | From monensin-treated hepatocytes | From chloroquine-treated hepatocytes | From monensin-treated hepatocytes |
| 0.1 M Tris, 18 h | 2.17 ± 0.17 | 4.95 ± 0.95 | 2.25 ± 0.35 |
| 0.1 M NH₂OH, 18 h | 38.83 ± 1.86 | 14.40 ± 0.90* | 14.00 ± 0.20* |
| 1 M Tris, 1 h | 1.83 ± 0.44 | 3.55 ± 0.65 | 0.80 ± 0.50 |
| 1 M NH₂OH, 1 h | 42.93 ± 1.62 | 17.80 ± 2.20* | 18.80 ± 0.70* |

*Values significantly different from the control (total ASGP-Rs) are indicated: p < 0.005.

ASGP-Rs and ASGP-Rs in solution after treatment with NH₂OH. The use of Tween 20 as a blocking agent and its inclusion in the ligand-binding step improved the ¹²⁵I-ASOR binding avidity and reduced nonspecific binding.

To exclude the possible effects of other factors such as pH changes and temperature on ASGP-R activity, the treatment of ASGP-Rs with NH₂OH or Tris was carried out on ice in BIC20 containing 0.025% Triton X-100 at pH 7.4. In initial studies, we found that purified total ASGP-Rs progressively lost up to 50% activity after storage in BIC10 containing 0.05% Triton X-100 at 4°C for more than 1 week. Furthermore, the rate of ASGP-R activity loss rapidly increased when the temperature was ≥20°C. Freezing and thawing also caused loss of activity. We suspect that the State 2 ASGP-R activity is being lost under these conditions. For this reason, we used only freshly purified ASGP-Rs in all the experiments reported here (<12 h old) to minimize any initial loss of ASGP-R activity.

Treatment of ASGP-Rs with 1 M NH₂OH caused ASGP-R inactivation in a time-dependent manner, whereas similar treatment with milder nucleophiles such as 1 M Tris or methyamine (not shown) had no significant effect on ASGP-R activity (Fig. 1A). The kinetics of ASGP-R inactivation in the presence of 1 M NH₂OH were quite biphasic. The rate of inactivation during the initial 60 min was greater than that during incubation after 60 min. About 50% of the ASGP-Rs were inactivated within 1 h; extended incubation times of up to 18 h resulted in a progressive inactivation of the remaining ~50% of ASGP-Rs. A possible reason for ASGP-R inactivation might be that under these conditions NH₂OH could cause cleavage of ASGP-R subunits that results in the loss of activity. To assess this possibility, the NH₂OH-treated ASGP-Rs were analyzed by SDS-PAGE under reducing conditions (Fig. 1B). Within the first 2 h (during which time ~50% ASGP-R inactivation occurs), NH₂OH treatment did not degrade any of the three ASGP-R subunits (Fig. 1B). Prolonged incubation times after 2 h indeed caused a progressive increase in ASGP-R degradation: prominent fragments of 40 and 24 kDa were observed. This result indicates that inactivation of the first ~50% of ASGP-Rs was not due to peptide cleavage, whereas inactivation of the second ~50% ASGP-Rs could be explained by the observed peptide degradation.

In another experiment, purified total ASGP-Rs were incubated with 1 M NH₂OH on ice for 1 h (this treatment caused an inactivation of ~50% ASGP-Rs as shown in Fig. 1A), and NH₂OH was then removed by repeated dilution of the mixture and subsequent concentration using a Centricon-10 device. Active ASGP-Rs were then separated from inactive ASGP-Rs by purification on ASOR-Sepharose. About 50% of the total ASGP-Rs could still bind to ASOR, the other ~50% had lost their ligand binding activity without detectable peptide cleavage (data not shown). This result supports the conclusion that under mild conditions, NH₂OH inactivates one population of receptors without affecting the other population. Hydroxylamine sensitivity of ASGP-Rs was identical whether a solid-phase or solution-based assay was used to assess ligand binding activity.

