Szego, C. M., Seeler, B. J., & Smith, R. E. (1976) *Eur. J. Biochem. 69*, 463.
Vedeckis, W. V., Schrader, W. T., & O'Malley, B. W. (1978) in *Biochemical Actions of Hormones* (Litwack, G., Ed.) Vol. V, p 321, Academic Press, New York.
Vedeckis, W. V., Schrader, W. T., & O'Malley, B. W. (1979) in *Steroid Hormone Receptor Systems* (Leavitt, W. W., & Clark, J. H., Eds.) p 309, Plenum Press, New York.
Vedeckis, W. V., Schrader, W. T., & O'Malley, B. W. (1980) *Biochemistry* (following paper in this issue).
Wrange, Ö., & Gustaffson, J.-Å. (1978) *J. Biol. Chem. 253*, 856.

Progesterone-Binding Components of Chick Oviduct: Analysis of Receptor Structure by Limited Proteolysis†

Wayne V. Vedeckis,‡ William T. Schrader,* and Bert W. O'Malley

ABSTRACT: An endogenous calcium-activated sulfhydryl protease in chick oviduct cytosol has been utilized to study the structure of the chick oviduct progesterone receptor subunits, progestophilins A (79000 g/mol) and B (117000 g/mol). The protease is not a normal component of the native progesterone receptor aggregate (6 and 8 S) complexes. Both receptor protein subunits (A and B) can be cleaved to two hormone-binding fragments, form IV (43000 g/mol) and meroreceptor (23000 g/mol). The meroreceptors obtained from the A and B proteins are indistinguishable from each other on the basis of both size (gel filtration chromatography) and charge (isoelectric focusing, p*I* 8.3). These findings suggest a structural similarity between the A and B proteins. The discovery of a weak deoxyribonucleic acid (DNA) binding activity for the B protein suggests an even greater similarity between B and A subunits, since the A subunit has previously been shown to bind to DNA. The proteolytic fragments do not bind to DNA–cellulose, implying that the hormone- and DNA-binding regions of the A and B proteins exist in separate domains.

The chick oviduct progesterone receptor has been studied extensively in our laboratories and in others [for a review, see Vedeckis et al. (1978)]. Two nonidentical hormone-binding macromolecules can be extracted from oviduct cells. The two proteins, progestophilins A and B, have molecular weights of 79000 and 117000, respectively, and are both single polypeptide chains. We have purified these individually to apparent homogeneity (Kuhn et al., 1975; Schrader et al., 1977a; Coty et al., 1979), and their associations with nuclear constituents have been determined (O'Malley et al., 1971; Schrader et al., 1972; Kuhn et al., 1975, 1977; Coty et al., 1979). We have also identified a 6 S entity in tissue extracts which appears to be a dimer of these two proteins. This notion is supported by chromatographic analyses (Schrader et al., 1975, 1977b; Buller et al., 1976) and chemical cross-linking studies (O'Malley et al., 1977; Birnbaumer et al., 1979).

Due to the similarity of the hormone-binding sites of these two subunits, we have been interested in whether or not they contain the same polypeptide core. Because they differ in size, the possibility remains that a precursor–product relationship may exist for these two proteins. We have chosen to study the structural features of these two subunits by examining the proteolytic fragments generated from each. Proteolytic digests of the two proteins would also serve to map the hormone- and genomic-binding sites.

An endogenous protease is present in crude chick and hen oviduct cytosol which is capable of hydrolyzing the progesterone receptor proteins (Sherman et al., 1976, 1978; Sherman & Diaz, 1977; Vedeckis et al., 1979, 1980). This enzyme is a calcium-activated protease very similar to the estrogen "receptor transforming factor" (RTF)[1] in calf uterine cytosol characterized by Puca and co-workers (Puca et al., 1972, 1977; Sica et al., 1976). In the oviduct cytosol, the protease was shown (Sherman et al., 1974, 1976; Sherman & Diaz, 1977) to digest the B receptor protein to a hormone-binding fragment termed the meroreceptor (M_r 23000; Stokes radius = 2.1 nm; sedimentation coefficient = 2.6 S) while the hormone-binding cleavage product of the A protein appeared to be a larger fragment, form IV (M_r 43000; Stokes radius = 2.7 nm; sedimentation coefficient = 3.6 S). Since the proteolytic fragments appeared to be different for the two proteins, it was not clear whether the A and B subunits shared identical, or merely kinetically similar, hormone-binding regions.

We have now undertaken a more detailed study of the proteolytic digestion of proteins A and B by using this endogenous protease. Both crude protease extracts and partially purified enzyme (Vedeckis et al., 1980) were used. Contrary to other studies (Sherman & Diaz, 1977), we report that both receptor proteins are digested to form IV and ultimately to

† From the Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030. *Received September 11, 1978.* This research was supported by National Institutes of Health Grants HD-07857 and HD-07495 to the Baylor Center for Population Research and Studies in Reproductive Biology. W.V.V. is a recipient of a U.S. Public Health Service postdoctoral fellowship. This is the 19th in a series of articles relating to the characterization of chick oviduct progesterone-binding components. Vedeckis et al. (1980) is the preceding article in the series.
‡ Present address: Department of Biochemistry, Louisiana State University Medical Center, New Orleans, LA 70112.

[1] Abbreviations used: RTF, receptor transforming factor; DES, diethylstilbestrol; Na_2EDTA, disodium ethylenediaminetetraacetate; DEAE, diethylaminoethyl; [^{3}H]progesterone, [1,2-$^3H_2$]progesterone; [^{3}H]A, [^{3}H]progesterone–progestophilin A complex; [^{3}H]B, [^{3}H]progesterone–progestophilin B complex.

0006-2960/80/0419-0343$01.00/0

the meroreceptor. The fragments from A and B are similar with respect to size and charge. Therefore, the A and B proteins appear to contain a similar 43 000 g/mol hormone-binding region, suggesting a great deal of structural similarity between these two proteins.

Materials and Methods

Tissues. Oviducts (1–2 g) were obtained from White Leghorn chicks which had been implanted with DES pellets (Coty et al., 1979).

