Progesterone-Binding Components of Chick Oviduct: Partial Purification and Characterization of a Calcium-Activated Protease Which Hydrolyzes the Progesterone Receptor†

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ABSTRACT: A calcium (Ca^{2+})-activated protease has been purified from laying hen oviducts. This enzyme can catalyze the limited proteolysis of the native chick oviduct progesterone receptor subunits, A and B, to smaller hormone-binding fragments. The protocol used has resulted in a 2000-fold purification of the enzyme in 40% yield from hen oviduct postmitochondrial supernatant fractions. This resulted in an active, purified protease preparation which can be used as an analytical tool for studying receptor protein structure. Characterization of the purified enzyme has shown that it is activated by Ca^{2+} (0.5–1 mM), has a molecular weight of 113 000, and has a sedimentation value of 6 S. No effect of calmodulin (Ca^{2+}-dependent regulator) could be shown on the enzymatic activity of the protease. The enzyme has a K_m of 1.04×10^{-8} M for the receptor protein substrate. The protease is inactivated by sulfhydryl attacking reagents and thus can be classified as a sulfhydryl protease. This protease exhibits remarkable similarities to the "receptor transforming factor (RTF)", a Ca^{2+}-activated protease which performs a limited proteolysis on the calf uterine estrogen receptor [Puca, G. A., Nola, E., Sica, V., & Bresciani, F. (1977) *J. Biol. Chem. 252*, 1358].

The progesterone receptor of chick oviduct has been extensively studied as a mediator of the hormone's effect on gene expression [for a review, see Vedeckis et al. (1978)]. The receptor exists in two distinct molecular forms, A and B, which are believed to be subunits of a native 6S complex (Schrader & O'Malley, 1972; Schrader et al., 1975; Birnbaumer et al., 1979). Although the A (M_r 79 000) and B (M_r 117 000) receptor proteins have been purified to apparent homogeneity (Kuhn et al., 1975; Schrader et al., 1977a; Coty et al., 1979) and have been shown to be separate polypeptide chains, they contain hormone-binding sites which are indistinguishable based upon ligand specificity and hormone-binding kinetics (Schrader & O'Malley, 1972; Hansen et al., 1976).

We have recently studied the action of an endogenous Ca^{2+}-activated protease on the chick oviduct progesterone receptor proteins (Vedeckis et al., 1979, 1980). This enzyme cleaves both the A and B receptor proteins to two smaller hormone-binding fragments, termed form IV (M_r 43 000) and meroreceptor (M_r 23 000) by Sherman and co-workers (Sherman et al., 1976, 1978; Sherman & Diaz, 1977).

We have undertaken the task of purifying the protease to the point at which it can be used as a tool for analyzing receptor structure. We report here the partial purification of the Ca^{2+}-activated protease to a degree compatible with these proposed studies. Concomitantly we have characterized this enzyme and its activity. In the companion paper, the enzymatic activity is used to analyze the structural organization of the progesterone receptor A and B proteins.

Materials and Methods

Animals. Female White Leghorn chicks were implanted with DES[1] pellets as described previously (Coty et al., 1979). After two implantations over 4 weeks the magnum portion of the oviducts weighed 1–2 g. All studies described here used oviducts which weighed at least 1 g.

Chemicals. [1,2-$^3H_2$]Progesterone (55.7 Ci/mmol) was obtained from New England Nuclear. Tris, sucrose, and ammonium sulfate were "ultrapure" grade from Schwarz/Mann. Phenylmethanesulfonyl fluoride (PMSF), iodoacetamide, and poly(ethylene glycol) (M_r 6000) were from Sigma, and *N*-ethylmaleimide (MalNEt) and 5,5′-dithiobis(2-nitrobenzoate) (DTNB) were from Calbiochem. All remaining chemicals were reagent grade and were obtained from J. T. Baker or Fisher Scientific.

Buffers. 10 mM Tris-HCl (pH 7.4 at 25 °C), 1 mM Na_2EDTA, and 12 mM 1-thioglycerol (buffer A) and 10 mM Tris-HCl and 1 mM Na_2EDTA (buffer B) were used. Potassium chloride was added to achieve the desired salt concentration as indicated. A saturated solution of ammonium sulfate was prepared in buffer A, and the pH was adjusted to 7.4 with ammonium hydroxide. Poly(ethylene glycol) (PEG) was prepared as a 40% (w/v) solution in buffer A.

Preparation of Cytoplasmic Supernatant Fraction (Cytosol) Containing Receptor. Oviduct magna were removed, trimmed of connective tissue, sliced longitudinally, and rinsed a number of times in cold 0.9% NaCl. Unless otherwise noted, all further procedures were performed at 0–4 °C. The oviducts were minced and homogenized with a Polytron PT10 (Brinkman) using 2–4 volumes (milliliter per gram fresh weight) of buffer A. The homogenate was centrifuged at 27000*g* for 10 min, followed by 1 h at 105000*g*. The resultant supernatant fraction, after aspiration of the floating lipid layer, was used as cytosol. In studies involving protease inhibitors, buffer B was used. Receptors were labeled by incubation of the cytosol with 2×10^{-8} M [^{3}H]progesterone for 2 h.

Ion-Exchange Chromatography. Phosphocellulose (Whatman P-11) and DNA–cellulose column chromatogra-

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[1] Abbreviations used: DES, diethylstilbestrol; Na_2EDTA, disodium ethylenediaminetetraacetate; DEAE, diethylaminoethyl; [^{3}H]progesterone, [1,2-$^3H_2$]progesterone; IAA, iodoacetic acid; IAM, iodoacetamide; MalNEt, *N*-ethylmaleimide; PMSF, phenylmethanesulfonyl fluoride; DTNB, 5,5′-dithiobis(2-nitrobenzoate).

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phies were performed on chick oviduct cytosol as described by Coty et al. (1979). The sizes of these columns are given under Results. Further purification of the hen oviduct protease was performed on a 60-mL (packed volume) DEAE-Sepharose CL-6B column (Pharmacia) equilibrated in buffer A.

Receptor Substrate Preparation. The substrates used for the analysis of protease activity were either [^{3}H]progesterone-labeled cytosol receptor, [^{3}H]progesterone receptor from cytosol precipitated at 35% saturation of $(NH_4)_2SO_4$, or partially purified ^{3}H-labeled receptor B protein.

