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Oligomeric Structure of Molybdate-Stabilized, Nontransformed 8S Progesterone Receptor from Chicken Oviduct Cytosol†

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ABSTRACT: Bioaffinity-purified, molybdate-stabilized, nontransformed 8S progesterone receptor (8S-PR) of chicken oviduct cytosol has been analyzed first by ion-exchange chromatography, second by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as revealed by silver staining and immunoblotting with anti-8S-PR and anti-B-PR-subunit polyclonal antibodies, and third by progestagen affinity labeling and cross-linking with glutaraldehyde. Two forms (I and II) of 8S-PR were detected as described by Puri and colleagues [Puri, R. K., Grandics, P., Dougherty, J. J., & Toft, D. O. (1982) *J. Biol. Chem. 257*, 10831–10837]. They contained the non-progestin-binding 90-kDa protein [Joab, I., Radanyi, C., Renoir, M., Buchou, T., Catelli, M. G., Binart, N., Mester, J., & Baulieu, E. E. (1984) *Nature (London) 308*, 850–853] and progesterone-binding proteins described by Schrader and colleagues [Schrader, W. T., Birnbaumer, M. E., Hughes, M. R., Weigel, N. L., Grody, W. W., & O'Malley, B. W. (1981) *Recent Prog. Horm. Res. 37*, 583–599], that is, A (~79 kDa) in form I or B (~110 kDa) in form II. Cross-linked 8S-PR molecules (I and II) displayed an ~8S sedimentation coefficient and ~7.0-nm Stokes radius, similar to the values reported for non-cross-linked purified 8S-PR, and the mass was found to be 260 kDa by SDS–PAGE. From these results, from specific activity measurements of purified 8S-PR, and from densitometric scanning after SDS–PAGE of 8S-PR I and II, we propose that each molecule of 8S-PR includes one molecule of the hormone-binding subunit A or B and two molecules of the non-hormone-binding 90-kDa protein. The biological significance of the 8S-PR structures purified from the oviduct cytosol is discussed with reference to recent immunohistochemical findings indicating the simultaneous presence of progesterone-binding and non-progesterone-binding components of progesterone receptor in target cell nuclei [Gasc, J. M., Renoir, J. M., Radanyi, C., Joab, I., Tuohimaa, P., & Baulieu, E. E. (1984) *J. Cell Biol.* (in press)].

Two high-affinity progesterone-binding proteins denoted A and B have been described in the chick oviduct cytosol (Schrader & O'Malley, 1972; Kuhn et al., 1975; Coty et al., 1979; Gronmeyer et al., 1983; Renoir et al., 1984). Their characterization and purification have been accomplished under conditions that lead to the "activation" or "transformation" of the progesterone receptor (PR), such as ammonium sulfate precipitation (Schrader et al., 1977) or exposure to high salt (Renoir et al., 1984). However, all specific high-affinity progestin-binding constituents of low-salt cytosol sediment as 8S species (Toft & Nishigori, 1979; Wolfson et al., 1980). It has been proposed that the 8S molecules (8S-PR)[1] represent the "native" PR in the absence of hormone (Baulieu et al., 1983), that is, a "nontransformed" form of the receptor showing low affinity for polyanions (Nishigori & Toft, 1980; Wolfson et al., 1980). Under cell-free conditions, 8S-PR dissociates into 4S-transformed or activated PR forms from which progestin dissociation rates are slower than from 8S-PR and which bind strongly to polyanions [Wolfson et al., 1981; see Moudgil (1983) for a recent review].

By use of the stabilizing property of molybdate ions, 8S-PR was recently purified from chick oviduct cytosol (Renoir et al., 1982a; Puri et al., 1982). Coomassie Blue staining of disc SDS–PAGE revealed a 90-kDa protein as its main constituent (Renoir et al., 1982a; Puri et al., 1982). Immunological and bioaffinity chromatography studies (Joab et al., 1983, 1984;

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[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; 8S-PR, nontransformed molybdate-stabilized 8S progesterone receptor; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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Baulieu et al., 1983; Renoir et al., 1984) demonstrated that this protein was not able to bind progestin. The A and B subunits that had not been detected initially in the constitution of the 8S-PR due to the low sensitivity of Coomassie Blue staining were subsequently demonstrated by silver staining and by immunoblotting after SDS–PAGE of the purified 8S-PR (Baulieu et al., 1983).

In this paper, by demonstrating and quantifying the presence of the 90-kDa, A and B components in the affinity chromatography purified 8S-PR, we characterize the forms 8S-PR I and 8S-PR II detected by Dougherty & Toft (1982) as containing A subunit and 90-kDa protein and B subunit and 90-kDa protein, respectively. These results have been obtained with the use of specific antibodies raised against the 8S-PR (Renoir et al., 1982b) and the B subunit (Tuohimaa et al., 1984) and by photoaffinity labeling studies. From specific activity determination, scanning of SDS–PAGE gels, and cross-linking experiments of purified 8S-PR, we conclude that each nontransformed 8S-PR molecule includes one progesterone-binding unit (A or B) per two 90-kDa protein molecules.

Experimental Procedures

Chemicals. [2,4,6,7-^{3}H]Progesterone ([^{3}H]P), 87–110 Ci/mmol, and [^{3}H]Org 2058 (16α-ethyl-21-hydroxy-19-nor-[6,7-^{3}H]pregn-4-ene-3,20-dione) (47 Ci/mmol) were from the Radiochemical Centre (Amersham Bucks, U.K.), and the synthetic progestin [^{3}H]R 5020 [[17-*methyl*-^{3}H]-17,21a-dimethylpregna-4,9(10)-diene-3,20-dione] (50 Ci/mmol) was a gift from Roussel-Uclaf (Romainville, France). Nonradioactive cortisol and progesterone (>95% pure) were obtained from Roussel-Uclaf. DEAE-Trisacryl was from Industrie Biologique Française (Gennevilliers, France), DEAE-Sephacel from Pharmacia (Uppsala, Sweden), and DEAE-cellulose DE-52 from Whatman. All other chemicals were of reagent grade and obtained from Merck (Darmstadt, West Germany), except when noted.