Chemicals. All chemicals were of reagent grade and obtained from Fisher or J. T. Baker Scientific. [1,2-$^3H_2$]-Progesterone (55.7 Ci/mmol) was purchased from New England Nuclear. Tris and ammonium sulfate were "ultrapure" grade from Schwarz/Mann. Ampholytes (pH 7–9 and 9–11) were obtained from LKB.

Buffers. Buffer A consisted of 10 mM Tris-HCl (pH 7.4 at 25 °C), 1 mM Na_2EDTA, and 12 mM 1-thioglycerol. KCl concentrations were adjusted as indicated under Results.

DEAE-cellulose Slurry Assay. The conditions for the assay for meroreceptor formation using the DEAE–cellulose slurry assay were the same as those described previously (Vedeckis et al., 1980). Details are given in the legend of Figure 1A.

Gel Filtration Chromatography. Receptor aggregates were partially purified by using an agarose A-15 m, 200–400 mesh (Bio-Rad), gel filtration column (2.6 × 36 cm) equilibrated in buffer A. Typically, 2-mL samples were applied and 1.5-mL fractions collected at a flow rate of 7.5 mL/h.

The proteolytic cleavage products were analyzed on a 2.6 × 36 cm Sephadex G-100 (Pharmacia) column which was equilibrated in buffer A containing 0.3 M KCl. Protein standards used for the calibration of the G-100 column were from a Combithek Protein Calibration Kit, Size II (Boehringer-Mannheim). The flow rate and sample and fraction sizes were the same as those for the A-15 m column.

Preparation of Substrates and Partially Purified A and B Subunits. Crude receptors were prepared as described previously (Vedeckis et al., 1980). Chick oviduct cytosol was used as starting material. This was treated with 10 mM iodoacetamide (30 min, 0 °C) to inhibit endogenous Ca^{2+}-activated protease. After the addition of 12 mM 1-thioglycerol, the receptors were precipitated at 35% saturation of $(NH_4)_2SO_4$ in buffer A.

The partially purified A and B subunits were prepared as follows. Saturated ammonium sulfate was added to cytosol to a final concentration of 35% saturation. After the mixture was allowed to stand for 30 min, the precipitate was collected by centrifugation at 27000*g* for 30 min. The supernatant was discarded, the tube walls were washed with cold distilled water, and the pellet was dissolved in the original cytosol volume of buffer A. This was then applied to a DEAE-cellulose column (Schrader, 1975) using 1 mL of packed resin per 10^6 cpm of added receptor-bound progesterone, and the column was washed with 5–10 column volumes of buffer A. Receptor A subunits labeled with [^{3}H]progesterone ([^{3}H]A) were step-eluted with 5 column volumes of buffer A containing 0.15 M KCl and collected in 1-mL fractions. Following this, [^{3}H]B receptors were step-eluted in the same manner by using buffer A containing 0.3 M KCl. The [^{3}H]A preparation contained no detectable B protein, whereas the [^{3}H]B sample was contaminated with up to 10% [^{3}H]A protein as shown by subsequent chromatography on phosphocellulose (PC). When the [^{3}H]B protein was to be used as a substrate for the protease, it was allowed to stand at 0 °C for 40 h prior to use. This resulted in a loss of residual contaminating Ca^{2+}-activated protease activity in the [^{3}H]B preparations. This protocol yielded a preparation which is referred to below as exogenous [^{3}H]B substrate. In experiments designed to analyze the cleavage products of the [^{3}H]B protein, the contaminating [^{3}H]A protein was completely removed by an additional PC chromatography step. The [^{3}H]B protein from DEAE-cellulose was diluted with buffer A to bring the KCl concentration to 0.03 M KCl and applied to a small PC column (10^6 cpm of receptor per mL of packed resin). After being washed with buffer A, the [^{3}H]B receptor was eluted with a linear KCl salt gradient from 0 to 0.4 M KCl in buffer A. The peak fractions eluting between 0.18 and 0.20 M KCl were pooled and used as the [^{3}H]B receptor.

DNA–Cellulose Chromatography. DNA–cellulose was prepared as described previously (Coty et al., 1979). Samples were applied to 2-mL DNA–cellulose columns equilibrated in buffer A and washed with 5–10 column volumes of the same buffer. Receptor bound to the column was eluted either by step elution with buffer A containing 0.5 M KCl or with a linear 0–0.35 M KCl gradient in buffer A (70 mL total gradient volume).

Isoelectric Focusing. Isoelectric focusing was performed on an LKB 2117 Multiphor apparatus using polyacrylamide slab gels (110 × 110 × 2 mm) prepared as described by Chua et al. (1978) with the following modifications: (1) the sucrose was replaced with 10% glycerol; (2) the ampholytes used were 2% of both pH 7–9 and 9–11 ranges. The gels were prefocused at 5 W for 1–2 h. Afterward, 20 μL of sample was applied in 0.5 × 0.5 cm wells cut into the gel and focusing was carried out at 1 °C for 2 h at 5 W or less. Cytochrome *c* was used as a marker to determine when focusing was complete. The gel was sliced into 0.5-cm fractions and placed into scintillation vials. After the gel slices were soaked overnight in scintillation cocktail, the samples were counted for tritium. Slices were also cut from the tracks in which the cytochrome *c* was run and were placed into 1 mL of distilled water. After the mixture was shaken for 16 h, the pH was determined by using a Radiometer Model 26 pH meter.

Radioactivity Measurements. Aqueous samples of 0.5 or 1 mL were counted in 4 or 8 mL, respectively, of ACS (Amersham) scintillation fluid, using a Beckman LS-233 scintillation counter. Efficiency for tritium was 26%.