For the purpose of obtaining the 35% $(NH_4)_2SO_4$ precipitated ^{3}H-labeled receptor substrate (hereafter called *substrate*, since this preparation was most commonly used), cytosol was prepared in buffer B as indicated above. After being labeled for 1 h with 2×10^{-8} M [^{3}H]progesterone, protease activity was destroyed by making the cytosol 10 mM in iodoacetamide (see Results). After 1 h the sulfhydryl attacking reagent was inactivated by the addition of 12 mM 1-thioglycerol. The cytosol was then brought to 35% saturation with saturated $(NH_4)_2SO_4$ in buffer A. After the mixture was allowed to stand for 30 min, the ^{3}H-labeled receptor was pelleted by centrifugation at 27000*g* for 30 min. The supernatant was discarded, the tube wall was rinsed with cold distilled water, and the pellets were stored at −20 °C.

The ^{3}H-labeled receptor B protein was obtained as follows. Cytosol was passed over phosphocellulose and DNA–cellulose columns, followed by labeling with [^{3}H]progesterone. The receptors were precipitated at 35% saturated $(NH_4)_2SO_4$, and the pellet was redissolved in the original cytosol volume of buffer A. This was applied to a DEAE-cellulose column. The column was washed with buffer A and eluted with a linear KCl gradient from 0 to 0.5 M. The B receptor protein eluted at 0.2 M KCl from this column (Schrader, 1975). The peak fractions were pooled, and the KCl was removed by dialysis vs. buffer A.

Sucrose Density Gradient Centrifugation. Samples (0.2 mL) were layered on 5–20% linear sucrose density gradients in buffer A. Centrifugation was performed in a Beckman SW 50.1 rotor for 16 h at 2 °C and 45 000 rpm. Fractions (0.2 mL) were collected by gravity using a Buchler gradient fractionator, and fractionation was performed at 0–4 °C when the preparations were to be used in subsequent experiments.

Gel Filtration Chromatography. The protease was purified, and its Stokes radius was determined, using a Pharmacia Sephadex G-200 column (2.6 × 95 cm) equilibrated in buffer A containing 0.3 M KCl. Fractions (3 mL) were collected at a flow rate of 18 mL/h.

The hormone-binding receptor fragments were analyzed on a Pharmacia Sephadex G-100 column (2.6 × 36 cm) equilibrated in buffer A–0.3 M KCl. A 2-mL sample was applied, and 1.5-mL fractions were collected at a flow rate of 12 mL/h.

Radioactivity Measurements. 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. Tritium efficiency was 26%.

Results

DEAE-cellulose Slurry Assay. Previous studies have used sucrose and glycerol density gradient centrifugation and gel filtration chromatography to monitor the conversion of the native progesterone receptor forms to their proteolytic products (Sherman et al., 1974, 1976, 1978; Sherman & Diaz, 1977). Unfortunately, these methods require a long time to run and are limited in the amount of samples which can be processed at any one time. However, it has also been noted (Schrader et al., 1977b) that the meroreceptor does not bind to DEAE-cellulose at pH 7.4 while all other receptor forms do bind. This observation was used as the basis for an assay of the Ca^{2+}-activated protease which is rapid and reproducible and allows the handling of a large number of samples.

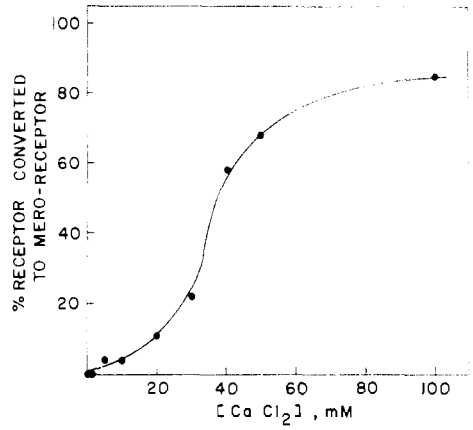

FIGURE 1: Effect of $CaCl_2$ concentration on meroreceptor formation. Chick oviduct cytosol (100 μL) was treated for 1–2 h at 0 °C with the indicated final concentration of $CaCl_2$. After addition of 1 mL of buffer A and 600 μL of a 50% DEAE-cellulose slurry (in buffer A), receptor was allowed to adsorb to the resin for 15 min. The resin was pelleted by a brief centrifugation and the supernatant counted.

Samples, usually 100 μL, were brought to 100 mM $CaCl_2$ for 1 h at 0 °C by the addition of 1.0 M $CaCl_2$ in water. The ionic strength was then reduced by dilution of the sample with 1 mL of buffer A, followed by the addition of 600 μL of a 50% (v/v) DEAE-cellulose (Whatman DE-52) slurry in buffer A. The resin was suspended every 5 min by gentle vortexing for a total of 15 min. The assay tubes were then spun in a tabletop centrifuge (Sorvall GLC) at 2600 rpm for 5 min at 4 °C to pellet the DEAE-cellulose. Finally, the supernatant was decanted into 20-mL scintillation vials and counted for ^{3}H in 8 mL of scintillation fluid. The amount of DEAE-cellulose slurry added and the total incubation time of the slurry with the sample are optimal, typically yielding >90% adsorption of native cytosol receptor–hormone complexes (data not shown). Control experiments using Sephadex G-25 chromatography demonstrated that Ca^{2+} additions resulted in less than 10% of the radioactivity appearing as free hormone. This was important because free hormone would be scored as meroreceptor with the DEAE-cellulose slurry assay.

By use of this assay, the reaction conditions involving the Ca^{2+}-activated proteolytic cleavage of the progesterone receptor were optimized. As shown in Figure 1, increasing levels of Ca^{2+} resulted in an increase in the total conversion of the cytosol receptor to meroreceptor after 1 h of treatment. Half-maximal activity was obtained at 40 mM Ca^{2+}, whereas 100 mM Ca^{2+} yielded ~80% conversion. Unless otherwise noted, 50 or 100 mM Ca^{2+} was used in the following experiments.

Figure 2 shows that, at 100 mM $CaCl_2$, meroreceptor formation was nearly linear for the first 10 min and thereafter progressed at a much lower rate, reaching a plateau at 60–120 min of incubation time. Therefore, 1 h was chosen as the standard reaction time for subsequent experiments. It should be noted that 20% conversion to meroreceptor was observed at the earliest time point in Figure 2. This is attributed to some conversion which occurred during the assay.