Another possibility is that the observed ASGP-R inactivation by NH₂OH is not really due to reactivity of NH₂OH, but rather to other minor components present in commercial NH₂OH preparations, such as heavy metal ions that could activate minor contaminating proteases that copurify with ASGP-Rs. To exclude this and several alternative possibilities, other controls were performed. Incubation of purified ASGP-Rs with divalent metal ions including Mg²⁺, Zn²⁺, Ni²⁺, Ca²⁺, and Mn²⁺ did not change their ligand binding activity. Furthermore, NH₂OH from various sources (Sigma, Aldrich, Fluka, and MC/B) all showed very similar kinetics of ASGP-R inactivation (not shown). The addition of the divalent metal ion chelators EGTA did not affect the ability of NH₂OH (1 M, 20 h) to inactivate up to ~50% of total ASGP-Rs, but did prevent...
Further inactivation and degradation of the remaining 50% of ASGP-R subunits (Fig. 2, A and B). This observation suggests that the activity loss of the less sensitive ASGP-R population is the consequence of subunit cleavage by NH$_2$OH. Addition of protease inhibitors such as PMSF, pepstatin, and leupeptin did not reduce the effectiveness of NH$_2$OH to inactivate these ASGP-Rs. Furthermore, after incubation of ASGP-Rs with Tris in the presence of Ca$^{2+}$ for more than 24 h, no detectable protein degradation could be observed (not shown).

The inactivation of ASGP-Rs by NH$_2$OH treatment (4°C for 4 h) is also dependent on NH$_2$OH concentration and this dose-response is biphasic (Fig. 3). The extent of ASGP-R inactivation was proportional to NH$_2$OH concentration in the range of 0–0.1 M, corresponding to 0 to 40% inactivation. ASGP-R inactivation was ~50% with 0.2 M NH$_2$OH. Increasing NH$_2$OH concentration in the range of 0.2–1.0 M only slightly increased ASGP-R inactivation. SDS-PAGE analysis showed no degradation products or decrease in size of RHL1, RHL2, and RHL3 after treatment with 0 to 0.2 M NH$_2$OH at 4°C for 4 h (not shown).

Biphasic kinetic and dose-responses were also observed for ASGP-R inactivation by NH$_2$OH using receptor immobilized on nitrocellulose, but inactivation occurred more slowly than in solution (not shown). The inactivation of ASGP-Rs was also pH- and temperature-dependent (Fig. 4). At pH 6.0 and 4°C, no significant inactivation was observed with 0.5 M NH$_2$OH for 1.5 h. A sharp increase in ASGP-R inactivation was seen between pH 6 and 7 and the extent of inactivation remained constant from pH 7 to 11. In comparison with treatment at 4°C, ASGP-R inactivation occurred more readily at 25°C, especially at basic pH (Fig. 4).

State 1 ASGP-Rs Are Less Sensitive to NH$_2$OH Treatment Than State 2 ASGP-Rs—The above results indicate that there are two populations of ASGP-Rs with respect to NH$_2$OH sensitivity. One subpopulation of total purified ASGP-Rs is much more sensitive to inactivation by NH$_2$OH treatment. Although the percentage of the sensitive population was variable (30–50%) from preparation to preparation, the biphasic activity loss with NH$_2$OH treatment was always observed (n > 10). Since our previous studies have shown that the State 1 and State 2 ASGP-R populations are roughly equal (50–50%), we surmised that the NH$_2$OH-sensitive subpopulation of ASGP-Rs may be the State 2 ASGP-Rs. State 2 ASGP-R activity has previously been shown to be inactivated by a variety of different treatments in isolated intact rat hepatocytes (26). We, therefore, are able to purify either total active ASGP-Rs (representing State 1 plus State 2) or State 1 ASGP-Rs. Presently, the State 2 ASGP-R population cannot be purified free of State 1 ASGP-Rs.