Results

Receptor–Protease Interaction. Previous studies have shown that chick oviduct cytosol prepared in low ionic strength buffer contains receptor in the form of 6S and 8S complexes; both the 6S and 8S aggregates contain the A and B receptor proteins. This has led to the hypothesis that progestophilins A and B are subunits of these aggregate forms (Schrader et al., 1975, 1977b; Buller et al., 1976; O'Malley et al., 1977; Birnbaumer et al., 1979) It has also been demonstrated that the Ca^{2+}-activated protease comigrated with the receptor upon isoelectric focusing and sucrose density gradient centrifugation of cytosol at low ionic strength (Sherman et al., 1974). Although the resolution of the receptor and protease activities by ammonium sulfate precipitation and high-salt sucrose density gradient centrifugation (Vedeckis et al., 1979b) demonstrated that these activities were not on the same polypeptide chain, the low K_m of the protease for the receptor (Vedeckis et al., 1980) and the results of Sherman et al. (1974) suggested that the enzyme and receptor might form a complex stable in low ionic strength. Thus, the 6S and 8S receptor aggregates could contain the protease as a subunit, rather than the association of receptor and protease occurring only just prior to cleavage. Therefore, experiments were undertaken to assay receptor aggregates for the presence of bound protease.

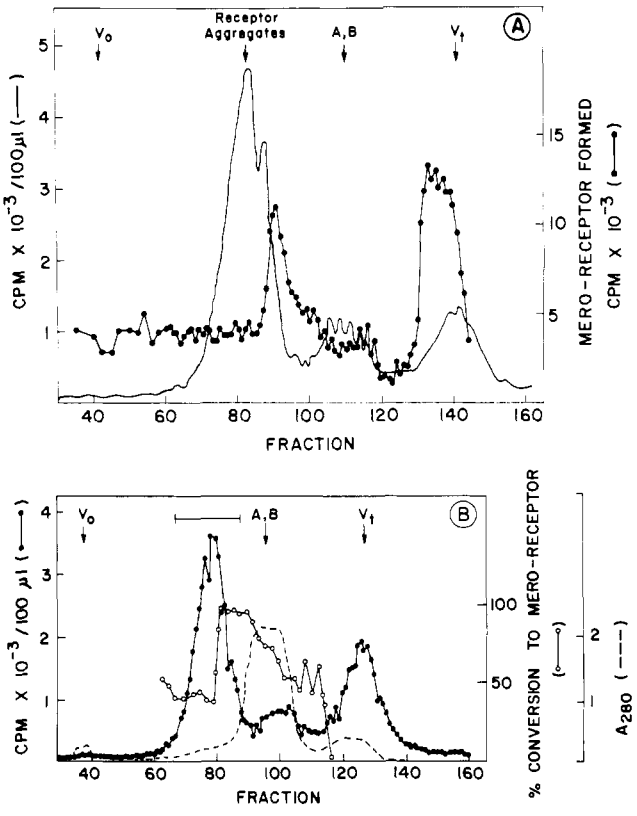

FIGURE 1: (A) Agarose A-15 m gel filtration chromatography of the Ca^{2+}-activated protease using exogenous substrate. 2 mL of unlabeled chick oviduct cytosol (2 mL of buffer per g fresh weight) was applied to an agarose A-15 m column (2.6 × 36 cm) equilibrated in buffer A, and 1.5-mL fractions were collected at a flow rate of 7.5 mL/h. 50-µL aliquots of each fraction were mixed with 50 µL of [^{3}H]B (18 500 cpm), which was prepared as described under Materials and Methods. Duplicate incubation mixtures received 100 mM $CaCl_2$ or water (control), and the DEAE-cellulose slurry assay was performed under the standard assay conditions (Vedeckis et al., 1980). The radioactivity in the supernatant for the control sample was subtracted from the Ca^{2+}-treated samples (●). No generation of supernatant counts occurred across the column for the control samples. A sample of [^{3}H]progesterone-labeled cytosol was chromatographed on the same column on a different occasion, and the profile obtained is superimposed for comparative purposes (—). V_0 = blue dextran; V_t = KCl; A and B indicate the elution positions of the A and B subunits of the chick oviduct progesterone receptor. (B) Agarose A-15 m gel filtration chromatography of the Ca^{2+}-activated protease using endogenous substrate. 2 mL of chick oviduct cytosol (2 mL of buffer per g fresh weight) was labeled with 3 × 10^{-8} M [^{3}H]progesterone for 2 h. The sample was chromatographed on an agarose A-15 m column as described in (A). 100-µL aliquots were treated with 100 mM Ca^{2+} at 0 °C for 1 h, and meroreceptor formation was analyzed by the DEAE-cellulose slurry assay. Radioactivity in the supernatant of the controls was subtracted from that of the Ca^{2+}-treated aliquot (○). An aliquot of each fraction was also counted to determine the elution position of the receptor (●). Absorbance was read at 280 nm (---). The rest is as in (A).

We have recently used agarose A-15 m gel filtration chromatography at low ionic strength (buffer A) to obtain a partially purified receptor aggregate preparation (Birnbaumer et al., 1979). The material has properties similar to native cytosol receptor aggregates. A typical elution profile of [^{3}H]progesterone–receptor complexes run on this column is shown in Figure 1A. The peak in fraction 80 sediments on sucrose gradients as intact 6S and 8S aggregates (Birnbaumer et al., 1979) and is purified about 15-fold over crude cytosol. This method was used to test for the presence of the protease in the receptor peak. An unlabeled cytosol receptor preparation was chromatographed on the column, and the protease elution profile was determined on 50-µL aliquots by using [^{3}H]B as substrate and the DEAE-cellulose slurry assay. Figure 1A shows that a peak of Ca^{2+}-activated protease eluted at the trailing edge of the receptor aggregate peak; the two were never coincident in repeated trials. The results show that the protease is not a required component of the receptor aggregate complex. The second peak of apparent Ca^{2+}-activated protease activity, eluting near the included volume, was not characterized further, but it is probably an artifact of the method, since only one peak of protease activity, sedimenting at 6 S, is observed by using sucrose density gradient centrifugation in high ionic strength (Vedeckis et al., 1980).

