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One-Step Immunoaffinity Purification of Active Progesterone Receptor. Further Evidence in Favor of the Existence of a Single Steroid Binding Subunit†

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ABSTRACT: A very high capacity immunoaffinity matrix for the purification of progesterone receptor was prepared by cross-linking a monoclonal antireceptor antibody to protein A–Sepharose through the Fc fragment. The monoclonal antibody was selected for its property of losing affinity for the receptor at pH 10.5, i.e., in conditions where the receptor remains stable for extensive periods of time. This made it possible to elute active receptor from the immunosorbent. From crude rabbit uterine cytosol the steroid–receptor complexes were purified in a single step. A 1-mL column (containing 7 mg of monoclonal antibody) bound 1600 pmol of steroid–receptor complexes of which 79.5% were eluted. The overall yield of purification was 49%. The specific activity of the purified steroid–receptor complexes was 6.71 ± 0.79 nmol of bound steroid/mg of protein (mean ± SE of four experiments). The purified receptor consisted of a mixture of 110 000- and 79 000-dalton forms. The latter appeared to be produced by proteolysis of the larger form during purification since immunoblot experiments showed that, at the start of purification, the 110 000-dalton form was present in overwhelming majority (80–95%) in the uterine cytosol and that the 79 000-dalton form only appeared during purification. This conclusion was also supported by the peptide analysis of both forms of receptor: the purified receptor was denatured and labeled with ^{125}I; the 110 000- and 79 000-dalton forms were isolated by gel electrophoresis in denaturing conditions and electroelution and were then submitted to mild or extensive digestions by trypsin, chymotrypsin, and protease V8 from *Staphylococcus aureus*. In all cases, the comparison of the patterns of peptides obtained suggested that the 79 000-dalton protein is a proteolytic product of the 110 000-dalton receptor. The rapidity and the mild elution conditions of this immunoaffinity method enabled us to purify active aporeceptor (ligand-devoid receptor). The purified receptor retained its original properties (affinity for the hormone and DNA-binding and density gradient sedimentation characteristics).

The structure and the biological function of steroid hormone receptors still remain poorly understood [recent review in Eriksson & Gustafsson (1983)]. This is mainly due to their fragility and their very low concentration, which makes it difficult to purify them in sufficient amounts and with a good yield. Moreover, all the purification procedures devised to date yield steroid–receptor complexes (Schrader et al., 1980; Puri et al., 1982; Renoir et al., 1982; Logeat et al., 1981; Greene et al., 1979; Wrange et al., 1979). Only very partial purifications of aporeceptors (ligand-devoid receptors) have been described (Maggi et al., 1981). For this reason, it has been impossible to study the effect of steroids on purified receptors.

We recently prepared monoclonal antibodies to the rabbit progesterone receptor (Logeat et al., 1983) and report here the use of these antibodies to purify rapidly and with a high yield the receptor. The efficiency of the method used relied mainly on two points: (1) The attachment of the antibody through the Fc fragment to protein A–Sepharose, leaving free all the antigen binding sites (Schneider et al., 1982). This ensured a very high capacity of the immunomatrix and also decreased the nonspecific binding of proteins. (2) The use of antibody that is very sensitive to pH conditions. Exposure of the immunosorbent to pH 10.5 allowed elution of the receptor. At this pH, the progesterone receptor was stable for long periods of time. The method was also used to purify steroid-free receptor.

Materials and Methods

Animals. New Zealand rabbits weighing 1 kg were injected during 8 days with diethylstilbestrol (100 μg in 0.5 mL of sesame oil/day) (Rao & Katz, 1977). On day 9, the rabbits were killed; the uteri were excised and rinsed in cold saline solution.

Antibodies. Monoclonal antibodies (IgG_{2a}) were purified from mouse ascite on protein A–Sepharose (Pharmacia). Elution of immunoglobulins was performed with 0.1 M sodium citrate pH 4 buffer. The characteristics of the Mi60-10 antireceptor monoclonal antibody used in this work have previously been described (Logeat et al., 1983). The mouse monoclonal antibodies used as controls and nonrelated to the receptor were a gift of Dr. P. Legrain (Institut Pasteur). They were antiidiotypic antibodies, raised against mouse myeloma

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antilevan antibodies (Legrain et al., 1983).

Proteolysis Inhibitors. Aprotinin, bacitracin, leupeptin, pepstatin, and phenylmethanesulfonyl fluoride were from Sigma.

Radioactive Steroids. [^{3}H]R5020 ([^{3}H]-17,2α-dimethyl-19-norpregna-4,9-diene-3.20-dione) (sp act. 87 Ci/mmol) was from New England Nuclear. [^{3}H]ORG.2058 ([^{3}H]-16α-ethyl-21-hydroxy-19-norpregn-4-ene-3.20-dione) (sp act. 47 Ci/mmol) was obtained from Amersham. In receptor purification experiments, both radioactive steroids were diluted with unlabeled compounds to lower specific activities.

Preparation of Uterine Cytosol. Uteri from 10–12 rabbits were excised and immediately homogenized in a cold room in 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.3–50 mM NaCl buffer (3 mL/g of uteri). The buffer contained a mixture of proteolysis inhibitors (pepstatin, 1 μg/mL; leupeptin, 0.1 mM; bacitracin, 100 μg/mL; aprotinin, 77 μg/mL; phenylmethanesulfonyl fluoride, 1 mM). Preliminary experiments showed that these compounds at these particular concentrations optimally but not completely inhibited the proteolysis of receptor.