To confirm that the NH$_2$OH-sensitive ASGP-R population corresponds to State 2 ASGP-Rs, we purified State 1 ASGP-Rs, after first inactivating State 2 ASGP-Rs in intact cells with either chloroquine or monensin as described under "Experimental Procedures." Treatment of isolated rat hepatocytes with chloroquine (1 mM, 60 min, at 37°C) or monensin (50 pM, 60 min, at 37°C) resulted in 40–60% inactivation of total ASGP-Rs as assessed by $^{125}$I-ASOR binding with permeable cells. In comparison with untreated cells, about 40–50% of active ASGP-Rs could be purified from treated cells, verifying that about half of the ASGP-Rs were indeed inactivated. The purified State 1 ASGP-Rs showed very similar subunit patterns to that of total ASGP-Rs by SDS-PAGE under reducing conditions (not shown). We then compared the sensitivity of State 1 ASGP-Rs and total (State 1 plus State 2) ASGP-Rs purified in parallel, to NH$_2$OH treatment. Kinetically, State 1 ASGP-Rs showed significantly less sensitivity to NH$_2$OH inactivation than the total ASGP-R pool (Fig. 5). During the first 30 min, 30–35% of the total ASGP-Rs were inactivated, whereas State 1 ASGP-R activity remained essentially unchanged. After 30 min incubation, a slow progressive loss of State 1 ASGP-R activity occurred that reached up to ~40% inactivation at 180 min. The difference in the rate of activity loss...
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**Fig. 4.** Effect of pH and temperature on ASGP-R inactivation by NH₂OH. Freshly purified, total active ASGP-Rs were incubated on ice or at 25 °C for 90 min with 0.5 mM NH₂OH (△), or 0.5 mM Tris (●) in 10 mM Hepes at the indicated pH containing 142 mM NaCl, 6.7 mM KCl, 20 mM CaCl₂, and 0.025% Triton X-100. The treated samples were diluted with 100 volumes of BIC20, loaded onto nitrocellulose, and ¹²⁵I-ASOR binding activity was assessed as described in the legend to Fig. 1.

**Fig. 5.** Kinetic difference between State 1 and total (State 1 plus State 2) ASGP-R inactivation by NH₂OH treatment. Active State 1 ASGP-Rs (●, ○), freshly purified either from chloroquine-treated (●), or monensin-treated hepatocytes (○), and active total ASGP-Rs (◆), freshly purified from nontreated cells, were incubated in BIC20 containing 0.025% Triton X-100 and 0.5 mM NH₂OH on ice for 0–3 h. At the indicated times, the ¹²⁵I-ASOR binding activity of samples was assessed. Each point is the mean ± S.E of four to six independent experiments. Values significantly different from the control (total ASGP-Rs) are indicated: * p < 0.001; †, p < 0.005; #, p < 0.05.

The dose dependence for ASGP-R inactivation by NH₂OH also showed a marked difference between the State 1 versus State 1 plus State 2 ASGP-R populations (Fig. 6). At ≤ 50 mM NH₂OH, no significant loss of State 1 ASGP-R activity was observed after incubation at 4 °C for 4 h, whereas ~25% of the activity of the total receptors was lost. The difference in the ability of NH₂OH to cause inactivation was more marked at a lower concentration range than at higher concentration (≤400 mM). The purified State 1 ASGP-Rs prepared from either chloroquine- or monensin-treated hepatocytes, showed essentially identical sensitivity to NH₂OH (both in kinetics and dose dependence; Figs. 5 and 6), indicating that treatment of isolated cells with chloroquine or monensin inactivated the same ASGP-R population (State 2), as reported previously (26). These results strongly support the conclusion that the NH₂OH-sensitive population is the State 2 receptor population.

NH₂OH Treatment of ASGP-Rs Bound to ASOR-Sepharose Readily and Selectively Dissociates the State 2 Population of ASGP-Rs—As an alternative way to assess the effect of NH₂OH treatment on ASGP-Rs, we determined the ability of NH₂OH at pH 7.4, 4 °C, to cause dissociation and inactivation of preformed ASGP-R-ASOR-Sepharose complexes in the presence of CaCl₂. Treatment with Tris (0.1 M, 18 h; or 1 M, 1 h) did not significantly dissociate purified ASGP-Rs from ASOR-Sepharose using either total (State 1 plus State 2) or State 1 ASGP-Rs (Table I), whereas NH₂OH treatment (0.1 M, 18 h; or 1 M, 1 h) dissociated ~40% of State 1 plus State 2 ASGP-Rs but <20% of State 1 ASGP-Rs from receptor-ASOR complexes. The dissociated ASGP-R subunits showed no degradation or decreased size as determined by SDS-PAGE (Fig. 7). To verify that the NH₂OH treatment reduced the affinity of ASGP-R for ASOR, which then caused the dissociation of the complexes, the ligand binding activity of the dissociated ASGP-Rs was assessed by using a dot-blot assay and by measuring their ability to rebind to ASOR-Sepharose. Both assays showed that ASGP-Rs, dissociated in the presence of NH₂OH, were subsequently inactive. Thus, receptor-ligand dissociation was due to inactivation of ASGP-Rs, rather than to an effect on the ASOR-Sepharose or to a transient reduction of the receptor affinity for ASOR.