In a similar manner, the A-15 m column fractions were also analyzed for protease activity when labeled cytosol was chromatographed as shown in Figure 1B. For this experiment endogenous receptor aggregate served as substrate. Although the substrate concentration varied across the receptor aggregate peak and was limiting in some fractions, the protease activity pattern paralleled that obtained by using exogenous substrate. Therefore, association of progesterone with the receptor also did not result in a change in the elution pattern of the protease, and the receptor protein does not appear to be associated with the enzyme under these conditions.

Products of Receptor Proteolysis. The two progesterone receptor subunits (A and B) are separate polypeptide chains (Kuhn et al., 1975; Schrader et al., 1977a; Coty et al., 1979). Studies of the hormone-binding cleavage products from each receptor subunit have shown, under certain reaction conditions, another proteolytic fragment designated form IV, with a molecular weight of 43 000 (Sherman et al., 1974, 1976). A later study indicated that form IV was a product of A subunit cleavage, whereas the B subunit yielded meroreceptor upon proteolysis (Sherman & Diaz, 1977).

In contrast to these results, our own studies had shown that exhaustive (2–3 h) treatment of crude cytosol with 100 mM $CaCl_2$ yielded meroreceptor quantitatively, supporting the notion that the A subunit could be converted to this form. However, it was imperative to obtain a definitive answer to the subunit digestion question. Therefore, the following experiment was performed. [^{3}H]A and [^{3}H]B (both free of Ca^{2+}-activated protease) were prepared as described under Materials and Methods. Cytosol (prepared in 2 mL of buffer per g of oviduct) was used as a source of protease. Receptors in this crude enzyme mixture were "blocked" from subsequent binding of [^{3}H]progesterone by incubation with 2 × 10^{-7} M unlabeled progesterone for 2 h, followed by dialysis for 1.5 h against three changes of 100 volumes of buffer A. Two milliliters of this cytosol was added to 2 mL of either [^{3}H]A or [^{3}H]B. After addition of 100 mM Ca^{2+} for 10 ([^{3}H]A) or 3 min ([^{3}H]B), the samples were desalted on a 20-mL Sephadex G-25 column equilibrated in buffer A. Two milliliters of the void volume peak was applied to a calibrated Sephadex G-100 column in buffer A containing 0.3 M KCl and chromatographed as described under Materials and Methods.

Figure 2 shows that, after digestion by the Ca^{2+}-activated protease, both A and B were converted individually to a peak eluting in fraction 80, coincident with authentic meroreceptor.[2]

[2] Although the molecular weights obtained by using the Sephadex G-100 column were 63 200 for form IV and 25 700 for meroreceptor, we have chosen to use the values published previously (Sherman et al., 1974, 1976) for these two fragments using Bio-Rad agarose gel filtration columns. As has been noted recently (Sherman et al., 1978), different values for the molecular weights are obtained depending upon the gel filtration matrix used. Only when the fragments are purified to homogeneity and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis will this conflict be resolved.

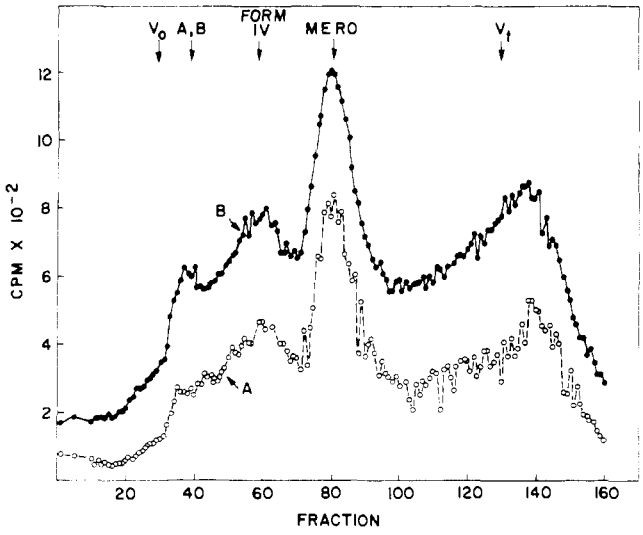

FIGURE 2: Proteolytic products of progesterone receptor A and B subunits analyzed by Sephadex G-100 gel filtration chromatography. Isolated [^{3}H]A (○) and [^{3}H]B (●) subunits were prepared as described under Materials and Methods and incubated with cytosol whose progesterone receptor was blocked with unlabeled hormone. 100 mM $CaCl_2$ was added for 10 ([^{3}H]A) and 3 min ([^{3}H]B), followed by desalting on a Sephadex G-25 column and subsequent G-100 chromatography. Details are given in the text. V_0 = blue dextran; A and B = elution position of A and B subunits; V_t = KCl. Protein standards eluted in the following fractions: bovine serum albumin (M_r 68 000), fraction 59; ovalbumin (M_r 43 000), fraction 67; chymotrypsinogen A (M_r 25 000), fraction 81; cytochrome *c* (M_r 12 500), fraction 95. Authentic meroreceptor and form IV prepared as described previously (Sherman et al., 1976) eluted at the positions shown by the arrows in fractions 80 and 60, respectively.