Receptor Proteolysis Generated by Elevated Temperature. The data in Figure 1 showed that relatively high (i.e., supraphysiological) concentrations of $CaCl_2$ were required for substantial receptor cleavage at 0 °C. Thus, experiments were performed to determine if reaction conditions could be found

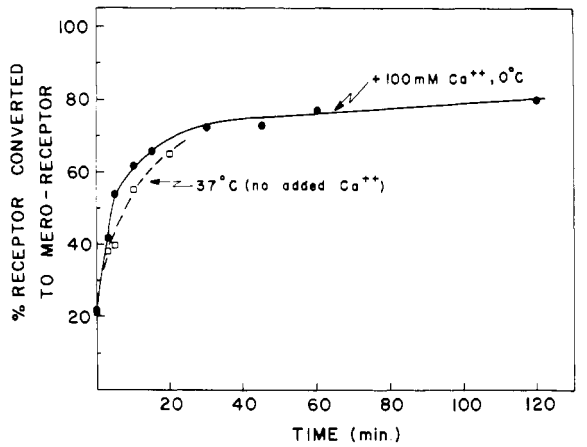

FIGURE 2: Time course of meroreceptor formation. Chick oviduct cytosol (100 μL) was treated for the indicated time with 100 mM $CaCl_2$ at 0 °C. Meroreceptor formation was assayed by using the DEAE-cellulose slurry assay as in Figure 1 (●). Data for the 37 °C incubation, with no added $CaCl_2$, were obtained by computing the area under the 2.6S region of the sucrose gradients in Figure 3 and expressing the meroreceptor as a percent of the total receptor-bound [^{3}H]progesterone (□).

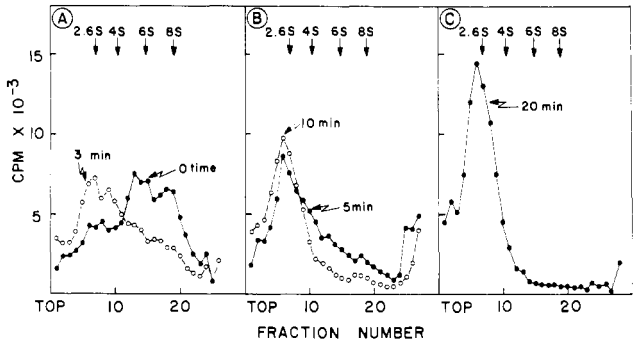

FIGURE 3: Generation of meroreceptor by incubation of cytosol at 37 °C. Chick oviduct cytosol was prepared in buffer A containing 1 mM PMSF (4 mL of buffer per g of oviduct), followed by labeling of the receptor with 1.6×10^{-8} M [^{3}H]progesterone for 2 h. 1 mL of cytosol was removed and incubated at 37 °C in a shaking water bath for each time indicated. Tubes were chilled for 30 min in an ice–water bath, and precipitated material was pelleted by a 15-min centrifugation at 4 °C. 200-μL aliquots of the supernatant were applied to 5–20% sucrose density gradients in buffer A and centrifuged, and fractions were collected as described under Materials and Methods. Arrows denote the sedimentation coefficients of progesterone receptor forms which were determined previously [see Vedeckis et al. (1978)].

which obviated the need for high Ca^{2+} concentrations. For a test of this, cytosol was warmed and assayed for meroreceptor production by the appearance of the 2.6S meroreceptor peak upon sucrose density gradient ultracentrifugation. Quantitation of the area under the 2.6S peak in Figure 3 allowed the measurement of meroreceptor formation which is plotted in Figure 2. It can be seen (Figure 2) that incubation of cytosol at 37 °C in the absence of added Ca^{2+} yielded similar reaction kinetics as those observed with 100 mM Ca^{2+} at 0 °C.

This assay method also allowed determination of the rate of conversion of both 6S and 8S cytosol receptor aggregates to meroreceptor as well as possible free hormone formation. Figure 3 shows that after 3 min at 37 °C meroreceptor formation was evident with a concomitant decrease in both the 6S and 8S receptor aggregate peaks. The conversion progressed with incubation time until at 20 min most of the receptor-bound progesterone migrated at 2.6 S (meroreceptor) on sucrose gradients. It is important to note that there was a relatively small increase in the amount of free hormone after 20 min at 37 °C, indicating that the hormone-binding activity

Table I: Tissue Distribution of Ca^{2+}-Activated Protease

tissue[a]	sp act. (units[b]/mg of protein)
oviduct	2.2
heart	3.9
liver	5.3
intestine	12.7
spleen	13.1
lung	16.5
thigh muscle	21.4
shell gland	105.8

[a] Each tissue was homogenized in 4 volumes (milliliter per gram fresh weight) of buffer A, and cytosol was prepared as for oviduct (see Materials and Methods). To 50 μL of cytosol was added 50 μL (87 700 cpm) of substrate. The reaction was started by the addition of 50 mM $CaCl_2$, and the DEAE-cellulose slurry assay was performed at 0, 1, 2, 5, 10, 20, 30, and 60 min after the start of the incubation. The initial rate of the reaction was then determined. [b] One unit of enzyme is that amount which catalyzes the conversion of 10 fmol of native receptor to meroreceptor per min.

is quite stable to the elevated temperature over this time interval. Finally, incubation of cytosol at 25 °C in 0.2 M KCl for 1–3 h also resulted in virtually quantitative conversion of native receptor to meroreceptor (data not shown). Thus, although very high Ca^{2+} concentrations are required for efficient reaction in crude cytosol at 0–4 °C, elevation of temperature can apparently effect the same conversion in the absence of added Ca^{2+}.

Tissue Distribution. Cytosol was prepared as described under Materials and Methods for a number of different chick tissues. Labeled receptor substrate was added to each followed by 50 mM $CaCl_2$. At various times after Ca^{2+} addition, aliquots were removed and subjected to the DEAE-cellulose slurry assay. The initial rate (slope) of meroreceptor formation was used to calculate the enzyme activity present in each cytosol sample. One unit of enzyme activity is defined as that which catalyzes the conversion of 10 fmol of native chick oviduct progesterone receptor to meroreceptor per min under the standard reaction conditions, that is, at 0 °C and 50 mM $CaCl_2$. The protein concentration of the cytosols was determined by using the method of Lowry et al. (1951).