Buffers. Buffer A contained 10 mM Tris/1.5 mM EDTA/10% (w/v) glycerol/12 mM 1α-thioglycerol, pH 7.4 at 25 °C. Na_2MoO_4 (20 mM) was added to buffer A to make buffer B. Buffer C was similar to buffer B, except that potassium phosphate (10 mM) replaced Tris.

Chickens and Oviduct Cytosol Preparation. The estrogen-stimulation schedule of Warren chickens, as well as the preparation of oviduct cytosol, has been described elsewhere (Wolfson et al., 1980; Renoir et al., 1982a).

Antibodies. Polyclonal immunoglobulins IgG-G3 obtained in a goat after immunization with purified 8S-PR have been described in Renoir et al. (1982b). Polyclonal immunoglobulins IgG-RB, obtained after immunization of a rabbit with purified B subunit, have been described in Tuohimaa et al. (1984).

Purification of Molybdate-Stabilized 8S-PR. Purification was performed as previously (Renoir et al., 1982a), but with the following modifications. (i) Two affinity columns (each ~30 mL) were used simultaneously to purify 8S-PR from the cytosol (~300 mL); after the cytosol was loaded, the gel was washed at a flow rate >100 mL/h successively with 3 column volumes of buffer B, then with 3 column volumes of buffer B containing 0.3 M KCl, 10 column volumes of buffer B, and 3 column volumes of buffer B containing 2.5 M urea. Under these conditions the receptor remains in an 8S form (Buchou et al., 1983). Finally, the affinity gel was equilibrated with 10 column volumes of buffer B. (ii) Specific elution of the receptor was performed by overnight exchange with [^{3}H]P or [^{3}H]Org 2058, specific activity of ~8 Ci/mmol, or [^{3}H]R 5020, specific activity of ~50 Ci/mmol. (iii) Affinity chromatography eluates (~120 mL) of the two affinity gel columns were loaded on top of 1 mL of DEAE-Sephacel packed in a 10-mL column (flow rate ~30 mL/h). The Ultrogel AcA-34 filtration step previously used (Renoir et al., 1982a) was omitted since no better purification was obtained and because it frequently led to losses of hormone binding.

Resolution of A and B Subunits from Purified 8S-PR by DEAE-Sephacel Chromatography. The affinity-purified molybdate-stabilized 8S-PR was chromatographed on DEAE-Sephacel 1- or 2-mL columns (flow rate ~30 mL/h) in order to detect A and B subunits (Schrader et al., 1972, 1977). After being loaded, the column was washed with 10 mL of buffer A. Elution was performed either by a linear 0–0.5 M KCl gradient (flow rate ~10 mL/h) or stepwise with 0.15 and 0.3 M KCl in buffer A.

Steroid Binding Activity. It was determined as in Renoir et al. (1984).

Density Gradient Ultracentrifugation. Sedimentation coefficients were measured by 10–35% glycerol gradient analysis as in Renoir et al. (1982b), with glucose oxidase, $s_{20,w}$ = 7.9, and peroxidase, $s_{20,w}$ = 3.6, as internal markers.

Affinity Labeling of Purified [^{3}H]R 5020 PR Complexes. The excess of ligand present in affinity chromatography eluates first was removed by charcoal adsorption. Then, 1-mL samples were placed in a Petri dish (liquid layer ~2 mm thick), at 1 cm from a refrigerated UV lamp (150-W mercury lamp, Hanau TQ 150, Heraeus, West Germany) shielded by Pyrex, so that wavelengths ≤300 nm were eliminated (Dure et al., 1980). The dish was kept at 0–2 °C. The samples were irradiated for 2.5 min, a time that gave us the maximum covalent attachment of [^{3}H]R 5020 with minimum protein degradation. Then, 50 μL of crude oviduct cytosol was added; this cytosol had been preincubated with 1 μM progesterone and 1 μM cortisol in order to prevent the binding of [^{3}H]R 5020 possibly released from purified receptor to PR or transcortin present in the added cytosol. The affinity-labeled samples were concentrated by precipitation with ammonium sulfate at 50% saturation, and after 18 h at 0 °C, the precipitate was collected by 20-min centrifugation at 1500*g*, redissolved in buffer A adjusted to 1% SDS and 1% β-mercaptoethanol, and boiled for 3 min prior to electrophoresis.

Electrophoresis, Gel Staining, Scanning, and Fluorography. Denaturing electrophoresis was performed in polyacrylamide slab gel according to Laemmli (1970). Samples contained 0.2–2 μg of purified PR or of molecular weight markers (MW-SDS-200 kit, Sigma, St Louis, MO). Gels were stained by silver nitrate (Wray et al., 1981). For scanning, each stained lane was cut out, and the absorbance was recorded in a DU8 Beckman spectrophotometer. The values corresponding to the area of the absorbance peaks were plotted for different amounts of the same PR preparation.

For analysis of cross-linked 8S-PR, the Laemmli (1970) technique was used in 5–7.5% acrylamide gradient. Electrophoreses were carried out at room temperature under 35 mA/gel for 4 h. Molecular weight values were determined by the method of Weber & Osborn (1969) by reference to calibration curves obtained with cross-linked hemocyanin from *Limulus polypherius* (Sigma; 70, 140, 210, and 280 kDa for the monomer and the three polymers, respectively).

Before fluorography, the gels were dried, enhanced 1 h, and exposed to a Kodak X-O-MAT-AR5 film at −70 °C for 4 weeks.