Cytosol was prepared in the cold room. Homogenization conditions were of special importance to further decrease the proteolysis. Initial homogenization was performed with a Waring Blendor (one stroke of 10 s at maximal speed). The homogenization was completed with an all-glass Potter Elvehjem apparatus kept in a mixture of ice and water (2 strokes at 1000 rpm at intervals of 10–20 s). Cytosol was obtained by a 65-min centrifugation at 48 000 rpm at 4 °C in a Ti 50.2 rotor. The whole procedure was performed as rapidly as possible. In most experiments, the cytosol was incubated for 30 min at 0 °C with 50 nM [^{3}H]R5020 (sp act. 10 Ci/mmol) or 50 nM [^{3}H]ORG.2058 (sp act. 10 Ci/mmol). Nonspecific binding was measured by a parallel incubation with 1 μM unlabeled R5020 or ORG.2058. The contact between hormone and receptor was continued during the ~8 h of chromatography.

Measurement of Steroid–Receptor Complexes. The dextran-coated charcoal method (Vu Hai & Milgrom, 1978) was used when the protein concentration was >1 mg/mL. In all other cases, it was the hydroxylapatite technique (Erdos et al., 1970). Correction for nonspecific binding was performed by subtracting bound radioactivity measured in presence of 1 μM unlabeled steroid.

Preparation of Immunosorbent. The method of Schneider et al. (1982) was followed without modification. Antibody attached to the protein A–Sepharose (Pharmacia) was 90–95% of input. Usually, gels containing 7 mg of antibody/mL were used.

Immunopurification of Receptor. The cytosol was chromatographed through three successive columns. The first column was made of protein A–Sepharose (7.5 mL, diameter 2.5 cm). The second consisted of nonspecific IgG_{2a}–protein A–Sepharose (2.5 mL, diameter 1 cm). These columns were destined to bind proteins having a nonspecific affinity to protein A or to immunoglobulins in general. The third column (1 mL, diameter 1 cm) contained Mi60-10 antibody–protein A–Sepharose. The flow rate was 12 mL/h. After passage of the cytosol, the last column was disconnected from the two others and washed with the following buffers: 10 mM Tris-HCl, pH 8.3–50 mM NaCl containing the mixture of protease inhibitors described above (50 mL); 10 mM Tris-HCl, pH 7.4, containing the protease inhibitors and 10% glycerol (50 mL); the same buffer without protease inhibitors (40 mL); 10 mM Tris-HCl, pH 7.4, 0.3 M NaCl, and 10% glycerol (40 mL); 10 mM Tris-HCl, pH 7.4, 1 M NaCl, and 10% glycerol (20 mL); 10 mM Tris-HCl, pH 6–10% glycerol (40 mL); finally, 10 mM Tris-HCl, pH 9–10% glycerol (40 mL).

The receptor was then eluted in 50 mM Tris-HCl pH 10.5 or pH 10.8 buffer containing 20% glycerol. Two successive elutions were performed: one with 2 mL and the other with 1 mL of buffer. The eluate was brought to pH 8 by addition of 0.2 M Tris-HCl pH 4.7 buffer. In some experiments where the purified receptor was dilute, a buffer containing either ovalbumin or bovine serum albumin was added to prevent the receptor from sticking to surfaces.

Measurement and Staining of Proteins. In the cytosol, proteins were measured by their optical absorption at 280 and 260 nm according to Kalckar (1947). In purified preparations, the protein concentration was very low. Great care was thus taken to obtain precise measurements by using the sensitive amido schwartz method (Schaffner & Neissman 1973) and by only taking into account measurements of >4 μg of protein. Bovine serum albumin was used as standard protein (standard curve 1–20 μg). In polyacrylamide gels, proteins were stained by either the silver (Wray et al., 1981) or the Coomassie blue (Laemmli, 1970) methods.

Immunoblot of Progesterone Receptor. Uterine cytosol (200 μg of protein and 0.4–0.5 pmol of receptor in 40 μL of buffer per electrophoresis lane) and purified receptor were electrophoresed in denaturing conditions as described by Laemmli (1970). The proteins were electrotransferred onto a nitrocellulose membrane (Transblot transfer medium, Bio-Rad Laboratories, Richmond, CA) with a Transblot cell apparatus (Bio-Rad) at 60 V and 350 mA for 150 min in a 25 mM Tris–192 mM glycine buffer, pH 8.4, containing 0.015% sodium dodecyl sulfate.

The membrane was saturated with proteins by incubating it for 90 min at room temperature, in a 3% solution of bovine serum albumin in phosphate-buffered saline (10 mM sodium phosphate pH 7.4 buffer containing 0.15 M NaCl). A volume of 20 mL/electrophoresis lane was used.

The purified Mi60-10 antireceptor monoclonal antibody [20 μg/lane in 5 mL of a solution of bovine serum albumin (1%) in phosphate-buffered saline] was incubated with the nitrocellulose membrane overnight at 4 °C. The membranes were washed 5 times in phosphate-buffered saline containing bovine serum albumin (0.5%) and Nonidet P-40 (0.5%).