As can be seen from Table I, all tissues assayed had a Ca^{2+}-activated protease activity which attacked the progesterone receptor. The highest amount of enzyme was found in the shell gland. Thigh muscle, lung, spleen, and intestine also had substantial activity while the lowest specific activity was found in oviduct tissue. However, since large quantities of this tissue are processed in the laboratory for receptor purification, for the protease isolation we utilized a fraction from the receptor preparation which is normally discarded. In addition, when the shell gland tissue is used for the purification of the Ca^{2+}-activated protease, the final specific activity obtained is not as good as that obtained from oviduct. Characterization of the shell gland enzyme demonstrated that it behaved identically with the oviduct protease upon DEAE-Sepharose and Sephadex G-200 chromatography (data not shown). The enzymatic activities of the other tissues were not further characterized.

Effect of Sulfhydryl Reagents on Crude Protease Activity. The results shown in Figure 3 confirm the previous observation (Sherman et al., 1974, 1978) that the Ca^{2+}-activated protease which cleaves the progesterone receptor is not a typical serine protease, since the incubation at 37 °C was performed in the presence of 1 mM PMSF, a potent serine protease inhibitor. Furthermore, neither 1 mM PMSF nor 5 mM 1,10-phenanthroline (an inhibitor of metalloexopeptidases and

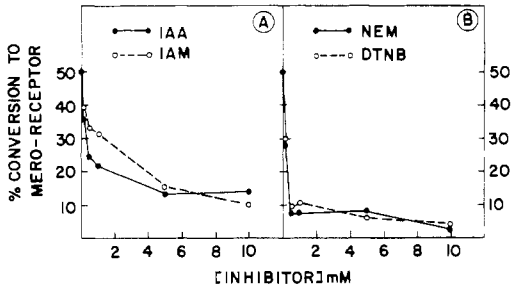

FIGURE 4: Inhibition of Ca^{2+}-activated protease by sulfhydryl attacking reagents. 100-μL aliquots of chick oviduct cytosol prelabeled with [^{3}H]progesterone were treated at the final concentration of reagent indicated. After 30 min the cytosol was made 100 mM in $CaCl_2$ and subjected to the DEAE-cellulose slurry assay as in Figure 1. The amount of conversion to meroreceptor was compared to controls treated with Ca^{2+} in the absence of sulfhydryl attacking reagents. All inhibitors were adjusted to pH 7.0 (at 25 °C) with KOH prior to addition to the cytosol samples. (A) IAA (●); IAM (○). (B) MalNEt (●); DTNB (○).

metalloendopeptidases) inhibited the conversion of cytosol receptor to meroreceptor by 100 mM $CaCl_2$ treatment (data not shown). However, reagents which attack sulfhydryl groups were very effective in inhibiting this enzymatic activity, as shown in Figure 4.

Solutions of various sulfhydryl attacking reagents were prepared in water, and the pH was adjusted to 7.0 with KOH. These were then added to cytosol prelabeled with [^{3}H]-progesterone and prepared in buffer B, which lacked thioglycerol. Iodoacetate and iodoacetamide effectively inhibited the Ca^{2+}-activated protease with maximal inhibition at 5–10 mM (Figure 4). There was no loss of receptor hormone-binding activity at any concentration tested (data not shown). MalNEt and DTNB caused maximal inhibition of activity at a 10-fold lower concentration of inhibitor (0.5 mM). However, a significant decrease in receptor-bound progesterone occurred at concentrations of 0.5 mM and greater for these latter two inhibitors. This is consistent with earlier observations on the effects of agents of this type upon receptor–hormone complexes (Sherman et al., 1970; O'Malley et al., 1971). Finally, since iodoacetamide-treated labeled receptor substrate can be hydrolyzed by the purified protease (see below), we conclude that the sulfhydryl attacking reagents inactivated the enzyme rather than rendered the receptor substrate resistant to hydrolysis. The studies described above demonstrate that the Ca^{2+}-activated protease responsible for receptor cleavage belongs to the general category of sulfhydryl proteases as classified by Hartley (1960).

Sedimentation Coefficient of the Crude Protease. Unlabeled cytosol was centrifuged on 5–20% sucrose density gradients as described under Materials and Methods, at either low or high (0.3 M KCl) ionic strength. Under both conditions the Ca^{2+}-activated protease sedimented at 6 S (Figure 5). Aliquots of these fractions were subsequently labeled overnight with 0.5×10^{-8} M [^{3}H]progesterone and assayed for progesterone receptor by using the DEAE-cellulose slurry assay. As has been shown previously (Schrader et al., 1975), high ionic strength buffer resulted in a shift in the sedimentation coefficient of the receptor from 6–8 to 4 S (Figure 5).

Since the sedimentation coefficient of the protease was not decreased in high ionic strength buffer whereas that of the receptor was diminished from 6 and 8 S to 4 S, it does not appear that the protease, in its native state, is a subunit of the progesterone receptor complex. Other attempts to analyze this possibility have yielded similar negative results (Vedeckis et al., 1980).

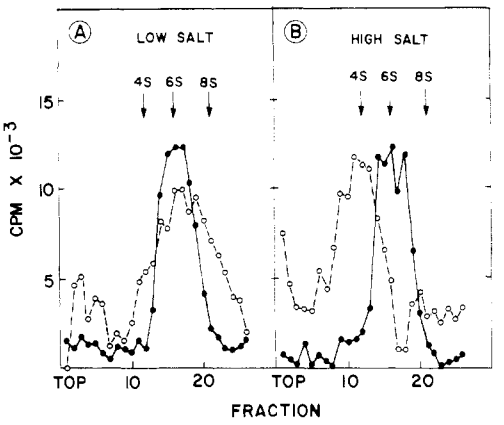

FIGURE 5: Sucrose density gradient centrifugation of the Ca^{2+}-activated protease. Unlabeled cytosol was centrifuged through 5–20% sucrose gradients as described under Materials and Methods and fractionated at 4 °C. Two 50-μL aliquots from each fraction were mixed with 50 μL of [^{3}H]B substrate (see Materials and Methods; total radioactivity per reaction = 20 500 cpm). One aliquot was made 100 mM in $CaCl_2$ while the other served as the control (water only added). The supernatant radioactivity in the control was subtracted from the $CaCl_2$-treated samples (●). 100-μL aliquots from each sucrose gradient fraction were labeled overnight with 18 000 cpm (0.5×10^{-8} M) of [^{3}H]progesterone. These samples were subsequently analyzed for hormone-binding activity (○). In both analyses the DEAE-cellulose slurry assay was utilized. Sucrose gradients were made up in either buffer A (panel A) or buffer A–0.3 M KCl (panel B).