Immunoblotting of PR. After SDS–PAGE (10% polyacrylamide), the proteins were transferred by diffusion to

nitrocellulose filters (Schleicher & Schüll; 0.45-μm pore size) according to Burnette (1980) and Bowen et al. (1980). The filters were incubated with IgG-G3 and IgG-RB (5–10 mg/mL). A second ^{125}I-labeled antibody ($\sim 3 \times 10^5$ cpm/mL) was used to detect anti-PR antibodies. Rabbit or goat IgG's obtained after 35% ammonium sulfate precipitation of the antisera raised in our laboratory were used for this purpose. The filters, after being extensively washed, were placed for 5 h at −70 °C, with Kodak X-O-MAT-AR5 film.

Protein Determination. The method of Schaffner & Weissmann (1973) was used with BSA as a standard. At least two different aliquots containing more than 4 μg of protein (2 times the lower detection limit of the assay) were measured for each sample. When buffer C was used, samples on nitrocellulose paper were washed with 1 M Tris, pH 7.4, and then with bidistilled water, to avoid formation of phosphomolybdenum complexes that would interfere with subsequent staining by amido-black.

Analytical Ultrogel AcA-22 Filtration. To determine the Stokes radius of PR samples, gel filtration was performed on Ultrogel AcA-22 columns (30 × 1.5 cm) equilibrated in buffer A containing 0.3 M KCl or in buffer B containing 0.15 M KCl and BSA (2 mg/mL). The flow rate was ~9 mL/h. Standard curves were plotted as described by Porath (1963): $K_d = V_e - V_0/V_t - V_0$, where V_e is the elution volume of the protein being studied and V_0 and V_t are the void and the total volumes of the column, respectively.

Cross-Linking of Purified 8S-PR. 8S-PR was purified in buffer C, according to exactly the same methodology as with buffer B, since cross-linking experiments with glutaraldehyde cannot be performed in the latter. The same yield of purification and specific activity were obtained with both buffers. Immediately after elution from the DEAE-Sephacel column, [^{3}H]progestin 8S-PR samples (400 μL) were cross-linked with glutaraldehyde (0.4% final concentration) at 0 °C. To determine the rate of the cross-linking reaction, buffered lysine (0.15 M final concentration) was added at different times to stop the reaction. After 30-min incubation at 25 °C, pure 8S-PR not covalently linked was dissociated by a 1-min incubation at 60 °C in the presence of electrophoresis buffer, and proteins were precipitated by overnight incubation at 25 °C followed by centrifugation (5 min at 10000*g*). The pellet was dissolved in 0.4 mL of 6 M urea and boiled for electrophoresis. For gel filtration and ultracentrifugation analyses, cross-linked samples (1 mL) treated for 1 h at 0 °C were layered directly on the top of the column or of the preformed glycerol gradients.

Counting of Radioactivity. Samples were mixed with 7 mL of Scintimix (0.4% in toluene) and counted in a Packard liquid scintillation spectrometer (~40% counting efficiency).

Results

Composition of Purified 8S-PR. (A) PAGE Analysis. Purified 8S-PR, obtained from molybdate-containing cytosol after affinity chromatography and ion-exchange chromatography (Renoir et al., 1982a), was analyzed in SDS slab gel electrophoresis and then proteins were revealed by silver staining. Three major bands were observed (Figure 1, lane 4). One corresponded to the 90-kDa protein previously detected by disc electrophoresis and Coomassie Blue staining (Renoir et al., 1982a) and was apparently the most abundant. The two other proteins migrated at 110 ± 4 kDa (mean ± SD; number of different preparations n = 15) and 79 ± 3 kDa (n = 15), respectively. The 90-kDa protein was not present in affinity chromatography eluates when, prior to purification, the cytosol was treated with 0.3 M KCl (in the absence of molybdate) [Figure 1, lane 2, and Renoir et al. (1984)] or when molybdate-containing cytosol was preincubated with 1 μM radioinert progesterone (Figure 1, lane 3). The 110- and 79-kDa proteins showing the same molecular size as the B and A subunits described by Schrader et al. (1981) were purified by affinity chromatography from KCl-treated cytosol (Figure 1, lanes 5 and 6) (Renoir et al., 1984) but were not found after "mock" purification in the presence of nonradioactive progesterone. These results demonstrated that the 90-kDa protein present in the 8S-PR is a nonhormone-binding protein [see also Baulieu et al. (1983) and Joab et al. (1983, 1984)] associated with A and/or B hormone-binding subunits in the 8S-PR. They suggested that molybdate ions stabilize the association and that the 90-kDa protein "follows" A and B during bioaffinity chromatography, while 8S-PR is dissociated by KCl (Yang et al., 1982) in the absence of molybdate and only A and B subunits are then retained by the bioaffinity column.

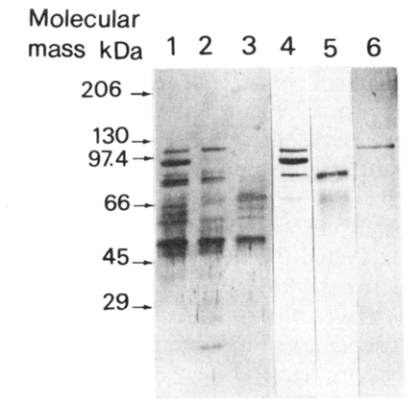

FIGURE 1: Chicken oviduct cytosol progesterone receptor: SDS–PAGE of 8S-PR, activated 4S-PR, and purified A and B proteins. Electrophoresis was performed in 7.5–15% polyacrylamide gradient (0.1% SDS) (Laemmli, 1970). 8S-PR and 4S-PR, partially purified after elution from affinity gel, were analyzed in lanes 1 and 2, respectively. While 110-kDa (B) and 79-kDa (A) bands are observed on both lanes, the 90-kDa protein strongly stained on lane 1 is practically undetectable on lane 2. (Lane 3) "Mock" purification of 8S-PR: low-salt cytosol containing 20 mM molybdate and 2 μM progesterone was passed through affinity gel, and after the usual KCl and urea washing, elution was performed with [^{3}H]P. Neither A, B, nor 90-kDa bands are detected. Control hormone-binding tests indicated that no receptor was eluted from the column under these conditions. (Lane 4) Purified 8S-PR eluted from DEAE-Sephacel column: A, B, and the major 90-kDa bands are observed. (Lanes 5 and 6) A and B proteins were purified as described in Renoir et al. (1984). Gels were stained with silver nitrate. Molecular mass of markers is indicated by arrows [for molecular mass references of markers, see Renoir et al. (1982a, 1984)].