The second antiserum (rabbit anti-mouse immunoglobulins, Sigma, purified on Protein A–Sepharose) was diluted 800-fold in a solution of phosphate-buffered saline containing 1% bovine serum albumin and 0.5% Nonidet P-40. The nitrocellulose membranes were incubated in this solution (4 mL/lane) for 2 h at room temperature and were then washed 5 times in the washing solution (see above).

Protein A (Pharmacia, Uppsala, Sweden) was iodinated with ^{125}I by Iodo Beads [as described in the technical bulletin of Pierce Chemical Co. (Rockford, IL)]. The nitrocellulose membrane was incubated in 6 mL of a solution of phosphate-buffered saline containing 1% bovine serum albumin and 0.5% Nonidet P-40 and (3–5) $\times$ 10^5 cpm/lane. Six washes were performed in the washing solution described above.

Filters were dried at room temperature and autoradiographed with the HS-90 film (Orwo, GDR). When quantitative data were needed, the Kodak No-screen NS-2T film (Eastman Kodak, Rochester, NY) was used. Exposure time was overnight.

RESULTS

Characteristics of Immunosorbent. (A) Binding of Receptor. Preliminary experiments had shown that filtration of

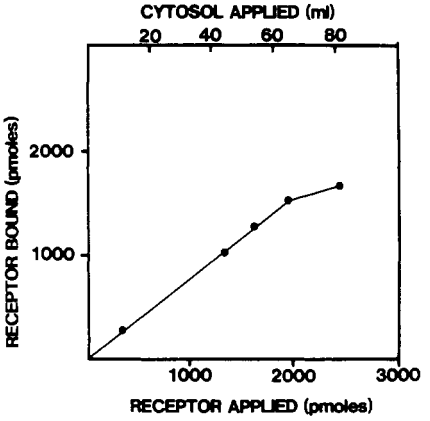

FIGURE 1: Binding of [^{3}H]R5020–receptor complexes to the immunosorbent. Cytosol containing [^{3}H]R5020–receptor complexes was chromatographed on three successive columns (protein A–Sepharose, nonspecific IgG–protein A–Sepharose, and antireceptor IgG–protein A–Sepharose) as described under Materials and Methods. Aliquots of the flow-through of the last column were used to measure the concentration of steroid–receptor complexes by the hydroxylapatite method (Erdos et al., 1970). Preliminary experiments have shown that when cytosol was chromatographed on the two nonspecific columns (protein A–Sepharose and nonspecific IgG–protein A–Sepharose), 85–90% of the complexes were recovered in the flow-through of the columns.

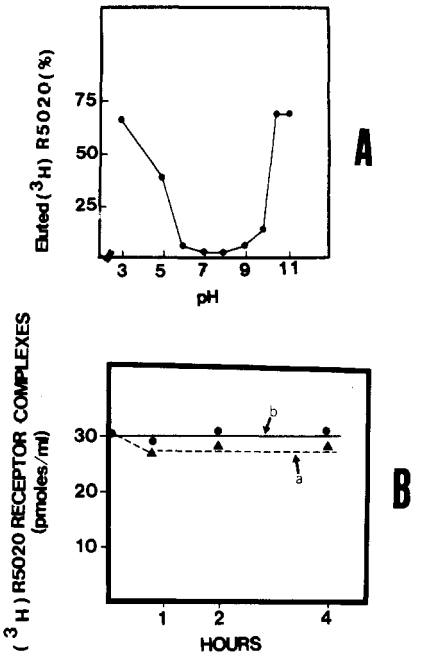

FIGURE 2: Effect of pH on the interaction between the immunosorbent and steroid–receptor complexes (A) and on the stability of steroid–receptor complexes (B). (A) Cytosol prepared in 10 mM Tris-HCl, pH 7.4–1.5 mM EDTA buffer was incubated for 2 h at 0 °C with 50 nM [^{3}H]R5020 (sp act. 16 Ci/mmol) and 1 μM unlabeled cortisol. An aliquot (4 mL) containing 100 pmol of steroid–receptor complexes was chromatographed through the immunomatrix (0.5 mL). After being washed with 10 mM Tris-HCl, pH 7.4–1.5 mM EDTA buffer (10 mL) and the same buffer containing 1 M NaCl and 10% glycerol (10 mL), the gel was resuspended in 10 mM Tris-HCl, pH 7.4–1.5 mM EDTA buffer (6 mL). Aliquots of 0.5 mL of the suspension were centrifuged, the buffer was drained, and the following buffers (200 μL) were added to various aliquots: 0.1 M sodium citrate, pH 3; 0.1 M sodium citrate, pH 5; 10 mM Tris-HCl, pH 6; 10 mM Tris-HCl, pH 7; 10 mM Tris-HCl, pH 8; 10 mM Tris-HCl, pH 10; 10 mM Tris-HCl, pH 10.5; 50 mM diethylamine, pH 11. After 10 min of incubation at 0 °C with agitation, the suspension was centrifuged. The supernatant was counted for radioactivity. (B) Cytosol was prepared and incubated with hormone as described in (A). Aliquots (0.5 mL) were diluted 2-fold with 1 M Tris-HCl, pH 11–20% glycerol buffer. The resulting pH was 10.5. After different periods of incubation at this pH (0–4 h), 0.3 mL of 50 mM Tris-HCl, pH 6–20% glycerol buffer was added to 0.1 mL of the incubate. This restored the pH to 7.4. The concentration of steroid–receptor complexes was measured by the dextran-coated charcoal method (Vu Hai & Milgrom, 1978). Correction for nonspecific binding was performed (see Materials and Methods). Other aliquots of the cytosol were submitted to a similar treatment except that all the buffers were at pH 7.4. (a) Stability of steroid–receptor complexes at pH 10.5; (b) stability of steroid–receptor complexes at pH 7.4.