Table II: Separation of Receptor from Protease by Ammonium Sulfate Precipitation

ammonium sulfate concn added[a] (% saturation)	receptor precipitated[b] (%)	conversion to meroreceptor[c] (%)
25	23	0
30	45	0
35	71	18
40	94	34
45	89	63
50	86	99

[a] Cytosol was brought up to the concentration of $(NH_4)_2SO_4$ listed with buffer-saturated $(NH_4)_2SO_4$ and pelleted by centrifugation, and the pellets were dissolved in the original cytosol volume of buffer A. [b] An aliquot of the redissolved pellet was counted and compared with the total cytosol receptor level as determined by a DEAE-cellulose slurry assay. [c] The percent conversion to meroreceptor was obtained by using a DEAE-cellulose slurry assay ($\pm Ca^{2+}$) under the standard conditions outlined in the text.

Isolation of Receptor Free of Protease Activity. Early observations by Sherman and her colleagues (1974) suggested that the meroreceptor was a subunit of the native progesterone receptor, liberated by a direct effect of calcium upon the protein. However, subsequent experiments with purified receptor showed that both A and B are single polypeptide chains (Schrader et al., 1977a; Coty et al., 1979). These results pointed to the fact that a protease activity was involved. For a test of whether the protease activity resided in the receptors themselves, the B protein, purified to apparent homogeneity, was treated with $CaCl_2$. No meroreceptor was produced (Kuhn et al., 1977). Furthermore, the receptor A protein could be separated from the activity by using DEAE-cellulose filters (Sherman & Diaz, 1977).

For verification that the activity responsible for meroreceptor formation resides in a macromolecule distinct from the receptor, cytosol was precipitated with varying concentrations of $(NH_4)_2SO_4$ and the receptor content and ability to generate

Table III: Purification of the Ca^{2+}-Activated Protease from Hen Oviduct

step	vol (mL)	protein (mg/mL)	total protein (mg)	total units[a]	sp act. (units/mg)	purifn (x-fold)	recovery (%)
low-speed supernatant	1050	55	57 800	37 200	0.64	1	100
PEG ppt	126	96	12 100				
phosphocellulose DT[b]	420	19	7 980	82 300	10.3	16	221
DNA-cellulose DT[b]	440	6	2620	80 300	30.6	48	216
ammonium sulfate ppt (40–60%)	500	2	1 000	78 800	78.8	123	212
DEAE-Sepharose pool (peak II)	74	0.92	68	22 700	334	522	61
Sephadex G-200 pool	61	0.19	12	15 500	1290	2020	42

[a] One unit of activity is defined as that amount of enzyme which catalyzes the conversion of 10 fmol of native progesterone receptor substrate to meroreceptor per min under the standard reaction conditions. [b] DT, dropthrough.

meroreceptor by Ca^{2+} treatment were both assayed. Table II shows that although 45% of the total cytosol receptor was precipitated at 30% saturated $(NH_4)_2SO_4$, no conversion to meroreceptor was obtained in the dissolved pellet. Furthermore, at an ammonium sulfate concentration which quantitatively precipitated receptor (40% saturation), only 34% of the receptor was converted to meroreceptor by Ca^{2+} treatment. A concentration of 50% saturated $(NH_4)_2SO_4$ was required for complete conversion to meroreceptor in this reaction time. Thus, it appears that a separate molecule (protease), which precipitates at a higher $(NH_4)_2SO_4$ concentration than the receptor, is required for the conversion of the native receptor to meroreceptor. This is very similar to the behavior of RTF in the calf uterine estrogen receptor system (Puca et al., 1977). Therefore, the receptor and the Ca^{2+}-activated protease can be effectively separated by differential precipitation with $(NH_4)_2SO_4$. These observations led us to an attempt to purify this enzyme.

Poly(ethylene glycol) (PEG) Precipitation of the Low-Speed Supernatant. The low-speed supernatant (27000*g*, 10 min) was used instead of cytosol (high-speed superntant) for the purification of the hen oviduct protease. This modification allows the preparation to be scaled-up considerably (10-fold).

One volume of 40% PEG was added to 3 volumes of low-speed supernatant to precipitate the protease. This was then centrifuged at 27000*g* for 30 min. After the supernatant was decanted, the pellet was dissolved in approximately one-tenth the original low-speed supernatant volume in buffer A, using an Ultra-Turrax (Janke and Kunkel) homogenizer. Insoluble material was pelleted by centrifugation at 27000*g* for 15 min.

When this preparation was analyzed for Ca^{2+}-activated protease activity, no activity could be detected. However, it was shown that after additional purification steps the enzyme activity was, in fact, present (Table III). Perhaps an inhibitor or some interfering substance (PEG?) is present in the sample.

Phosphocellulose Dropthrough (PC) and DNA–Cellulose Dropthrough Chromatography. The PC (360-mL packed volume) and DNA–cellulose (180-mL packed volume) columns were primarily run for the sake of the purification of the A receptor subunit (Coty et al., 1979). Because we were using a "byproduct" of the A receptor preparation from hen oviduct, no purification of the protease was sought during the procedures. However, Table III demonstrates an increase in the total amount of protease recovered after the PC dropthrough step as compared to the low-speed supernatant. We interpret these results to indicate that, perhaps, an inhibitor of the protease activity was removed via phosphocellulose adsorption. Another possibility is that some other competing substrate(s) was removed by the PC chromatography. The purification obtained from the phosphocellulose dropthrough step was 16-fold.

The recovery of protease activity in the DNA–cellulose dropthrough was 98% of the applied material, with a purification of threefold (Table III).

Ammonium Sulfate Precipitation. As shown in Table II, the oviduct progesterone receptor precipitates preferentially at 30–40% saturation of $(NH_4)_2SO_4$ while the protease precipitates between 40 and 60% saturation. Thus, after precipitation of the receptors at 40% saturated $(NH_4)_2SO_4$, the supernatant was centrifuged at 105000*g* for 1 h to remove any insoluble material. The 40% saturated $(NH_4)_2SO_4$ supernatant was brought to 60% saturation by using solid crystals of $(NH_4)_2SO_4$. Besides an excellent recovery of the total protease activity by this step (98%), it also removed most of the endogenous receptor substrate from the protease preparation. The step purification obtained was 2.6-fold. The 40–60% ammonium sulfate precipitate was dissolved in a quantity of buffer A which lowered the salt concentration to the equivalent of 0.15 M KCl or lower.