(B) Specific Activity Measurements. The specific activity of 8S-PR obtained after affinity and ion-exchange chromatography (Table I) was calculated on the basis of one hormone-binding site per protein of 100 kDa; we found that PR was 30 ± 8% pure (mean ± SD, n = 16), confirming the preponderance of the non-progesterone-binding 90-kDa protein in the purified preparations. The final yield was ~15 ± 5% (n = 16).

(C) Ion-Exchange Chromatography. The labeled 8S-PR obtained after bioaffinity chromatography was loaded on DEAE-Sephacel in molybdate-containing buffer B, and after this was washed with buffer A containing no molybdate, the PR was eluted with a KCl gradient, still in the absence of molybdate (Figure 2). Two radioactive peaks were eluted; the first one, obtained at 0.10–0.15 M KCl, represented <25% of the total eluted radioactivity (n = 7); the second radioactive peak was eluted at 0.20–0.25 M KCl. Those radioactive fractions migrated at an ~4S position in glycerol density

Table I: Purification of Chicken Oviduct Cytosol, Molybdate-Stabilized, 8S Progesterone Receptor[a]

	vol (mL)	binding act. (pmol/mL) (*a*)	proteins (mg/mL) (*b*)	sp act. (*a*/*b*)	yield (%)	*x*-fold purification
cytosol	220	34	17.2	1.98	100	
affinity chromatography	120	21	0.0118	1780	34	899
DEAE-Sephacel	17	89	0.0316	2816	20	1422

[a] A total of 16 experiments was performed. Elution from the affinity gel was obtained by [^{3}H]P (n = 12), [^{3}H]R 5020 (n = 3), or [^{3}H]ORG 2058 (n = 1), the final concentration of ligand being 0.5–2 μM. DEAE-Sephacel (n = 12), DEAE-cellulose DE-52 (n = 1), or DEAE-Trisacryl (n = 3) was used at the ion-exchange step. In each case, the two peaks I and II of the ion-exchange column were pooled. Purified 8S-PR was systematically submitted to SDS–PAGE and revealed by silver nitrate. A, B, and the 90-kDa proteins always wer found, and very minor bands were occasionally observed. The specific activity of original cytosol was 1.9 ± 0.6 pmol/mg of protein (n = 16), and the specific activity of the receptor after ion-exchange column was 2922 ± 980 pmol/mg of protein (n = 16). The purity, calculated as (final purification factor)/("theoretical" purification factor) × 100 was 30 ± 8% (n = 16). In the purification reported in detailed in the table, specific activity of the cytosol was 2 pmol/mg of protein, specific activity of the receptor was 2820 pmol/mg of protein, and purity was 28%.

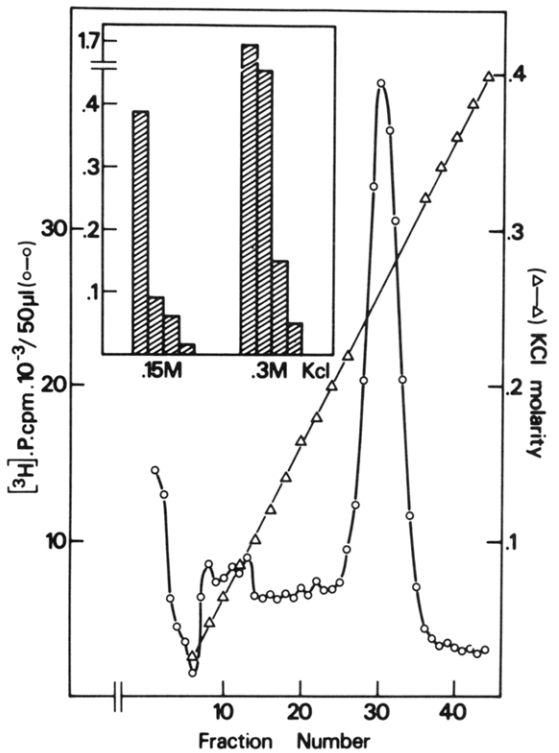

FIGURE 2: Identification by ion-exchange chromatography of A and B proteins in 8S-PR. After elution from affinity gel, the [^{3}H]8S-PR was loaded onto a 2-mL DEAE-Sephacel column equilibrated in buffer B and washed with 10 column volumes of buffer A. In the experiment reproduced in the main drawing, elution was obtained with a 0–0.5 M KCl gradient in buffer A (200-mL total volume). Fractions of 4 mL were collected. Two peaks, A and B, were obtained. In the experiment reported in the insert, stepwise elution was performed by 0.15 and 0.3 M KCl on 0.2-mL DEAE-Sephacel column loaded with $^1/_{100}$ of the affinity gel eluted fraction. Each bar represents the radioactivity eluted in 1-mL fractions. In both experiments, peak A was <15% of total eluted PR.