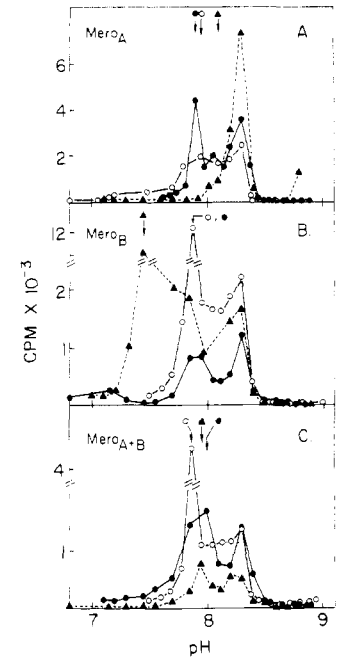

FIGURE 3: Isoelectric focusing of meroreceptors generated from the A and B proteins. The A and B receptor proteins were prepared by step elution from DEAE-cellulose after precipitation from cytosol at 35% saturated ammonium sulfate. The KCl was removed by dialysis of the eluates vs. buffer A. To 800 μL of ^{3}H-labeled receptor was added 200 μL of partially purified protease or 200 μL of cytosol whose progesterone receptor was blocked with unlabeled hormone. 100 mM $CaCl_2$ was added for 1 h. The samples were applied in a 0.5 × 0.5 cm well cut into the gel and applied at the indicated position (arrows) in the polyacrylamide gels containing 2% ampholytes (pH range 7–11). After being focused for 2 h at 5 W and 1 °C, the gels were cut into 0.5-cm fractions and counted for tritium. The pH gradient was determined from gel fractions soaked in 1-mL of distilled water and shaken for 16 h. Each curve represents a separate experiment. (Panel A) [^{3}H]A receptor plus purified protease (●, ▲) or blocked cytosol (○). (Panel B) [^{3}H]B receptor plus purified protease. (Panel C) Mixture of samples shown in panels A and B. In addition, one sample shown (●) is Ca^{2+}-treated, crude cytosol (50 mM Ca^{2+}, 2 h).

The two also gave significant amounts of material eluting in fraction 60, coincident with authentic form IV. Thus, both A and B subunits gave identical gel filtration profiles for the hormone-binding portions of the two proteins.

The gel filtration assay demonstrated that two hormone-binding proteolytic fragments from A and B were indistinguishable on the basis of the molecular size. Analytical slab gel isoelectric focusing was performed to test these fragments for their charge characteristics. When aliquots of Ca^{2+}-treated cytosol were analyzed after various reaction times, a peak of ^{3}H migrating to pH 8.3 increased with time, concomitant with an increase in meroreceptor as analyzed by using the DEAE-cellulose slurry assay (data not shown). Thus, authentic meroreceptor has an an isoelectric point of pH 8.3. We then used this method to assay receptor A and B digests. The results are shown in Figure 3.

For these experiments, a preparation of partially purified Ca^{2+}-activated protease was used to digest receptors A and B independently. The enzyme, isolated as described in the companion paper, had been observed to convert [^{3}H]B to meroreceptor as judged by gel filtration chromatography. Then it was used to digest both A and B as follows. When 380 μg/mL purified protease (Vedeckis et al., 1980) and Ca^{2+} (50 mM) were added to isolated [^{3}H]A and [^{3}H]B receptor subunit proteins, the resulting meroreceptors each had an isoelectric point of 8.3 (parts A and B of Figure 3). A mixture of meroreceptors obtained from separate reaction mixtures of [^{3}H]A and [^{3}H]B or Ca^{2+}-treated cytosol (which contains both A and B) gave identical results (Figure 3C). Thus, the meroreceptors from both the A and B receptor proteins are indistinguishable as analyzed by gel filtration chromatography and isoelectric focusing.

Form IV as an Intermediate. Since both proteins A and B yielded form IV and meroreceptor (Figure 2), it was of interest to investigate the pathway of hydrolysis. Because form IV is the larger fragment, it was a likely candidate as an intermediate in hydrolysis. The experiment shown in Figure 4 was performed to test this. The proteolysis of a mixture of A and B was limited by carrying out the reaction for a brief

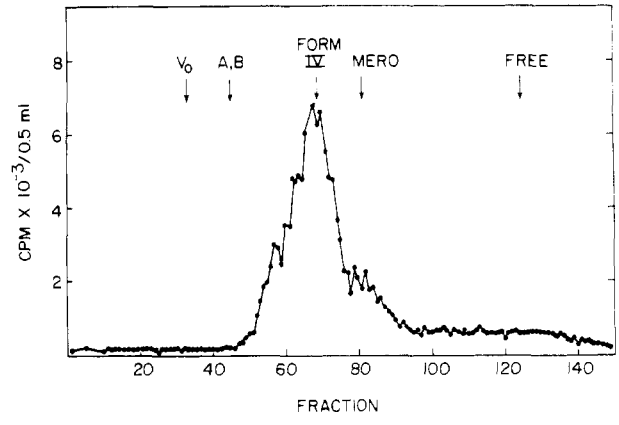

FIGURE 4: Limited proteolysis of native oviduct cytosol receptors to form IV. Chick oviduct cytosol was labeled with 2 × 10^{-8} M [^{3}H]progesterone for 2 h. The cytosol was made 5 mM in $CaCl_2$ and reacted for 5 min at 0 °C prior to desalting on a Sephadex G-25 column. Sephadex G-100 chromatography was carried out as described under Materials and Methods and in the legend to Figure 2. Elution positions indicated are the same as those in Figure 2. Free = the elution position of unbound [^{3}H]progesterone.

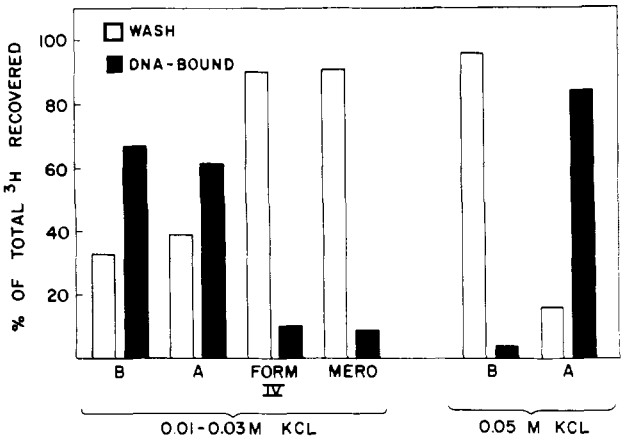

FIGURE 5: DNA–cellulose chromatography of receptor subunits and proteolytic cleavage products. The A and B subunits of the chick oviduct progesterone receptor were prepared by DEAE-cellulose chromatography as described under Materials and Methods. Form IV and meroreceptor were obtained by pooling the appropriate peak fractions after Sephadex G-100 chromatography of cytosol treated for 10 min at 4 °C with 100 mM $CaCl_2$. Details are given in the text. For the purpose of comparison, data are included for A and B subunits which were diluted to 0.05 M KCl, applied to DNA–cellulose, and washed with buffer A containing 0.05 M KCl prior to step elution of the bound material from the column [data from Kuhn et al. (1977)].

time in low calcium, as described in the figure legend. The results show complete hydrolysis of all the A and B protein and only 19% conversion to meroreceptor. Under these limiting conditions, over 80% of the hormone-binding fragments were form IV. In a separate experiment not shown here, the form IV region was pooled from a G-100 column, concentrated, and desalted by dialysis. Partially purified protease (Vedeckis et al., 1980) and 25 mM $CaCl_2$ were then added for 1 h, the $CaCl_2$ was removed, and the sample was applied to a G-100 column. All of the form IV material had been converted to meroreceptor by the protease. We conclude that form IV is an intermediate in the hydrolysis of both A and B to meroreceptor.