In contrast to total purified ASGP-Rs, only 14–19% of the State 1 ASGP-Rs were dissociated and inactivated by treatment with NH₂OH under the same conditions (Table I). The difference in inactivation between total versus State 1 ASGP-Rs was about 25% (p < 0.005) for both treatment conditions tested (0.1 mM NH₂OH, 18 h; 1 mM NH₂OH, 1 h). That only 14–19% of receptors were still released from State 1 ASGP-R-ASOR complexes by NH₂OH under conditions that removed almost 50% of total ASGP-Rs, is in agreement with the results shown in Figs. 5 and 6, and could be due to the presence of a small amount of State 2 ASGP-Rs in our State 1 ASGP-R preparations. We find that the extent of ASGP-R inactivation in isolated rat hepatocytes treated with chloroquine or monensin varies (40–60%) from cell preparation to preparation, even using the same conditions (26). At present, we cannot determine the percentage of State 2 ASGP-Rs that copurifies with State 1 ASGP-Rs by this method. Nonetheless, our results
The majority of hepatocytes contain two functionally different receptor populations: State 1 ASGP-Rs and State 2 ASGP-Rs. Preformed ASGP-R-ASOR-Sepharose complexes, prepared as described under "Experimental Procedures," were incubated with BIC20 containing 0.05% Triton X-100, and either 0.1 M Tris or 0.1 M NH$_2$OH at 4 °C for 18 h. After centrifugation the supernatant (s) and pellet (p), were washed once with BIC20-0.05% Triton X-100, and then analyzed by SDS-PAGE and silver staining as described in the legend to Fig. 1.

One potential disadvantage of using the dot-blot assay to quantitate changes in ASGP-R activity after treatment with NH$_2$OH is that only about 10% of immobilized ASGP-Rs are capable of binding ASOR (assuming that one ASGP-R binds one ASOR). The majority (~90%) of immobilized receptors are unable to bind ASOR, perhaps due to conformational inflexibility of immobilized molecules or inaccessibility to ligand. Differential adsorption or preferential conformational changes of active or inactivated ASGP-Rs during immobilization onto nitrocellulose could complicate interpretation of the results. Several controls were performed to address this possibility.

Using specific antireceptor antibody to quantitate nontreated or NH$_2$OH-treated ASGP-Rs after immobilization, we found that NH$_2$OH treatment did not change the adsorption of receptor. Most importantly, the finding that ~50% of ligand-bound ASGP-Rs are more readily dissociated and inactivated by NH$_2$OH (Table I) corroborates the results obtained from the dot-blot assay. These results also argue against a difference in adsorption ability or in conformational changes of immobilized active versus inactivated ASGP-Rs.

Figure 7. Effect of NH$_2$OH on preformed ASGP-R-ASOR complexes. Preformed ASGP-R-ASOR-Sepharose complexes, prepared as described under "Experimental Procedures," were incubated with BIC20 containing 0.05% Triton X-100, and either 0.1 M Tris or 0.1 M NH$_2$OH at 4 °C for 18 h. After centrifugation the supernatant (s) and pellet (p), were washed once with BIC20-0.05% Triton X-100, and then analyzed by SDS-PAGE and silver staining as described in the legend to Fig. 1.

Discussion

Our previous studies have demonstrated that rat hepatocytes contain two functionally different receptor populations: State 1 ASGP-Rs and State 2 ASGP-Rs. Although present in approximately equal numbers, the State 2 ASGP-Rs mediate the large majority (~80%) of ligand processing (i.e. endocytosis, segregation, and degradation) in hepatocytes. Functionally, therefore, State 2 receptors are roughly about 4 times more active than State 1 receptors. In contrast to State 1 ASGP-Rs, the State 2 ASGP-Rs can be modulated in their cellular distri-

Fig. 8. Mild treatment with NH$_2$OH releases all of the metabolically incorporated [3H]palmitate from ASGP-Rs. Cultured hepatocytes (2 x 10$^6$ cells/dish) were labeled with 400 µCi/ml [3H]palmitate for 4 h and active ASGP-Rs were purified as described under "Experimental Procedures." The purified ASGP-Rs were incubated with BIC20 alone (lane 1), or containing 1 M Tris (lane 2), or 1 M NH$_2$OH (lane 3) on ice for 2 h prior to SDS-PAGE. The gel was subjected to fluorography (26) for 25 days.