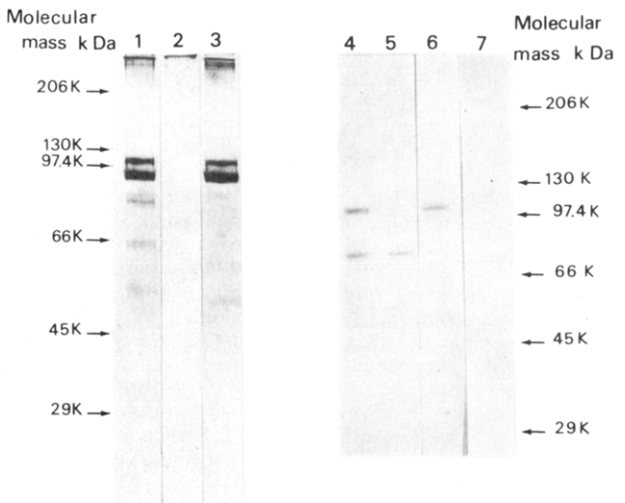

FIGURE 3: Photoaffinity labeling of purified 8S-PR and activated 4S-PR forms. The [^{3}H]R 5020–8S-PR eluted from the affinity gel was loaded on a DEAE-Sephacel column, and stepwise elution was used, as in the insert of Figure 2, in order to concentrate the receptor prior to UV irradiation. The affinity gel eluate (lane 1) and the fractions eluted at 0.15 M KCl (lane 2) and 0.30 M KCl (lane 3) from the DEAE-Sephacel were loaded on 7.5–15% polyacrylamide gradient SDS–PAGE and stained with silver nitrate. Corresponding samples (lanes 4–6), UV irradiated at 0 °C for 2.5 min and then treated as described under Experimental Procedures, were loaded on 10% polyacrylamide SDS–PAGE and fluorographed. Before UV treatment, an aliquot (1 mL) of PR eluted at 0.30 M KCl was treated with 2 μM cold P during 2 h at 25 °C, in order to exchange the radioactive ligand (lane 7). Note that the 90-kDa band, which is seen by silver staining (lanes 1 and 3), was never detected by fluorography. B and A (lane 4), A (lane 5), and B (lane 6) are detected by fluorography while isotopic dilution eliminated R 5020 affinity labeling (lane 7).

gradients (not shown). By SDS–PAGE, the 79-kDa peptide was found in the first fraction, plus some contaminants (Figure 3, lane 2); the second peak (Figure 3, lane 3) contained both 90- and 110-kDa proteins. Further experiments demonstrated that the elution profiles of 90-kDa protein and of the B subunit of PR are only partially superimposed: the 90-kDa protein, detected by immunoassay, was eluted slightly before the 110-kDa subunit (data not shown). These results of ion-exchange chromatography supported the conclusion that the 79- and the 110-kDa proteins were the A and B subunits of PR, respectively.

(*D*) *Affinity Labeling.* [^{3}H]R 5020–8S-PR, obtained after the bioaffinity chromatography step, and the two radioactive fractions separated stepwise by DEAE-Sephacel chromatography in the absence of molybdate were submitted to UV irradiation under the above-described conditions. Analysis by SDS–PAGE (Figure 3) followed by fluorography indicated after bioaffinity chromatography (lane 4) photolabeled bands at the level of A and B and after ion-exchange chromatography only A in the first fraction (lane 5) and only B in the second fraction (lane 6). The 90-kDa protein was present in this latter fraction and was revealed by silver staining (Figure 3, lane 3), but no progestin labeling was found at its level. The same results were obtained whether cytosol was added before UV irradiation or cytosol was added after UV irradiation. Incubation of [^{3}H]R 5020 labeled preparations with an excess of nonradioactive progesterone prior to UV irradiation abolished the labeling of binding subunits by [^{3}H]R 5020 (Figure 3, lane 7, for example), confirming that only the specific progestin-binding proteins were labeled by [^{3}H]R 5020 in UV-irradiated preparations.

Polymorphism of 8S-PR. (*A*) *Separation of Forms I and II.* Molybdate-stabilized 8S-PR can be resolved by ion-ex-

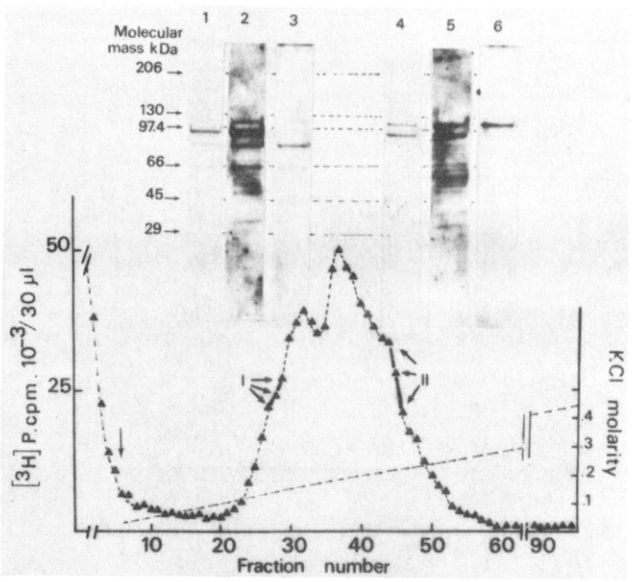

FIGURE 4: Polymorphism of 8S-PR. 8S-PR eluted from affinity gel was chromatographed on DEAE-Sephacel in the presence of molybdate and eluted with a 0–0.5 M KCl gradient. Two overlapping radioactive peaks were observed. Fractions of the ascending part (27–29) and of the descending part of the radioactive peak (44–46) were studied separately by SDS–PAGE and immunoblotting. (Lanes 1 and 4) Silver staining performed after transfer onto two nitrocellulose papers; (lanes 2 and 5) immunoblotting of the corresponding paper with IgG-G3; (lanes 3 and 6) immunoblotting with IgG-RB. The presence of the 90-kDa protein in 8S-PR I and 8S-PR II is revealed by silver staining and by IgG-G3 in lanes 1 and 2 and in lanes 4 and 5, respectively. The A protein in 8S-PR I and B protein in 8S-PR II are detected by the three techniques.