cytosol through columns of protein A–Sepharose and nonreceptor-related immunoglobulin–protein A–Sepharose increased the capacity of the specific immunosorbent to retain the receptor and decreased the concentration of contaminating proteins in the eluate. The capacity of the immunosorbent was thus studied in these conditions. As shown in Figure 1, a 1-mL column containing 7 mg of Mi60-10 antireceptor antibody could bind 65% of the 2500 pmol of [^{3}H]R5020–receptor complexes contained in 85 mL of cytosol. After chromatography of this volume of cytosol, the column was still not saturated, but the proportion of receptor bound was decreasing (55% of the receptor was present in the filtrate of the column in the last fraction). Therefore, it could have been possible to increase the amount of purified receptor but at the expense of a decrease in yield.

(*B*) *Elution of Receptor.* The Mi60-10 antibody–receptor interaction is very sensitive to pH conditions (Figure 2A). In acid pH conditions, the radioactive steroid bound to receptor is only eluted from the immunomatrix at pH 3–5, i.e., in conditions where the receptor is not stable (not shown). In alkaline pH conditions the elution is observed at pH 10.5–11. In these conditions the steroid–receptor complexes are stable for up to 4 h (Figure 2B). In further experiments the receptor was thus eluted from the immunosorbent at pH 10.5–10.8.

Purification of Receptor. (*A*) *Purification of Steroid–Receptor Complexes.* Cytosol was incubated for 30 min at 0 °C with 50 nM [^{3}H]R5020 or [^{3}H]ORG.2058 in presence of a cocktail of protease inhibitors. The incubation was continued during chromatography. The cytosol was then passed on three successive columns: protein A–Sepharose, nonspecific antibody–protein A–Sepharose, antireceptor antibody–protein A–Sepharose. The latter column was washed as described under Materials and Methods, and receptor was eluted with alkaline pH: 61 ± 2% (mean ± SE, six experiments) of the chromatographed steroid–receptor complexes were bound by the immunosorbent, and 79 ± 5% (mean ± SE, six experiments) of the bound complexes were eluted. Thus, the overall yield (complexes eluted divided by complexes present in cytosol) was 49 ± 3% (mean ± SS, six experiments). When applied to a hydroxylapatite column, 80% of the eluted complexes were bound. The specific activity of the purified receptor–steroid complexes was 6.71 ± 0.79 nmol of bound steroid/mg of protein (mean ± SE of six experiments).

Electrophoresis in denaturing conditions (Figure 3, lane 1) showed the presence of two protein bands of 110 000 and 79 000 daltons. Western blot analysis (Figure 3, lane 2) confirmed that both bands interacted with the antireceptor monoclonal antibodies. A very faint band was also seen at 65 000 daltons. In the cytosol from which the receptor was purified, the 110 000-dalton protein represented 80–95% of the antibody-bound proteins (Figure 3, lane 3). Comparison between the starting material (cytosol) and the purified receptor thus suggested that the 79 000-dalton receptor species was formed by proteolysis during purification.

(*B*) *Purification of Receptor in the Absence of Steroid.* The same purification procedure was used with one exception: washing with pH 6 buffer was omitted. Analysis of the pu-

Table I: Purification of Receptor

	vol (mL)	protein (mg)	steroid–receptor complexes (pmol of bound steroid)	sp act. (pmol of bound steroid/mg of protein)	yield (%)	purification (measured by change in sp act.)[a]	purification (measured by yield of protein)[b]
			Purification in the Presence of Ligand				
cytosol	96	1461	3360	2.3			
eluate from immunosorbent	4	0.22	1575	7159	47	3112	6640
			Purification in the Absence of Ligand				
cytosol	90	1229	2581	2.1			
eluate from immunosorbent	4	0.18					6827

[a] Specific activity in eluate divided by specific activity in cytosol. [b] Amount of protein in cytosol divided by amount of protein in eluate.

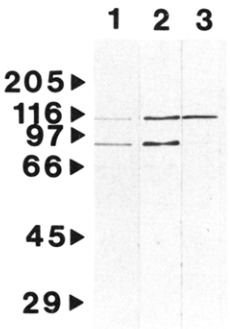

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of purified receptor: (lane 1) silver staining of purified receptor (0.1, μg); (lane 2) immunoblot analysis of purified receptor (1 μg); (lane 3) immunoblot of the uterine cytosol (250 μg of protein) used for the purification. Molecular weights ($\times 10^{-3}$) of marker proteins (Sigma) are shown by arrows.