DEAE-Sepharose Chromatography. The redissolved $(NH_4)_2SO_4$ precipitate was applied to a 60-mL DEAE-Sepharose column, and, after collecting the dropthrough fractions, the column was washed with 3 column volumes of buffer A. Elution was performed by using a 470-mL linear KCl gradient in buffer A containing 0–0.6 M KCl. Fractions (5.9 mL) were collected, and every second fraction was assayed for the protease by using the DEAE-cellulose slurry assay. Gradient elution of the column resulted in two peaks of Ca^{2+}-activated protease activity (Figure 6).

DEAE-Sepharose chromatography resulted in an apparent fourfold purification of the protease activity in the peak II material (Table III). Recovery of the protease activity was only 29% of that applied to the column. An undetermined amount may have eluted in the dropthrough and wash of the column, and a considerable amount of the activity was present in peak 1 (Figure 6). However, since peak II was more highly purified it was used in the subsequent steps. Peak II from the DEAE-Sepharose chromatography was brought to 60% saturated ammonium sulfate (with solid crystals) to concentrate the protease. After centrifugation (27000*g*, 30 min) the pellet was redissolved in 3 mL of buffer A.

Sephadex G-200 Chromatography. Gel filtration chromatography of the redissolved pellet on Sephadex G-200 is shown in Figure 7. The purification obtained from this step was approximately fourfold, and the recovery of applied protease activity was 68%. The G-200 pool (hereafter referred to as "purified protease") was precipitated at 60% saturated ammonium sulfate and stored at 4 °C. This preparation was stable under these conditions for at least 3 months (data not shown). Thus, following a number of steps to eliminate the major contaminants of hen oviduct cytosol, we have obtained a very active preparation of Ca^{2+}-activated protease (Table

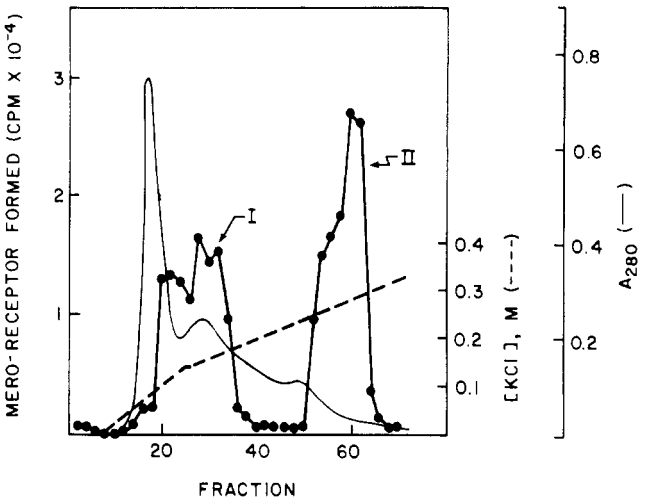

FIGURE 6: DEAE-Sepharose chromatography of the Ca^{2+}-activated protease from hen oviduct. 500 mL of the redissolved 40–60% ammonium sulfate precipitate was applied to a 60-mL (packed volume) column of DEAE-Sepharose CL-6B. The column was washed with 3 column volumes of buffer A and the protease eluted with a 470-mL linear KCl gradient in buffer A containing 0–0.6 M KCl. Aliquots (50 μL) of every second fraction (5.9 mL) were assayed by using the DEAE-cellulose slurry assay. The substrate used was the labeled 35% ammonium sulfate pellet, and 50 mM Ca^{2+} was added for 30 min. The absorbance was read at 280 nm. Fractions 52–63 were pooled, precipitated at 60% saturated $(NH_4)_2SO_4$ by the addition of solid crystals, and centrifuged to pellet the precipitated protein. The pellets were redissolved in a total of 3 mL of buffer A. (●) Protease activity; (—) A_{280}; (- - -) [KCl], molarity.

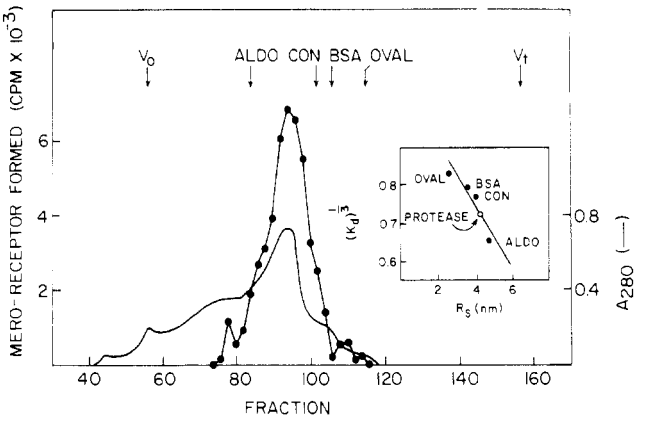

FIGURE 7: Sephadex G-200 chromatography of the Ca^{2+}-activated protease. The concentrated (3 mL) pool from DEAE-Sepharose was applied to a 2.6 × 95 cm Sephadex G-200 column equilibrated in buffer A–0.3 M KCl. 3-mL fractions were collected, and 10 μL from every fifth fraction (fractions 40–60 and 125–140) or every second fraction (fractions 62–120) was assayed for protease activity as indicated in the text and the legend to Figure 6. Absorbance was read at 280 nm. Fractions 84–102 were brought to 60% saturated $(NH_4)_2SO_4$ by the addition of solid crystals and stored at 4 °C. The protein standards used were as follows: rabbit muscle aldolase (R_s = –4.97 nm); conalbumin (R_s = 4.07 nm); bovine serum albumin (R_s = 3.63 nm); ovalbumin (R_s = 2.8 nm). (●) Protease activity; (—) A_{280}. The inset shows the determination of the Stokes radius of the protease by using these protein standards. $K_d = (V_e - V_0)/(V_t - V_0)$, where V_e = elution position of the protease or standard proteins, V_0 = void volume determined by using blue dextran (M_r 2 000 000), and V_t = total volume of the column determined by the elution position of KCl.

III). The final protease preparation was purified more than 2000-fold with a 42% yield compared to the low-speed supernatant fraction.

Characterization of the Digestion Product Produced by the Purified Protease. The authenticity of the purified protease was shown by incubating ^3H-labeled receptor B protein with the purified protease in the presence of 50 mM Ca^{2+} (Figure 8A). The digested receptor eluted from the G-100 column primarily as meroreceptor. Very little free progesterone was generated as evidenced by Sephadex G-25 chromatography, eliminating the possibility of the purification of a "nonspecific" protease.