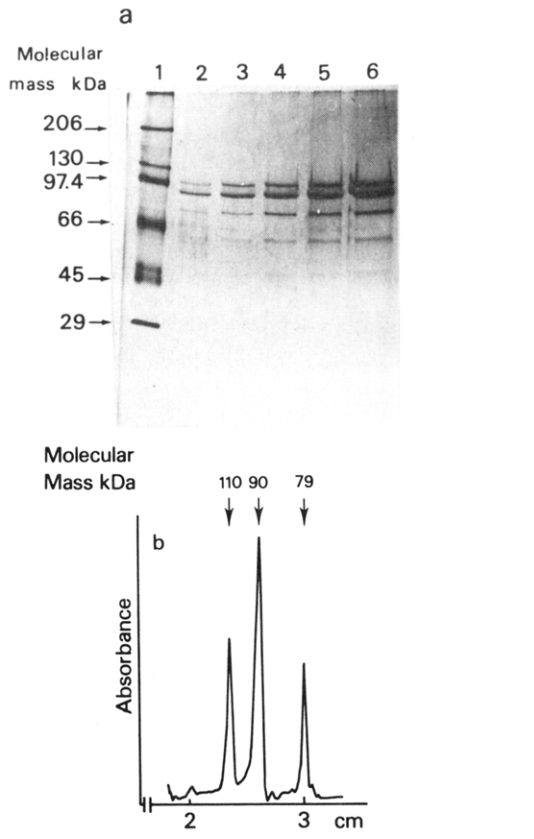

FIGURE 5: Scanning of SDS–PAGE of 8S-PR. (a) SDS–PAGE (7.5–15% polyacrylamide gradient) was loaded with different aliquots of 8S-PR eluted from DEAE-Sephacel chromatography and silver stained (lanes 2–6) 25, 50, 75, 100, and 150 µL of 8S-PR (38% purity; 17 µg of PR/mL); (lane 1) markers. (b) Scanning of lane 4 in the DU8 spectrophotometer.

change chromatography into two forms called I and II according to Dougherty & Toft (1982) and Puri et al. (1982). We have also observed two peaks of 8S-PR, when applying a shallow KCl gradient in elution from the DEAE column (Figure 4). However, the two peaks were not completely separated, irrespective of the buffer used (Tris or phosphate) or of the column material (DEAE-Sephacel, DEAE-Trisacryl, or DEAE-cellulose). The first peak was eluted at ~0.1 M KCl and the second peak at ~0.16 M KCl, the same as obtained with crude preparations (Dougherty & Toft, 1982).

(*B*) *Analysis by SDS–PAGE.* 8S-PR I and II were analyzed by SDS–PAGE. Due to the overlapping elution of the two forms, samples for analysis were not taken from the summit but from the rising part of the first peak and from the descending part of the second peak as shown in Figure 4. Silver staining indicated predominantly the presence of A in 8S-PR I and of B in 8S-PR II, while the 90-kDa protein was present as the strongest band in both PR I and PR II (Figure 4, lanes 1 and 4). Immunoblotting experiments with IgG-G3 confirmed the presence of the 90-kDa protein in both form I and form II. Immunoblots done both with IgG-G3 and with IgG-RB showed that the A subunit was more represented than the B subunit in 8S-PR I and that only the B subunit was present in 8S-PR II. IgG-G3 also revealed the presence of some B protein in the analyzed fractions of peak I, probably due to imperfect separation of the two peaks. This antibody showed also other weak bands (contaminants or proteolytic fragments).

SDS gels loaded with different amounts of total 8S-PR (I + II), eluted from DEAE-Sephacel, and revealed by silver staining (Figure 5a, lanes 2–6) were scanned (Figure 5b); proportionality was established between the amount of 8S-PR protein loaded onto the gels and the densitometric assay for the 90-kDa protein and the sum of A + B subunits (data not shown). The ratio of the 90-kDa protein per binding units (A + B) was 2.2 ± 0.3 (three different preparations, five aliquots in each). When 8S-PR I and II were analyzed separately, the ratio of the 90-kDa protein to A in I was 1.9 ± 0.2 (three aliquots), while the ratio of the 90-kDa protein to B in II was 2.1 ± 0.3 (three aliquots).

Chemical Cross-Linking of Purified 8S-PR with Glutaraldehyde. (*A*) *Analysis by SDS–PAGE.* Purified cross-linked 8S-PR (see Experimental Procedures) was analyzed by SDS slab gel electrophoresis in a 5–7.5% polyacrylamide gradient. After silver staining, two diffuse bands were detected already after 5-min incubation with glutaraldehyde, and their intensity increased with time of cross-linking treatment. Concomitantly, we observed the disappearance of the three proteins initially present in the purified 8S-PR (Figure 6). The minor cross-linked band corresponded to ~190 kDa, and the more intense one to ~260 kDa. These values are not precise due to the width of the bands, which can be explained by the number and length of (poly)glutaraldehyde chains and/or the number of charged lysine groups surrounding the cross-linked products. Possibly also for these reasons, no further immunological characterization was possible. Attempts to obtain fluorograms of cross-linked [^{3}H]R 5020 photolabeled purified 8S-PR were unsuccessful (too low density of radioactivity). The same two bands as those seen in Figure 6 were observed when 8S-PR I and 8S-PR II obtained from ascending and descending parts of the radioactive peak from ion-exchange chromatography were cross-linked separately (not shown).

(*B*) *Hydrodynamic Parameters.* The sedimentation coefficient and Stokes radius of cross-linked purified 8S-PR were $s_{20,w}$ = 8 and R_s = 7.0 nm, identical with those of purified

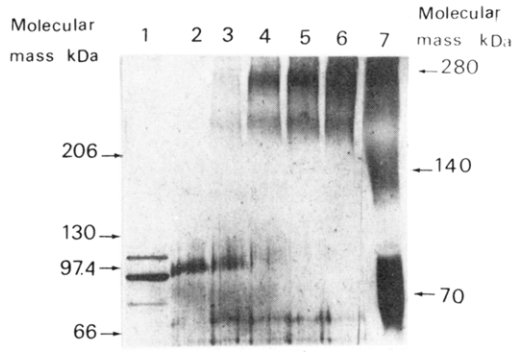

FIGURE 6: Cross-linking of purified 8S-PR with glutaraldehyde. 8S-PR was purified by affinity and ion-exchange chromatography in phosphate buffer containing molybdate. The receptor was immediately treated at 0 °C with glutaraldehyde (0.4% final concentration). After 0-, 1-, 5-, 15-, 30-, and 60-min incubation with glutaraldehyde, cross-linked PR samples were analyzed on 5–7.5% polyacrylamide gradient SDS–PAGE (lanes 1–6). Lane 7 shows cross-linked hemocyanin used as marker for cross-linked samples (lanes 2–6). Non-cross-linked standard proteins are indicated by arrows on the left.

non-cross-linked 8S-PR measured in buffer B (Baulieu et al., 1983), whether the cross-linked 8S-PR was analyzed in the presence or absence of 0.3 M KCl without molybdate. Calculation according to Siegel & Monty (1966), using partial specific volume $\bar{V} = 0.74$ cm^3/g, gave ~240 kDa.