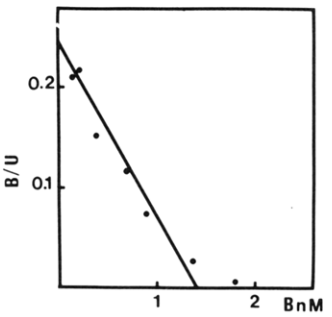

FIGURE 4: Binding of the progestin [^{3}H]ORG.2058 to purified progesterone aporeceptor. Aporeceptor was eluted from the immunomatrix with 50 mM Tris-HCl, pH 10.8–20% glycerol buffer. It was diluted 10-fold with 10 mM Tris-HCl, pH 8.3–30% glycerol buffer containing 5 mg/mL ovalbumin (Sigma). Aliquots (300 μL) were incubated for 2 h at 0 °C with [^{3}H]ORG.2058 (sp act. 47 Ci/mmol, concentration varying between 1.3×10^{-10} and 9.5×10^{-8} M). A series of incubations were also performed in presence of 3×10^{-6} M unlabeled ORG.2058 to correct for nonspecific binding. Bound hormone (B) was measured by the hydroxylapatite method (Erdos et al., 1970). U = unbound hormone.

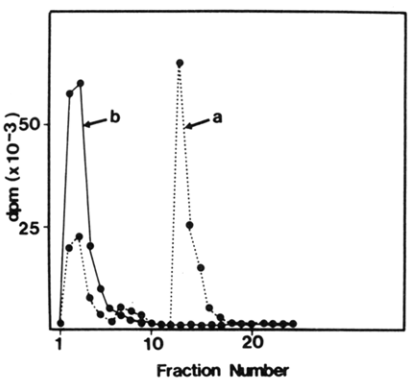

FIGURE 5: Binding to DNA–cellulose of purified [^{3}H]ORG.2058 receptor complexes. (a) Receptor–[^{3}H]ORG.2058 (sp act. 10 Ci/mmol) complexes were purified as described. An aliquot (330 pmol of bound steroid) was diluted with 1 volume of 10 mM Tris-HCl, pH 7–2 mM dithiothreitol buffer containing bovine serum albumin (6 mg/mL). The incubate was chromatographed on a column (0.7 mL, diameter 1 cm) of DNA–cellulose (Alberts & Herrick, 1971) (1 mg of DNA/mL of cellulose). The DNA–cellulose was washed with 10 mL of 10 mM Tris-HCl, pH 7.4, 2 mM dithiothreitol, and 30% glycerol buffer containing bovine serum albumin (3 mg/mL). Receptor was eluted with the same buffer containing 1 M NaCl. Fractions (1 mL) were collected, and aliquots (50 μL) were counted for radioactivity. (b) A parrellel chromatography was performed with a sample containing the same concentration of [^{3}H]ORG.2058 and the same buffer with bovine serum albumin to study a possible nonspecific (nonreceptor) binding of hormone to DNA–cellulose. DNA-bound receptor was eluted at fraction 13.

rified fraction by polyacrylamide gel electrophoresis followed by either silver staining or Western blot analysis showed the same pattern as that observed after purification of steroid–receptor complexes (not shown). Thus, the purity of receptor was similar when it was purified in either the presence or absence of steroid.

The protein yield (Table I) was also similar to that obtained with hormone-incubated cytosol (starting material: 90 mL of cytosol containing 1.23 g of protein; elution from the immunosorbent: 180 μg of protein). After incubation with radioactive hormone and measurement of steroid–receptor complexes, a specific activity of 3.17 nmol of bound steroid/mg of protein was found. Thus, if receptor purity is identical when purified in either the presence or absence of steroid, it indicates that slightly less than half of the purified aporeceptor molecules are in the native form, i.e., able to bind steroid.

Characterization of Purified Receptor. The affinity toward the hormone could only be studied in steroid-free receptor preparations. As shown in Figure 4, the purified aporeceptor exhibited the same affinity for its ligand (K_D = 5 nM) as when receptor was studied in crude cytosol (Fleischmann & Beato, 1978).

The property of binding DNA was studied by DNA–cellulose chromatography (Figure 5): 65% of the immunopurified steroid–receptor complexes were retained on the column from which 52% were eluted by high salt. Western blot analysis showed that both 110000- and 79000-dalton forms were bound by DNA–cellulose. No difference was seen in the proportion of both forms in the applied and salt-eluted receptors (not shown).

Density gradient ultracentrifugation was performed in both low- and high-salt conditions (Figure 6). In 0.3 M KCl, a peak of receptor was seen at 3.8 S, with some complexes sedimenting at the bottom of the tube. At low ionic strength a small peak was observed at 5.2 S, but most of the steroid–receptor complexes migrated as polydisperse aggregates.