The molecular basis for the inactivation-reactivation cycle of State 2 ASGP-Rs is related to fatty acylation. We previously demonstrated the existence of two receptor populations in purified ASGP-Rs.

The inactivation of State 2 ASGP-Rs is transient and reversible, we suggested that receptor inactivation could be the mechanism by which cells achieve efficient ligand dissociation and subsequent segregation of ligand from receptor (34). During receptor-mediated endocytosis, the concentration of ligand in endosomes can be increased up to 10$^4$-fold over the extracellular concentration (2). Cell surface ASGP-Rs are also concentrated about 50-fold in endosomal membranes. Under these conditions of such high concentrations, dissociated ligand molecules would likely rebind to active receptors even at the lower pH of early endosomes. Any ligand rebound to receptor would not be shuttled to lysosomes for degradation, but rather nonproductively recycled back to the cell surface. Such "futile" receptor cycles would, of course, be wasteful. Most significantly, futile ligand recycling would increase as the extracellular ligand concentration increased, and the endocytic machinery would function less efficiently when the need for ligand clearance was greatest. This situation, which could be physiologically deleterious, is avoided by inactivating ASGP receptors so that ligand rebinding does not occur.

The cumulative evidence indicates that the ASGP-R is a hetero-oligomeric complex, composed of RHL1 and RHL2/3 subunits. (i) Both RHL1 and RHL2/3 gene products are required for expression of a functional ASGP-R capable of binding ASOR in transfected cells (35). (ii) Subunit-specific antibodies communoprecipitate all three RHL subunits of the ASGP-R (16, 36). (iii) Studies with a chemical affinity derivative of 125I-ASOR showed identical cross-linking patterns for State 1 or State 2 ASGP-Rs (16). (iv) Affinity-purified ASGP-Rs from cells with active State 1 or State 1 plus State 2 ASGP-Rs show identical subunit patterns in SDS-PAGE. These above results indicate that functional differences between State 1 and State 2 ASGP-R are not due to different subunit compositions.

This and the accompanying paper (28) establish for the first time a structural difference between the two populations of receptor and the possible role of this structural difference in the functional differences between State 1 and State 2 ASGP-Rs. The molecular basis for the inactivation-reactivation cycle of State 2 ASGP-Rs is related to fatty acylation. We previously

3 D. D. McAbee, P. A. Haynes, F.-Y. Zeng, J. A. Oka, and P. H. Weigel, unpublished data.
showed that palmitoyl-CoA rapidly and quantitatively reactivates inactivated State 2 ASGP-Rs, suggesting that either ASGP-R subunits or other unknown regulatory proteins are palmitoylated and that a palmitoylation-depalmitoylation cycle may regulate ASGP-R activity (9). These results prompted us to examine more directly if ASGP-Rs are modified by fatty acylation. As detailed in the accompanying paper (28), all three RHL subunits contain covalently attached palmitate and stearate. These fatty acids were found using gas chromatography-mass spectrometric analysis of purified RHL subunits after SDS-PAGE of active ASGP-Rs. Our results in the present study provide further evidence to support this conclusion; metabolic labeling with \[^{3}H\]palmitate also confirms that RHL1, RHL2, and RHL3 are fatty acylated in intact cells.

Surprisingly, only the State 2 ASGP-Rs, not State 1 ASGP-Rs, are palmitoylated in a metabolic labeling experiment. Essentially all of the palmitic acid metabolically incorporated into RHL1, RHL2, and RHL3 is released by mild hydroxylamine treatment that inactivates only the State 2 ASGP-Rs. We conclude that State 2 ASGP-Rs are clearly fatty acylated. However, further studies are needed to determine whether State 1 ASGP-Rs are fatty acylated; it is quite possible that they are not.