DNA-Binding Properties of Native Receptor Forms and Proteolytic Fragments. Meroreceptor which was obtained by treating crude cytosol with $CaCl_2$ did not bind to DNA–cellulose (Vedeckis et al., 1978). This fact suggested that the protease might hydrolyze the receptors in such a way that the DNA-binding domain is excised from the hormone-binding region. The following studies were performed in order to begin mapping of functional regions to the various receptor forms and proteolytic fragments.

Cytosol was treated for 10 min with 100 mM $CaCl_2$, desalted over a Sephadex G-25 column, and applied to a Sephadex G-100 column in buffer A containing 0.3 M KCl. Fractions from the form IV and meroreceptor peaks were pooled and dialyzed against buffer A until the conductivity was equivalent to 0.03 M KCl. The samples were then applied to DNA–cellulose columns (2-mL packed volume), which were then washed with 10 column volumes of buffer A. Adsorbed receptor was step-eluted with buffer A containing 0.5 M KCl and the amount of bound receptor calculated. Figure 5 shows that neither form IV nor meroreceptor was capable of binding to DNA–cellulose. Under these conditions, the A subunit bound to a level of 61% while, surprisingly, 67% of the B subunit was bound to the column. Binding was 4% to cellulose lacking DNA. Previous studies (Schrader et al., 1972; Kuhn et al., 1977) had failed to detect binding of the B subunit. This dilemma was resolved when the DNA–cellulose column, to which B subunit had been adsorbed, was eluted with a KCl

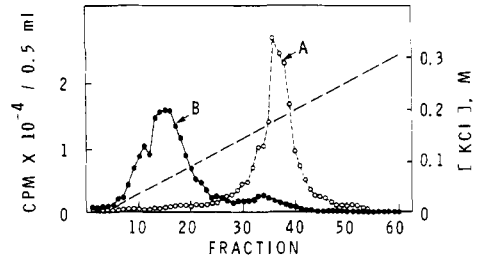

FIGURE 6: DNA–cellulose chromatography of progesterone receptor A and B subunits. [^{3}H]A and [^{3}H]B subunits were prepared as described under Materials and Methods, diluted to 0.01 M KCl with buffer A, and applied to 2-mL DNA–cellulose columns. The column was then washed with 10 mL of buffer A. The absorbed receptors were eluted with a linear 0–0.35 M KCl gradient (in buffer A) and 1-mL fractions collected. 78 and 56% of the applied radioactivity were bound to the column for the A and B subunits, respectively.

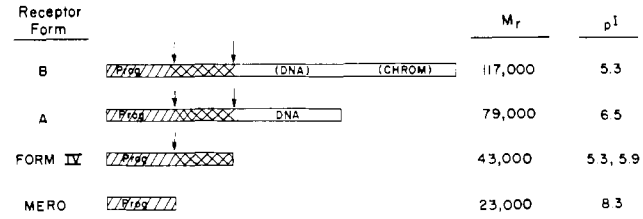

FIGURE 7: Possible structural relationships between the native progesterone receptor subunits and the proteolytic fragments. The hatched areas represent the regions of all four molecules which appear to be similar. The cross-hatched area is the region present in all forms but the meroreceptor. "Prog" indicates the region responsible for the binding of the steroid hormone progesterone. The A subunit contains a DNA-binding site which is apparently lost from the hormone-binding region after proteolysis. The weak DNA-binding activity of the B subunit may be partially occluded in the native conformation. "Chrom" refers to the chromatin-binding activity of the B subunit (Schrader et al., 1972; Jaffe et al., 1975; Kuhn et al., 1977). Although many orientations of these regions are possible, only the simplest is presented, and by no means is this representation any more valid than others not depicted here. The isoelectric points were determined as described in the text and the legend to Figure 3.

gradient. The B subunit eluted at very low salt concentrations, at ~0.06 M KCl (Figure 6). All earlier studies had been performed by loading the B protein onto the DNA–cellulose column in buffer A containing 0.05 M KCl, followed by washing in the same buffer. Thus, the very weak DNA-binding activity of the B subunit was missed in these earlier studies.

Thus, either a DNA-binding portion of the fragments exists in a conformation which is inaccessible to interact with DNA or, more likely, the DNA-binding regions of the native A and B subunits have been removed from the hormone-binding region by the proteolytic cleavages. The changes in electrostatic charge which accompany receptor proteolysis suggest that the latter may be the case.

Relationships between Receptor Forms and Proteolytic Fragments. The above results suggest that the A and B subunits contain a large portion of their polypeptide chains which are indistinguishable from each other. The putative relationships between the various receptor forms are summarized in Figure 7. Both subunits may contain a similar hormone-binding region equal to at least 43 000 g/mol (form IV). This is equivalent to 54% of the A subunit (M_r 79 000) and 37% of the B subunit (M_r 117 000). Since form IV does not bind to DNA–cellulose, it is likely that the DNA-binding region of the A subunit resides on a 36 000 g/mol fragment which is physically separate from the hormone-binding region. Alternatively, the DNA-binding region may be present in form IV (and meroreceptor) but due to conformational alterations

during proteolysis is inaccessible or no longer capable of interaction with DNA.