Comparison by Enzymatic Proteolysis of the 110000- and 79000-Dalton Species of Receptor. Western blot experiments have shown that the cytosol contained in great majority the

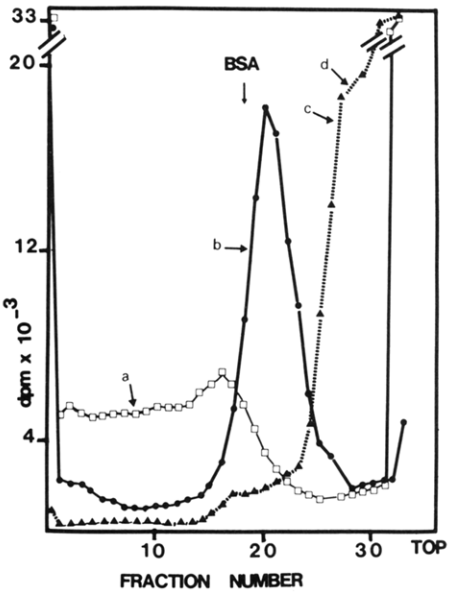

FIGURE 6: Glycerol gradient ultracentrifugation analysis of purified receptor. (a) [^{3}H]ORG.2058 (sp act. 10 Ci/mmol)–receptor complexes were purified as described. An aliquot (200 μL containing 66 pmol of steroid–receptor complexes) was diluted with 3 volumes of 10 mM Tris-HCl, pH 7.4–2 mM dithiothreitol buffer containing bovine serum albumin (3 mg/mL). Two hundred microliters was layered on a 10–30% glycerol gradient prepared in 50 mM Tris-HCl pH 7.4 buffer. Centrifugation in a SW 50.1 rotor lasted 16 h at 48 000 rpm at 4 °C. (b) The same experiment was performed, but the glycerol gradient was prepared in high ionic strength (the buffer contained 0.3 M KCl). (c and d) Nonspecific binding was studied by incubating the same buffer containing bovine serum albumin with the same concentration of [^{3}H]ORG.2058. Aliquots were centrifuged on low ionic strength (c) or high ionic strength (d) glycerol gradients. No difference was observed between curves c and d.

110 000-dalton species of receptor, whereas in the purified preparation both 110 000- and 79 000-dalton forms were found. The simplest interpretation of this observation was that the 79 000-dalton protein was a fragment of receptor arising by proteolytic cleavage from the 110 000-dalton form during purification. To test this hypothesis, we labeled the denatured purified receptor with ^{125}I and isolated both forms of receptor by gel electrophoresis in denaturing conditions. The isolated proteins were submitted to partial proteolysis by trypsin, chymotrypsin, and protease V8 of *Staphylococcus aureus*. The radioactive peptides derived from the 79 000- and 110 000-dalton forms of receptor were compared by gel electrophoresis. As shown in Figure 7, in all cases similar peptides were observed. The experiment was performed with short or long incubations, yielding large or small peptides. In all cases, comparison of peptides from both forms of receptor showed similar patterns (see, for instance, Figure 7C,D). These experiments also led to the conclusion that the 79 000-dalton form derives from the 110 000-dalton receptor.

Discussion

Although recent progress has been accomplished, especially in affinity chromatography methods, steroid–receptor purification has remained a very difficult task [see review in Eriksson & Gustafsson (1983)]. We have taken advantage of the availability of monoclonal antibodies to use immunoaffinity chromatography for the one-step purification of the rabbit progesterone receptor.

Cross-linking of the antibody through the Fc fragment to protein A–Sepharose (Schneider et al., 1982) allowed us to prepare an immunosorbent with a high capacity (over 1600 pmol of receptor/mL of gel). This property has been ascribed to the fact that the antigen binding sites are all kept functional as they are not involved in the bridging of the antibody to the matrix.