\(\text{NH}_{2}\text{OH} \) treatment decreases ASGP-R activity in a time- and dose-dependent manner (Figs. 1 and 2). Inactivation of ASGP-Rs is kinetically biphasic; 50% of ASGP-Rs are very sensitive to \(\text{NH}_{2}\text{OH} \) and the other 50% are much less sensitive. During the loss of \(\text{NH}_{2}\text{OH} \)-sensitive ASGP-R activity, no protein degradation occurs, indicating that loss of activity is not due to peptide cleavage. Although the percentage of \(\text{NH}_{2}\text{OH} \)-sensitive ASGP-Rs ranged from 30 to 60% with different ASGP-R preparations, the phenomenon of biphasic ASGP-R inactivation by \(\text{NH}_{2}\text{OH} \) was always observed.

Our results in this study suggest that ASGP-Rs are acylated in vivo. Many membrane proteins and receptors are palmitoylated, often at Cys residues near transmembrane domains (37-42). The subunits of the rat and human ASGP-Rs have a Cys-Ser sequence close to the cytoplasmic membrane junction (14, 43). This same sequence in the transferrin receptor (44) and the HLA-D-associated invariant chain (45) is palmitoylated at Cys. It is possible, therefore, that the same position in one or more of the ASGP-R subunits is also palmitoylated.

Inactivation of ASGP-R by \(\text{NH}_{2}\text{OH} \) is likely due to the removal of covalently bound fatty acids from one or more of the ASGP-R subunits. Since all three RHL subunits contain covalently bound fatty acids (palmitic acid and stearic acid) and ASGP-Rs are hetero-oligomeric, there are many possible partially deacetylated receptor species whose ligand binding activity could be affected.

We also find that \(\text{NH}_{2}\text{OH} \) treatment, but not Tris treatment, results in the formation of dimeric RHL subunits (based on SDS-PAGE analysis under nonreducing conditions) indicating that deacylation with \(\text{NH}_{2}\text{OH} \) generates free thiol groups that then form disulfide bonds in a time-dependent way.\(^4\) This formation of new disulfide bonds upon \(\text{NH}_{2}\text{OH} \) treatment has been demonstrated in other palmitoylated proteins such as vesicular stomatitis virus G glycoprotein (38) and human tissue factor (46). \(\text{NH}_{2}\text{OH} \) has been widely used to remove thioester bond-linked palmitate from many palmitoylated proteins, such as transferrin receptor (37), rhodopsin (19), and virus glycoproteins (38). Under mild conditions, deacylation by \(\text{NH}_{2}\text{OH} \) did not greatly affect the conformational structure of rhodopsin as determined by circular dichroism (39). Although \(\text{NH}_{2}\text{OH} \) has usually been used as a specific chemical to release ester and thioester-linked fatty acids from fatty acylated proteins (40, 41), it also has a number of other chemical effects on proteins. \(\text{NH}_{2}\text{OH} \) (usually \(\geq 1 \text{ m}, \text{ pH } \geq 9.0, \geq 37 °C \)) cleaves susceptible Asn-Gly bonds in many proteins (47). 2 n \(\text{NH}_{2}\text{OH} \) at pH 9 and at 45 °C has been used specifically to cleave at the C-terminal side of succinimidyl esters in proteins (48). \(\text{NH}_{2}\text{OH} \) is also known to remove O-acetyl groups from tyrosine (49).