Discussion

This paper demonstrates that the 8S-PR of the chicken oviduct cytosol is heterogeneous in its protein composition and that it is polymorphic, since distinct proteins are present in the structure of the 8S-PR I and 8S-PR II forms. A tentative model that involves, per molecule of 8S-PR, one molecule of a progestin-binding protein and two molecules of a non-progestin-binding protein is proposed on the grounds of our quantitative estimations.

8S-PR molecules of crude cytosol are stabilized by molybdate ions, which enable them to resist the dissociating effect of increased ionic strength. Under specific conditions, using, first, homogenization in 0.15 M KCl that approaches physiological ionic strength and, second, molybdate ions added to stabilize the complexes, we have obtained strong suggestion that 8S-PR is not an artifact due to low salt and/or molybdate conditions (Baulieu et al., 1983). The protein composition of the 8S-PR has been a matter of controversy. The presence of a 90-kDa protein in purified 8S-PR, largely preponderant as visualized by SDS–PAGE, has been reported by us (Renoir et al., 1982a) and by others (Puri et al., 1982). This was in contradiction with already demonstrated A and B progesterone-binding 4S subunits purified from ammonium sulfate precipitates of the cytosol (Schrader et al., 1977, 1981). We have found later that a monoclonal antibody BF4 (Radanyi et al., 1983), obtained after immunization of a rat with purified 8S-PR, reacted with a non-hormone-binding 90-kDa component of crude or purified 8S-PR (Joab et al., 1983, 1984). In addition, we have observed that, when purifying PR from high salt (0.3 M KCl) treated cytosol with the same affinity column that yielded 8S-PR from low-salt cytosol, we obtained only the A and B proteins and not the 90-kDa component (Renoir et al., 1984). The present data indicate the presence of A, B, and 90-kDa proteins in 8S-PR. Several techniques concur to identify them: SDS–PAGE indicating molecular weight, immunoblotting with IgG-G3 revealing the three different peptides and with IgG-RB detecting B (it has been raised against the B protein) and A but not the 90-kDa protein, photoaffinity labeling of A and B but not of 90-kDa unit, and ion-exchange chromatography showing the same elution pattern of radioactive components included in the purified 8S-PR as for A and B separately purified. Silver staining also revealed other bands of variable although always low intensity, especially at ~55, 45, and 20 kDa; their significance remains unknown. Specific activity determinations of purified 8S-PR (16 different purifications) suggested, on the basis of one binding site per 100 000 daltons, that ~$^2/_3$ of the protein included in purified 8S-PR does not bind hormone.

Cross-linking experiments, of interest to investigate the structure of oligomeric proteins (Richards & Knowles, 1968), also argue in favor of a very close interaction of the 90-kDa protein with the A and/or B hormone-binding units and against the concept of a randomly contaminating protein. The 90-kDa protein is not artificially selected by the bioaffinity column as suggested by Birnbaumer et al. (1984), since, in presence of an excess of nonradioactive progesterone, even if there are molybdate ions present, not only A and B but also the 90-kDa protein are *not* retained. The same lack of so-called artifactual retention of this 90-kDa protein by the affinity chromatography column is observed when KCl-treated cytosols are studied and only the A and B subunits are retained (Renoir et al., 1984). Urea treatment used during affinity chromatography has been very important to eliminate the non-PR-associated 90-kDa protein that is present in excess in the cytosol over the amount of A and B PR subunits (Baulieu et al., 1983). Finally, since the BF4 monoclonal antibody reacts with crude 8S-PR even in the absence of molybdate (Joab et al., 1984), it does not appear that molybdate is responsible for the interaction of the 90-kDa protein with A and/or B, although it stabilizes this association and the 8S-PR structure. The presence of non-hormone-binding protein(s) in the 8S form of bovine and porcine uterus cytosol estradiol receptor (Murayama et al., 1980a) of the chick oviduct PR (Murayama et al., 1980b) and in the androgen receptor of the rat prostate Dunning tumor (Colvard & Wilson, 1981) has been reported, but the lack of physicochemical and immunological characterization of purified material precludes further comparison with the present data.

Dougherty & Toft (1982) recently demonstrated the polymorphism of 8S-PR since they could resolve in crude cytosol two entities ("I" and "II") by ion-exchange chromatography. We confirm their finding with purified 8S-PR, although we could not separate completely form I from form II, and we could analyze their protein composition by using SDS–PAGE revealed by silver staining and by immunoblotting with specific antibodies. We show that 8S-PR I and 8S-PR II both contain the 90-kDa protein and the A and B subunit, respectively. Scanning of 8S-PR I and 8S-PR II preparations after SDS–PAGE and silver staining gave results compatible with a stoichiometry of two 90-kDa molecules for one progesterone-binding A or B subunit, respectively.

Cross-linking experiments performed for the first time with highly purified 8S-PR preparations demonstrated ~260 kDa after SDS–PAGE analysis. Hydrodynamic parameters of cross-linked 8S-PR, $s_{20,w} \sim 8$ and $R_s \sim 7.0$ nm, indicated that it is hormone binding and likely represents the original 8S-PR. These values are in agreement with values found for crude and purified non-cross-linked 8S-PR (Wolfson et al., 1980; Renoir et al., 1982a; Puri et al., 1982). The mass calculated on the basis of these parameters (240 kDa) is an aproximation, but it agrees well with the value obtained by SDS–PAGE.