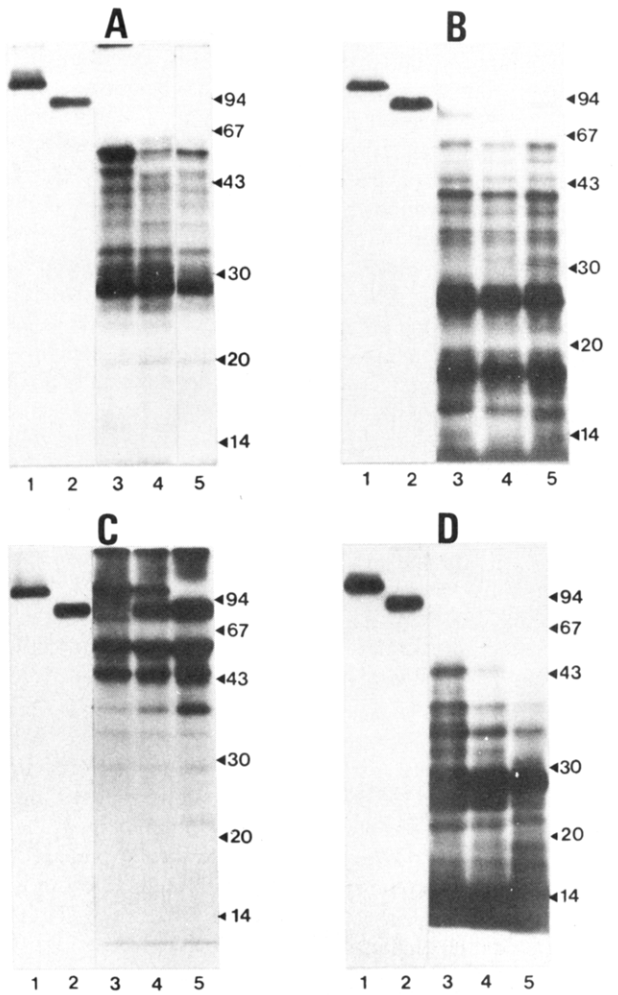

FIGURE 7: Comparison by enzymatic proteolysis of the 110 000- and 79 000-dalton forms of the progesterone receptor. The immunopurified receptor (120 pmol) was first denatured in 200 μL of 200 mM Tris-HCl pH 7.0 buffer containing 2% SDS, at 90 °C for 2 min, and was labeled with ^{125}I (1 mCi) by using Iodo Beads (Pierce Chemical Co., Rockford, IL) at room temperature for 15 min. After gel filtration through a G-25 Sephadex column (20 mM Tris-HCl pH 7.2 buffer containing 0.1% ovalbumin) and denaturation in Laemmli's (1970) sample buffer, the iodinated receptor (sp act. 4 μCi/pmol) was run on a 7.5% acrylamide slab gel. The 110 000- and the 79 000-dalton bands were identified by autoradiography at −80 °C and were sliced out with a razor blade. Each form was then electroeluted and electrodialyzed as described (Hunkapiller et al., 1983) in presence of 100 μg of protease-free bovine serum albumin (Bethesda Research Laboratories) as carrier protein, for 16 h. Partial proteolysis was performed as follows: 200 000 cpm of each form of the receptor was incubated at 37 °C with 0.05 μg of trypsin (TPCK-treated, from Sigma) (A), α-chymotrypsin (from Miles Laboratory) (B), or *S. aureus* V8 protease (from Miles Laboratory) (C and D), in 100 μL of 200 mM Tris-HCl pH 7.4 buffer containing 50 μg of protease-free bovine serum albumin. Incubation times were 2 min (A–C) or 30 min (D). The iodinated proteolytic peptides were analyzed by electrophoresis on 12.5% acrylamide gels, in denaturing conditions. (Lane 1) Nonproteolyzed 110 000-dalton form (20 000 cpm); (lane 2) nonproteolyzed 79 000-dalton form (20 000 cpm); (lane 3) proteolyzed 110 000-dalton form (100 000 cpm); (lane 4) mixture of the two proteolyzed forms (50 000 cpm of each one); (lane 5) proteolyzed 79 000-dalton form (100 000 cpm). Molecular weights ($\times 10^{-3}$) of marker proteins (from Pharmacia) are shown by arrows.

During chromatography of the uterine cytosol, the specific immunosorbent was preceded by two nonspecific gels: one simply made of protein A–Sepharose and the other involving a nonspecific IgG–protein A–Sepharose complex. This treatment enabled the elimination of proteins having an affinity

either to protein A or to IgG in general.

Use of immunoaffinity methods of purification often yields denatured proteins since drastic conditions are necessary to elute the antigen. However, monoclonal antibodies constituting a homogeneous population of molecules whose binding to antigen may be dependent on the ionization of a specific function in the antibody or in the antigen may be highly sensitive to ionic strength or pH conditions. A systematic search showed that receptor Mi60-10 antibody interaction was abolished at pH 10.5, in conditions where receptor retained its capacity to bind the hormone. The use of these mild elution conditions made it possible to prepare receptor in the native state, i.e., able to bind its ligand and to interact with DNA. It also made possible the purification of steroid-free receptor. The latter preparation will be very useful in studying the effect of hormones or antihormones on various functions of purified receptor as for instance binding to specific regions of cloned genes (Bailly et al., 1983). Such experiments have been impossible until now since receptor purification procedures, including affinity chromatography, have always yielded steroid–receptor complexes and the removal of ligand from pure steroid–receptor complexes without alteration of receptor having proved to be very difficult.

The purity of the receptor eluted from the immunosorbent was examined by two methods. Electrophoresis on polyacrylamide gels in denaturing conditions followed by staining showed two bands (110 000 and 79 000 daltons) both interacting with the antireceptor monoclonal antibody in Western blot experiments. Specific activity of the receptor preparation was 6.71 nmol of bound steroid/mg of protein. Theoretical maximal specific activity of an equimolar mixture of 110 000- and 79 000-dalton binders is 10.5 nmol/mg of protein. By this criterium, 64% of the protein species present in the eluate of the immunoaffinity chromatography were steroid binding species. The immunosorbent retains steroid–receptor complexes but also free and denatured receptor. Some dissociation of steroid–receptor complexes may possibly occur during the purification since the latter is performed in the absence of free ligand.

The purified receptor consists mainly of two molecular species of 110 000 and 79 000 daltons. In the case of chicken (Birnbaumer et al., 1983) or human receptors (Lessey et al., 1983), similar species have been observed, and it has been proposed that they represent different subunits of the progesterone receptor. The existence of two such subunits would be unique among steroid hormone receptors since purified estrogen and glucocorticoid receptors consist of a single subunit (Greene et al., 1979; Wrange et al., 1979). Our results favor the existence of a single subunit in the rabbit progesterone receptor, the other forms being due to proteolysis. One site in the molecule seems to be especially accessible to proteolytic enzyme(s), yielding the 79 000-dalton fragment. Several lines of evidence support this conclusion: previous studies (Loosfelt et al., 1984) and also the present work show that when the cytosol is cautiously prepared in the presence of protease inhibitors, only the 110 000-dalton form of receptor is observed. During the purification, the 79 000-dalton form appears. The same conversion of the 110 000-dalton receptor into a mixture of 110 000- and 79 000-dalton proteins is observed when the receptor is precipitated by ammonium sulfate (40% saturation). When proteolysis inhibitors are added during the precipitation, this conversion is prevented (Loosfelt et al., 1984). Ammonium sulfate precipitation enhances proteolysis by two possible mechanisms: increase of ionic strength (Sherman et al., 1983) and coprecipitation of intracellular proteases (Maeda et al., 1984; Nelson & Traub, 1982; Singh & Kalnitsky, 1978). Finally, a direct evidence of the relationship between the 110 000- and 79 000-dalton forms of receptor was obtained by comparing peptides generated by trypsin, chymotrypsin, and protease V8 from *S. aureus*. The conclusion that the existence of two (or more) subunits of progesterone receptor in other mammalian species is also due to proteolytic degradation is supported by recent immunoblot studies of receptors from guinea pigs, rats, hamsters, and humans (R. Pamphile, F. Logeat, and E. Milgrom, unpublished observations).