Discussion

The present study characterizes the limited proteolytic digestion of chick oviduct progesterone receptor subunits. Limited proteolysis is an important regulatory mechanism in other biochemical processes, the most dramatic of which are proenzyme activation and the clotting of blood [for a review, see Walsh (1975)]. It is apparent that limited proteolysis of steroid hormone receptor proteins is a general phenomenon, and it has now been observed for estrogen (Puca et al., 1977), aldosterone (Sherman et al., 1978), and glucocorticoid (Carlstedt-Duke et al., 1977; Sherman et al., 1978; Wrange & Gustaffson, 1978) receptors.

We have been unable to demonstrate a stable association of the protease with the receptor as would have been expected if it were a subunit of the 6S and 8S native cytosol receptor aggregates. Thus, although the protease associates with the receptor during hydrolysis, it does not appear to be a stable component of these native complexes.

The most important use to date for the oviduct enzyme has been as a probe for the molecular structure of the chick oviduct progesterone receptor. The present study provides the first definitive evidence that A and B subunits of the receptor can be cleaved to form IV and the meroreceptor. Thus, the limit digest of both subunits is the meroreceptor. Preliminary evidence suggests that form IV may be an intermediate in the conversion of the A and B subunits to meroreceptor. By limiting the $CaCl_2$ concentration, it has been possible to convert all of the native progesterone receptor to form IV. Additionally, a kinetic analysis has demonstrated that various proportions of native receptor, form IV, and meroreceptor can be obtained (data not shown). Although not definitive, these results suggest that form IV is a possible, but not necessarily obligatory, intermediate in the conversion of the A and B progesterone receptor subunits to meroreceptor.

An unexpected result was the discovery that the B subunit does exhibit a weak, but finite, DNA–cellulose-binding activity. Thus, the A and B subunits both contain a DNA-binding domain, although whether or not they are identical remains to be determined. Nevertheless, the weak DNA-binding activity of the B subunit raises the possibility that this protein contains part or even all of the remainder of the A subunit structure. Thus, one could imagine that the entire A subunit polypeptide chain is present within the B subunit, plus an extra portion which occludes the DNA-binding region and confers the ability of the B subunit to bind to target cell chromatin (Schrader et al., 1972; Jaffe et al., 1975; Kuhn et al., 1977).

Tentative assignment of the regions of acidic and basic residues can be made, based upon the results of ion-exchange chromatography and isoelectric focusing. The meroreceptor exhibited a relative lack of charged residues based upon ion-exchange chromatography (Vedeckis et al., 1978) and its poor migration on polyacrylamide gels (data not shown). Since progesterone is a very hydrophobic molecule, the meroreceptor binding site would be expected to be a very hydrophobic pocket, largely devoid of charged amino acid residues. Form IV binds to DEAE-cellulose, indicating that the next 20 000 g/mol portion must contain numerous acidic residues (data not shown). This was confirmed by the determination of the isoelectric point of form IV, which yielded two forms with p*I* values of 5.3 and 5.9 (data not shown). Based upon these data, it is likely that some heterogeneity exists for the form IV fragment. The additional 36 000 g/mol peptide which constitutes the remainder of subunit A must contain numerous exposed basic residues to account for the binding of both A and B to phosphocellulose. This fragment is also a likely candidate to contain the DNA-binding domain; involvement of basic residues in the DNA-binding domain is thereby a prediction of these tentative assignments. The separation of the hormone- and DNA-binding regions of the glucocorticoid receptor by limited proteolysis has been claimed in a recent report (Wrange & Gustaffson, 1978). The remaining 38 000 g/mol portion (in the B subunit) is likely to have additional exposed acidic residues, since B is more acidic than A.

Attempts have been made to demonstrate the generation of A and B subunits by proteolytic processing. The larger molecular forms of the receptor (6 and 8 S) are readily converted to 4S monomers by high concentrations of monovalent cations, consistent with the simple dissociation of ionic interactions between subunits rather than a proteolytic event. One would need to postulate that monovalent cations also activate a protease which gives rise to the A and B proteins. Furthermore, since a wide variety of experimental procedures routinely yields equal amounts of A and B subunits, it is difficult to construct a mechanism whereby one-half of the precursor receptor was converted to each subunit, unless the subunits originally existed as a single polypeptide chain containing two hormone-binding sites.

We have performed a large number of experiments, using many experimental manipulations, in an attempt to demonstrate B and A conversion of a precursor receptor to both subunits. Treatments included high salt, ammonium sulfate precipitation, heat treatment, low Ca^{2+} levels, and protease inhibitors. These experiments were performed on crude oviduct homogenates, the postmitochondrial supernatant fraction, cytosol, and partially purified B protein. The assay methods used were those which could differentiate between the A and B proteins (DEAE-cellulose, gel filtration, and DNA–cellulose chromatography). All of these results were negative. Thus, although proteolytic processing still remains a possibility, perhaps occurring in vivo soon after receptor synthesis, we have as yet been unable to demonstrate its existence.

The function of the proteases which perform a limited cleavage of steroid hormone receptors is unknown. Presumably, it is not degradative, since these are cases of limited proteolysis with high affinity for the receptor as substrate. It has been suggested that RTF cleavage of the calf uterine estrogen receptor is the "activation" mechanism whereby the proteolytic fragments, and not the native cytosol receptor, can enter the nucleus (Puca et al., 1972, 1977; Sica et al., 1976). Since neither the meroreceptor nor form IV binds to DNA, this is probably not the case for the chick oviduct progesterone receptor system. Rather, the conversion of the receptor A and B subunits to non-DNA-binding forms could be a mechanism whereby the progesterone-binding region of the receptor is released from its site of action on the genome.

Acknowledgments

We thank Marinan Freeman for expert technical assistance.

References

Birnbaumer, M. E., Schrader, W. T., & O'Malley, B. W. (1979) *Biochem. J. 181*, 201.

Buller, R. E., Schwartz, R. J., Schrader, W. T., & O'Malley, B. W. (1976) *J. Biol. Chem. 251*, 5178.