\(^4\) F.-Y. Zeng and P. H. Weigel, manuscript in preparation.
13. Drickamer, K. (1987) Kidney Int. 32, 5167–5180
14. Halberg, D. F., Wager, R. E., Farrel, D. C., Hildreth, J., IV, Quesenberry, M. S., Loeb, J. A., Holland, E. C., and Drickamer, K. (1987) J. Biol. Chem. 262, 9828–9838
15. Weigel, P. H. (1993) in Subcellular Biochemistry (Bergeron, J. J. M., and Harris, J. R., eds) 19, pp. 125–161, Plenum Press, New York
16. Halberg, D. F., Wager, R. E., Farrell, D. C., Hildreth, J., IV, Quesenberry, M. S., Loeb, J. A., Holland, E. C., and Drickamer, K. (1987) J. Biol. Chem. 262, 9828–9838
17. Weigel, P. H. (1993) in Subcellular Biochemistry (Bergeron, J. J. M., and Harris, J. R., eds) 19, pp. 125–161, Plenum Press, New York
18. Weigel, P. H. (1993) in Subcellular Biochemistry (Bergeron, J. J. M., and Harris, J. R., eds) 19, pp. 125–161, Plenum Press, New York
19. Herzig, M. C. S., and Weigel, P. H. (1990) Biochemistry 29, 6437–6447
20. Schmidt, M. F. G. (1989) Biochim. Biophys. Acta 988, 411–426
21. Weigel, P. H. (1991) J. Biol. Chem. 266, 20118–20223
22. Schmidt, M. F. G. (1989) Biochim. Biophys. Acta 988, 411–426
23. Weigel, P. H. (1991) J. Biol. Chem. 266, 20118–20223
24. Weigel, P. H., and Oka, J. A. (1982) J. Biol. Chem. 257, 1201–1207
25. Clarke, B. L., Oka, J. A., and Weigel, P. H. (1987) J. Biol. Chem. 262, 17384–17392
26. Seglen, P. O. (1973) Exp. Cell Res. 82, 391–398
27. Weigel, P. H., and Oka, J. A. (1983) J. Biol. Chem. 258, 5089–5094
28. McAbee, D. D., Lear, M. C., and Weigel, P. H. (1991) J. Cell. Biochem. 45, 59–68
29. Ray, D. A., and Weigel, P. H. (1985) Anal. Biochem. 145, 37–46
30. Zeng, F-Y., and Weigel, P. H. (1994) Anal. Biochem. 213, 213–219
31. Zeng, F-Y., and Weigel, P. H. (1994) Anal. Biochem. 213, 213–219
32. Oka, J. A., and Weigel, P. H. (1991) Arch. Biochem. Biophys. 289, 362–370
33. McAbee, D. D., Clarke, B. L., Oka, J. A., and Weigel, P. H. (1990) J. Biol. Chem. 265, 629–635
34. McAbee, D. D., and Weigel, P. H. (1988) Biochemistry 27, 2061–2069
35. McPhaul, M., and Berg, P. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 8863–8867
36. Sawyer, J. T., Sanford, J. P., and Doyle, D. (1988) J. Biol. Chem. 263, 10534–10538
37. Omary, M. B., and Trowbridge, I. S. (1981) J. Biol. Chem. 256, 4715–4718
38. Magee, A. I., Koyama, A. H., Maiter, C., Wen, D., and Schlesinger, M. J. (1984) Biochim. Biophys. Acta 798, 156–166
39. Traylor, K. D., and Dewey, T. G. (1994) Biochemistry 33, 1718–1723
40. Sefton, B. M., and Buss, J. E. (1987) J. Cell Biol. 104, 1449–1453
41. Olson, E. N. (1988) Prog. Lipid Res. 27, 177–197
42. Grand, R. J. A. (1989) Biochem. J. 258, 625–638
43. Spies, M., Schwartz, A. L., and Lodish, H. F. (1985) J. Biol. Chem. 260, 1797–1982
44. Adam, M., Turbide, C., and Johnson, R. M. (1988) Arch. Biochem. Biophys. 264, 553–563
45. Koch, N., and Hammerling, G. J. (1986) J. Biol. Chem. 261, 3434–3440
46. Bach, R., Kingsburg, W. H., and Nenerson, Y. (1988) Biochemistry 27, 4227–4231
47. Boedtker, H., and Balian, G. (1977) Methods Enzymol. 47, 85–145
48. Kwong, M. Y., and Harris, R. J. (1994) Protein Sci. 3, 147–149
49. Tildon, J. T., and Oglivie, J. W. (1972) J. Biol. Chem. 247, 1265–1271
50. Wedegartner, P. B., and Bourne, H. R. (1994) Cell 77, 1063–1070
51. Hancock, J. F., Paterson, H., and Marshall, C. J. (1990) Cell 63, 133–139
52. Sudo, Y., Valenzuela, D., Beck-Sickinger, A. G., Fishman, M. C., and Strittmatter, S. M. (1992) EMBO J. 11, 1079–1087
53. Alvarz, E., Girones, N., and Davis, R. J. (1990) J. Biol. Chem. 265, 16644–16655