From a practical point of view, the obtention of a mixture of the 110 000- and 79 000-dalton forms of receptor is not a major problem in structural studies since electrophoretic separation followed by electroelution allows the isolation of each form in good yield (H. Loosfelt and F. Logeat, unpublished observations). However, although both forms bind hormone and DNA, other biological properties may be different, and we are currently testing various methods to inhibit completely the proteolysis of receptor or to separate the two forms by a nondenaturing process.

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Nicole Malpoint typed the manuscript.

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Equilibrium Constant for Binding of an Actin Filament Capping Protein to the Barbed End of Actin Filaments†

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ABSTRACT: Depolymerization of treadmilling actin filaments by a capping protein isolated from bovine brain was used for determination of the equilibrium constant for binding of the capping protein to the barbed ends of actin filaments. When the capping protein blocks monomer consumption at the lengthening barbed ends, monomers continue to be produced at the shortening pointed ends until a new steady state is reached in which monomer production at the pointed ends is balanced by monomer consumption at the uncapped barbed ends. In this way the ratio of capped to uncapped filaments could be determined as a function of the capping protein concentration. Under the experimental conditions (100 mM KCl and 2 mM $MgCl_2$, pH 7.5, 37 °C) the binding constant was found to be about 2×10^9 M^{-1}. Capping proteins effect the actin monomer concentration only at capping protein concentrations far above the reciprocal of their binding constant. Half-maximal increase of the monomer concentration requires capping of about 99% of the actin filaments. A low proportion of uncapped filaments has a great weight in determining the monomer concentration because association and dissociation reactions occur at the dynamic barbed ends with higher frequencies than at the pointed ends.

During the last years a number of proteins have been isolated that bind selectively either to the barbed or to the pointed ends of actin filaments. Most of the known proteins cap the barbed ends (Isenberg et al., 1980, 1983; Kilimann & Isenberg, 1982; Maruta & Isenberg, 1983). Proteins binding to the pointed ends of actin filaments have also been isolated (Maruyama et al., 1977; Southwick & Hartwig, 1982). These capping proteins have two effects. They inhibit association of monomers and dissociation of filament subunits at the ends where they bind. Furthermore, capping proteins shift the concentration of actin monomers coexisting with actin filaments (Brenner & Korn, 1979). When consumption of actin monomers at the lengthening barbed end of treadmilling actin filaments is blocked by capping proteins, filaments continue to produce monomers by release of subunits at the pointed ends. The actin monomer concentration increases. [For a recent review of treadmilling of actin, see Neuhaus et al. (1983).] Another group of actin filament binding molecules, the actin filament fragmenting proteins, has similar functions. They are able to insert themselves between the subunits in the actin filaments, causing them to disassemble. The actin filament fragmenting proteins cap the barbed ends of the broken filaments and inhibit them for reannealing (Yin & Stossel, 1979; Bretscher & Weber, 1980; Craig & Powell, 1980; Hasegawa et al., 1980; Hinssen, 1981).

In this study, we analyzed quantitatively the enhancement of the actin monomer concentration by a capping protein isolated from bovine brain. On the basis of known kinetic parameters of treadmilling of actin, it was possible to determine the equilibrium constant for binding of the capping protein to the barbed ends of actin filaments.

Materials and Methods

(*a*) *Preparation of the Proteins.* Actin was prepared according to the method of Rees & Young (1967). The protein was applied to a Sephacryl S-200 column (2.5 × 90 cm). Part of the protein was modified with *N*-ethylmaleimide at cysteine-374 and subsequently with 7-chloro-4-nitro-2,1,3-benzoxadiazole at lysine-373 to produce a fluorescently labeled actin (Detmers et al., 1981). The concentration of unmodified actin was determined photometrically at 290 nm by using an extinction coefficient of 24 900 M^{-1} cm^{-1} (Wegner, 1976). Fluorescently labeled actin was determined by the Lowry method (Lowry et al., 1951).

Capping protein was isolated from bovine brain according to the method of Kilimann & Isenberg (1982) with the following alterations: After hydroxylapatite chromatography the capping protein was concentrated on a DEAE-cellulose column (1.5 × 10 cm). Following each chromatography step the capping activity of the fractions was tested by the depolymerizing effect of the capping protein. Aliquots of the capping protein fractions were added to samples to give final concentrations of 2 μM polymeric actin (5% fluorescently labeled

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