Ca^{2+} Requirement and the Effect of Calmodulin on Purified Protease Activity. Studies above using a crude cytosol enzyme preparation (Figure 1) indicated that the optimal Ca^{2+} concentration for the reaction was 50–100 mM. This extraordinarily high Ca^{2+} concentration was unusual, since many Ca^{2+}-dependent enzymes are inhibited by such high concentrations (Szego et al., 1976). This experiment was repeated by using the purified enzyme, as shown in Figure 9. In this case, the Ca^{2+} requirement was drastically reduced to between 0.5 and 1 mM Ca^{2+}.

The product of the purified protease activity was then analyzed at a lower (2 mM) Ca^{2+} concentration. When analyzed by Sephadex G-100 chromatography, a peak was observed which corresponded to meroreceptor (Figure 8B). Therefore, the proteolytic fragments produced by the crude and purified protease at both high (50 mM) and low (2 mM) Ca^{2+} levels were the same (meroreceptor). This validates the use of the DEAE-cellulose slurry assay with the purified protease at low Ca^{2+} concentrations (Figure 9). High concentrations, though not required, were used with the purified enzyme to obtain maximal reaction kinetics and total conversion.

It is not known by what mechanism the $CaCl_2$ requirement was lowered. However, a number of apparent Ca^{2+}-dependent enzyme activities have been found to require the presence of a small (M_r ~20000) calcium-binding protein called calmodulin [calcium-dependent regulator (CDR)]. We therefore added 10 μg/mL pure rat testis calmodulin (Dedman et al., 1977) to the purified protease and measured the rate of meroreceptor formation at various Ca^{2+} concentrations. As can be seen in Figure 9, calmodulin did not increase the rate of the reaction at any Ca^{2+} concentration utilized. Therefore, a role for calmodulin in the action of the Ca^{2+}-activated protease could not be demonstrated.

Characterization of the Purified Protease. Under the reaction conditions used, the conversion of substrate to meroreceptor by the purified protease was linear for the first 10 min of the reaction time and the rate of conversion was dependent upon enzyme concentration (data not shown). The Stokes radius of the protease was determined by a comparison of its elution position on Sephadex G-200 to various standard proteins (Figure 7, inset). This analysis yielded a value of 4.3 nm for the Stokes radius (R_s). As shown earlier (Figure 5), sucrose density gradient centrifugation of cytosol demonstrated that the sedimentation coefficient of the enzyme is 6 S at either low or high ionic strength. In addition, the purified protease sediments at 6 S (data not shown). Using the Stokes radius and the sedimentation coefficient, it was possible to calculate the molecular weight (113 000) and the frictional ratio (1.24) of the protease (Siegel & Monty, 1966).

When various concentrations of the ^3H-labeled receptor substrate were incubated with the protease, the affinity of the enzyme for its substrate could be assessed. These experiments resulted in the value of 1.04×10^{-8} M for the Michaelis constant of the enzyme for its substrate.

Comparison of the Crude and Purified Enzymes. The studies summarized in Table IV were performed to test whether the crude and purified enzymes behaved similarly. Of particular importance was the fact that we had shown that the product of receptor hydrolysis in both cases was the

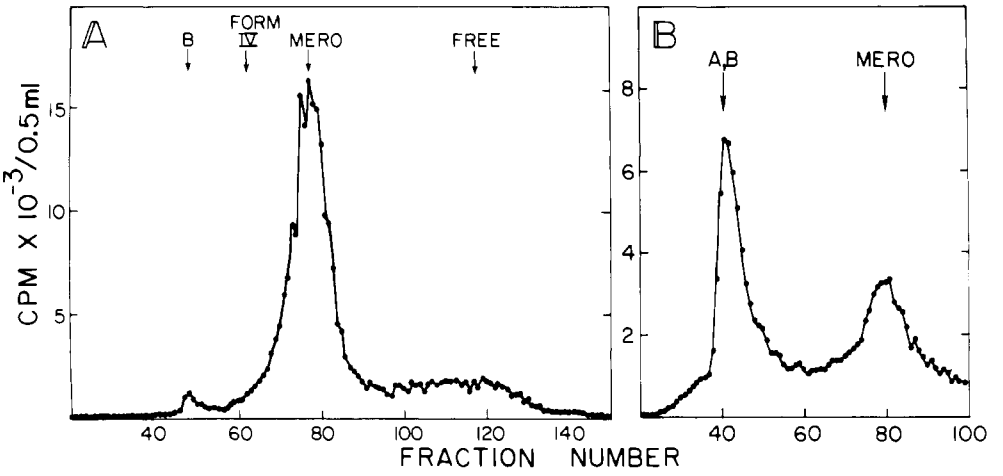

FIGURE 8: (A) Conversion of [^{3}H]B receptor protein to meroreceptor by the purified protease. 4 mL of [^{3}H]B receptor (2650000 cpm) was mixed with 200 μL of purified protease. After 30 min at 50 mM $CaCl_2$ the mixture was desalted on a 20-mL Sephadex G-25 column. 2 mL of the excluded receptor-bound [^{3}H]progesterone (850000 cpm) was chromatographed on a Sephadex G-100 column as described under Materials and Methods. 500 μL of the 1.5-mL fractions was counted. Elution positions were as follows: B = authentic B receptor protein; form IV (M_r 43000); mero = meroreceptor (M_r 23000); free = uncomplexed [^{3}H]progesterone. (B) Reaction product formed by the purified protease at low Ca^{2+}. 1 mL of receptor substrate was mixed with 1 mL of purified protease. After 5 min of reaction at 2 mM Ca^{2+}, the sample was applied to a Sephadex G-100 column and chromatography was performed as in (A).