It is difficult to reconcile our data with either a tetramer structure (Miller et al., 1975; Sherman et al., 1984) or a 6S

dimer structure (Schrader et al., 1975) [incidentally, our results are compatible with those of Birnbaumer et al. (1979), indicating an ~8S sedimentation coefficient for crude cross-linked PR, which, however, does not correspond to the A–B dimer structure suggested by the same group]. The weak 190-kDa band in SDS–PAGE that we observed after glutaraldehyde treatment may be due to the linkage of two of the three molecules constituting 8S-PR. Since no evidence for a corresponding radioactive peak has been obtained in ultracentrifugation and gel-filtration experiments with cross-linked 8S-PR, it is possible that the cross-linked 190-kDa structure is composed of two 90-kDa units, dissociated from 8S-PR and forming a dimer. This should lead to a transformation of the corresponding amount of progestin-binding units from the 8S to 4S form, and indeed, a small 4S peak of bound [^{3}H]-progesterone was detected in glycerol density gradient centrifugation of glutaraldehyde cross-linked preparations (not shown). Preliminary experiments have suggested the presence of such nonreceptor-associated 90-kDa dimers in crude cytosol (C. Radanyi, unpublished results).

The biological significance of the polymeric structure of the 8S-PR awaits further experiments. A preliminary paper has indicated distinct protein kinase activities of different purified PR preparations (Garcia et al., 1983). The sequence similarities (Birnbaumer et al., 1983a,b; Gronmeyer et al., 1983) and immunological cross-reactivity (Tuohimaa et al., 1984) suggest much analogy between A and B, raising an important question related to the previous data reporting difference of DNA binding ability of the two subunits (Schrader et al., 1981). The possibility of different mechanisms implicated in receptor activation by different agents, in the presence or absence of hormone (Yang et al., 1982), also remains the matter of further investigation.

Finally, it is important to mention the recently obtained immunohistochemical data (Baulieu et al., 1983; Gasc et al., 1983, 1984); they demonstrate the simultaneous presence of 90-kDa and A and/or B proteins in the nucleus of target cells of the oviduct, in the presence or absence of progesterone. Although these immunohistochemical observations are not quantitative and do not prove the interactions between the visualized antigens, they suggest that the 8S structure may be natural and present in the nucleus of target cells where it would be the "native" "nonactivated" PR. Our findings are in agreement with more recently published immunohistochemical data for estradiol receptor localization (King & Greene, 1984) and with results of Welshons et al. (1984) that indicated that cytosoluble steroid receptor may represent an "extraction artifact". Finally, although if the 90-kDa protein is present in an excess in the chick oviduct cytosol over the amount of steroid-binding proteins (Baulieu et al., 1983; Birnbaumer et al., 1984), it remains of interest to know why PR subunits A and B interact preferentially with it and whether and how this association is in any way functional in the cell.

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Microtubule-Associated Proteins Connect Microtubules and Neurofilaments in Vitro†

Eric J. Aamodt and Robley C. Williams, Jr.*

ABSTRACT: Neuronal intermediate filaments (neurofilaments) prepared from brain form a viscous, sedimentable complex with microtubules under suitable conditions [Runge, M. S., Laue, T. M., Yphantis, D. A., Lifsics, M. R., Saito, A., Altin, M., Reinke, K., & Williams, R. C., Jr. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1431–1435]. Under the same conditions, neurofilaments prepared from spinal cord did not form such a complex. Brain neurofilaments were shown to differ from spinal cord neurofilaments in part by having proteins that resemble microtubule-associated proteins (MAPs) attached to them. MAPs became bound to spinal cord neurofilaments when the two structures were incubated together. The resulting MAP-decorated neurofilaments formed a viscous complex with microtubules, showing that some component of the MAPs mediated the association between the two filamentous organelles. By means of gel filtration, the MAPs were separated into two major fractions. The large Stokes radius fraction was active in producing neurofilament–microtubule mixtures of high viscosity, while the small Stokes radius fraction was not. The dependence of the viscosity of neurofilament–microtubule mixtures upon the concentration of MAPs was found to possess a maximum. This result suggests that the MAPs serve as cross-bridges between the two structures. Neurofilaments, with and without bound MAPs, were allowed to adhere to electron microscope grids. The grids were then exposed to microtubules, fixed, and stained. The grids prepared with MAP-decorated neurofilaments bound numerous microtubules, each in apparent contact with one or more neurofilaments. The grids prepared with untreated neurofilaments lacked microtubules. These results show that one or more of the MAPs mediates association between microtubules and neurofilaments.

The cytoplasm of eukaryotic cells contains at least three types of filamentous organelles: microtubules, intermediate filaments, and microfilaments, connected together to form the cytoskeleton. The axons of vertebrate neurons have provided a convenient system in which to study the interaction of these cytoskeletal elements and their involvement in growth and transport. Within the axon, the chief cytoskeletal elements appear to be microtubules and the intermediate filaments of neurons, the neurofilaments (Peters et al., 1976). The neurofilaments and microtubules are aligned roughly parallel to the long axis of the axon and appear to be linked to each other and to membranous organelles through thin cross-bridges (Wuerker & Palay, 1969; Bertolini et al., 1970; Wuerker, 1970; Smith, 1971; Smith et al., 1977; Ellisman & Porter, 1980; Hodge & Adelman, 1980; Rice et al., 1980; Metuzals et al., 1981; Hirokawa, 1982; Schnapp & Reese, 1982). At least some of these bridges must break and re-form as the cell transports material and moves. The cross-bridges seen in axons resemble the cross-connecting filaments of 20–40-Å diameter seen in numerous cell types (Wolosewick & Porter, 1976; Porter et al., 1979; Heuser & Kirschner, 1980).

Apparently as a result of this cross-bridging, neurofilaments and microtubules move together in the slowest component of

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