Carlstedt-Duke, J., Gustaffson, J.-Å., & Wrange, Ö. (1977) *Biochim. Biophys. Acta 497*, 507.

Chua, K. E., Crossman, E. J., & Gilmour, C. A. (1978) *Sci. Tools 25*, 9.

Coty, W. A., Schrader, W. T., & O'Malley, B. W. (1979) *J. Steroid Biochem. 10*, 1.
Jaffe, R. C., Socher, S. H., & O'Malley, B. W. (1975) *Biochim. Biophys. Acta 399*, 403.
Kuhn, R. W., Schrader, W. T., & O'Malley, B. W. (1975) *J. Biol. Chem. 250*, 4220.
Kuhn, R. W., Schrader, W. T., Coty, W. A., Conn, P. M., & O'Malley, B. W. (1977) *J. Biol. Chem. 252*, 308.
O'Malley, B. W., Toft, D. O., & Sherman, M. R. (1971) *J. Biol. Chem. 246*, 1117.
O'Malley, B. W., Vedeckis, W. V., Birnbaumer, M. E., & Schrader, W. T. (1977) in *Molecular Endocrinology* (MacIntyre, I., & Szelke, M., Eds.) p 135, Elsevier/North-Holland Biomedical Press, Amsterdam.
Puca, G. A., Nola, E., Sica, V., & Bresciani, F. (1972) *Biochemistry 11*, 4157.
Puca, G. A., Nola, E., Sica, V., & Bresciani, F. (1977) *J. Biol. Chem. 252*, 1358.
Schrader, W. T. (1975) *Methods Enzymol. 36*, 187.
Schrader, W. T., Toft, D. O., & O'Malley, B. W. (1972) *J. Biol. Chem. 247*, 2401.
Schrader, W. T., Heuer, S. S., & O'Malley, B. W. (1975) *Biol. Reprod. 12*, 134.
Schrader, W. T., Kuhn, R. W., & O'Malley, B. W. (1977a) *J. Biol. Chem. 252*, 299.
Schrader, W. T., Coty, W. A., Smith, R. G., & O'Malley, B. W. (1977b) *Ann. N.Y. Acad. Sci. 286*, 64.
Sherman, M. R., & Diaz, S. C. (1977) *Ann. N.Y. Acad. Sci. 286*, 81.
Sherman, M. R., Atienza, S. B. P., Shansky, J. R., & Hoffman, L. M. (1974) *J. Biol. Chem. 249*, 5351.
Sherman, M. R., Tuazon, F. B., Diaz, S. C., & Miller, L. K. (1976) *Biochemistry 15*, 980.
Sherman, M. R., Pickering, L. A., Rollwagen, F. M., & Miller, L. K. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 167.
Sica, V., Nola, E., Puca, G. A., & Bresciani, F. (1976) *Biochemistry 15*, 1915.
Vedeckis, W. V., Schrader, W. T., & O'Malley, B. W. (1978) in *Biochemical Actions of Hormones* (Litwack, G., Ed.) p 321, Academic Press, New York.
Vedeckis, W. V., Schrader, W. T., & O'Malley, B. W. (1979) in *Steroid Hormone Receptor Systems* (Leavitt, W. W., & Clark, J. H., Eds.) p 309, Plenum Press, New York.
Vedeckis, W. V., Freeman, M. R., Schrader, W. T., & O'Malley, B. W. (1980) *Biochemistry* (preceding paper in this issue).
Walsh, K. A. (1975) in *Proteases and Biological Control*, p 1, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Wrange, Ö., & Gustaffson, J.-Å. (1978) *J. Biol. Chem. 253*, 856.

Raman Scattering in Bilayers of Saturated Phosphatidylcholines. Experiment and Theory†

David A. Pink,* Trevor J. Green, and Dennis Chapman

ABSTRACT: Raman spectroscopy has been applied to a model biomembrane structure in order to obtain information about phospholipid hydrocarbon chain ordering. The intensity of the 1130-cm^{-1} Raman line obtained from a dipalmitoylphosphatidylcholine (DPPC) coarse aqueous dispersion has been measured as a function of temperature. The intensities of this line and a reference line were taken as peak areas. The main phase transition together with the pretransition was observed. A theory of chain conformations as a function of temperature and rules for the assignment of Raman scattering intensities for this line have been constructed. Good agreement with the DPPC experimental data has been obtained. Predictions for the intensity of this line as a function of temperature from dimyristoyl- and distearoylphosphatidylcholine dispersions have also been made.

Information about hydrocarbon chain order and mobility in model biomembrane structures is of importance in understanding the perturbation which intrinsic molecules such as cholesterol or proteins can produce when present within the lipid bilayer. Recent studies using the physical techniques electron spin resonance and deuterium nuclear magnetic resonance spectroscopy give contradictory answers (unless time-scale arguments are used) to the question of whether intrinsic proteins cause either an ordering or an immobilization of adjacent lipids or, alternatively, a disordering and fairly rapid exchange between lipid environments [see Chapman et al. (1979) for a recent review].

It is thus useful to examine these perturbation effects with a probeless technique which operates on a very rapid time scale compared to magnetic resonance experiments. In principle Raman spectroscopy should provide additional information on lipid hydrocarbon chain ordering, although previously there have been differing approaches to the quantitative interpretation of experimental data.

The technique of Raman spectroscopy has found use in the study of lipids in both model and biological membranes. Several workers have made use of the temperature dependence

† From the Institut für Neurobiologie der Kernforschungsanlage, D-517 Jülich, Federal Republic of Germany (D.A.P), and the Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine, University of London, 8 Hunter Street, London WC1N 1BP, England (T.J.G. and D.C.). *Received June 11, 1979; revised manuscript received September 10, 1979.* This work was supported in part by the National Science and Engineering Research Council of Canada, the Science Research Council and the Wellcome Trust.

* Permanent address and author to whom correspondence should be addressed: Theoretical Physics Institute, St. Francis Xavier University, Antigonish, Nova Scotia B2G 1C0, Canada.

0006-2960/80/0419-0349$01.00/0