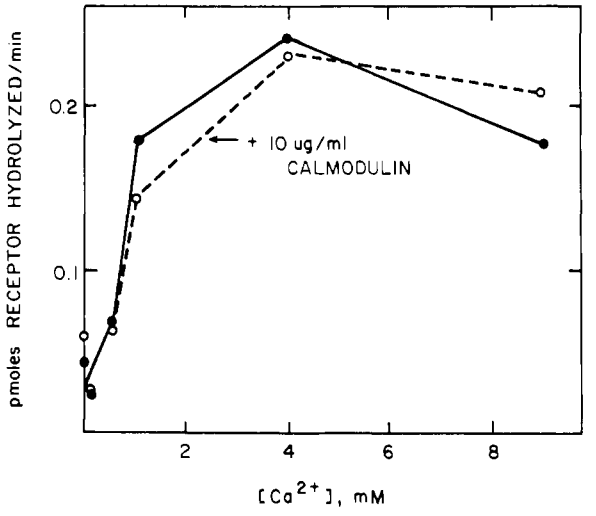

FIGURE 9: Effect of $CaCl_2$ concentration on the enzymatic activity of the purified protease. Purified protease (50 μL) and substrate (50 μL) were mixed and various concentrations of $CaCl_2$ added. The initial rate of the conversion of substrate to meroreceptor was determined by performing the DEAE-cellulose slurry assay at various times after Ca^{2+} addition. (●) Protease alone; (○) protease plus 10 μg/mL purified rat testis calmodulin.

meroreceptor [see parts A and B of Figure 8 and Vedeckis et al. (1979a)]. Table IV also shows that the sedimentation coefficient and response to protease inhibitors were the same for both the crude and purified materials although the purified enzyme was somewhat more sensitive to the inhibitors. In addition, the 40–60% saturated $(NH_4)_2SO_4$ precipitate eluted in the same position from the Sephadex G-200 column as did the purified protease. Finally, both the crude and purified proteases demonstrated an irreversible inactivation of enzymatic activity by Ca^{2+} (Table IV). Removal of $CaCl_2$ by dialysis did not restore activity (data not shown).

Table IV: Comparison of the Crude and Purified Protease Activities

parameter	protease[a] crude	protease[a] purified
sedimentation coeff	6 S	6 S
Stokes radius[b]	4.3 nm	4.3 nm
mol wt[c]	113000	113000
ID_{50} for iodoacetamide[d]	3 mM	0.8 mM
ID_{50} for sodium fluoride[d]	9 mM	5 mM
inactivation time[e]	30 min	30 min
half-maximal [Ca^{2+}]	40 mM	0.8 mM

[a] The crude enzyme source was cytosol, and the purified enzyme was the Sephadex G-200 pooled material as prepared in Table III. [b] For this determination the crude protease was precipitated from cytosol at 40–60% saturation in $(NH_4)_2SO_4$. [c] Determined from s and R_s (Siegel & Monty, 1966). [d] Concentration of inhibitor which yielded one-half of the enzyme activity without any inhibitor. [e] Time after 50 mM $CaCl_2$ addition at which no protease activity remained when analyzed by the addition of substrate.

Table V: Characteristics of the Ca^{2+}-Activated Protease

parameter	
mol wt	113000
sedimentation coeff	6 S
Stokes radius	4.3 nm
frictional ratio (f/f_0)	1.24
K_m for receptor	1.04×10^{-8} M
functional requirement	reduced sulfhydryl group
activators[a]	$Ca^{2+} > Mn^{2+} = Sr^{2+} > Ba^{2+}$
inhibitors	iodoacetate, iodoacetamide, MolNEt, DTNB, leupeptin,[b] antipain[b]

[a] Sherman et al. (1974). [b] Sherman et al. (1978).

Discussion

The protocol described above resulted in a substantial purification of a Ca^{2+}-activated protease which can catalyze limited proteolysis of the chick oviduct progesterone receptor proteins. A summary of the characteristics of this enzyme is given in Table V. The purification obtained (2000-fold) and the yield (42%) are compatible with planned studies on progesterone receptor protein structure. At present we have no way of assessing the absolute purity of the enzyme preparation. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the purified protease demonstrated clearly that it was not homogeneous (data not shown). Approximately 20 protein bands were discernible on the gel. Of these there were six major bands which constituted ~60–70% of the total protein and ranged in molecular weight from 18000 to 86000. It is not known whether any of these major bands represents the

protease, subunits of the enzyme, or various components responsible for peaks I and II obtained by DEAE-Sepharose chromatography.

The purified hen oviduct protease has characteristics identical with those of the chick oviduct enzyme (Vedeckis et al., 1979). Additionally, the purified chick and hen oviduct enzymes have the same elution profile as crude chick oviduct protease when analyzed by Sephadex G-200 chromatography. Finally, the purified hen oviduct protease produces the same limit digest (meroreceptor) as the crude enzyme does in cytosol (Vedeckis et al., 1979, 1980). Thus, these properties of the enzyme have not undergone drastic alterations during the purification.

The purified oviduct protease has a Michaelis constant for progesterone receptor which is quite low (1.04×10^{-8} M). This is significant, because the receptor exists in the cell at a concentration similar to the K_m of the enzyme. Thus, it is at least kinetically possible for this protease to have some physiological function in vivo with respect to the receptor proteins. No direct in vivo functional tests have yet been undertaken for this enzyme. We have also found that the purified enzyme requires much lower levels of Ca^{2+}. Since we have no way of knowing the milieu of the enzyme within the cell, we cannot as yet speculate as to the normal role of Ca^{2+} for this enzyme activity. However, the highest levels of this enzyme have been found in the chick shell gland, which is the major Ca^{2+}-mobilizing tissue in this animal and also contains progesterone receptor. It is not clear why high levels of Ca^{2+} do not inhibit the enzyme as has been found with other Ca^{2+}-activated proteases. However, RTF is also not inhibited by Ca^{2+}, at least up to 12 mM (Puca et al., 1977).

The effect of Ca^{2+} appears to be activation of the enzyme rather than alteration of the substrate conformation in a fashion rendering it susceptible to hydrolysis. When Ca^{2+} was added to the enzyme in the absence of receptor substrate, a time-dependent, irreversible decrease in enzymatic activity was observed (Table IV). One interpretation of this is an activation of the enzyme, followed by autoproteolysis, similar to results obtained with the RTF in calf uterine extracts (Puca et al., 1972, 1977).

Finally, it has not been possible to demonstrate that the Ca^{2+} effect on enzyme activity is mediated by calmodulin, although the presence of endogenous calmodulin in the purified protease prevents the exclusion of this possibility.

The characterization of this enzyme has yielded some interesting observations. The protease has a molecular weight of 113 000, a sedimentation value of 6 S, a Stokes radius of 4.3 nm, and a K_m for receptor substrate of 1.04×10^{-8} M, and it is a sulfhydryl (thiol) protease. These values are remarkably similar to the Ca^{2+}-activated sulfhydryl protease (RTF) found in calf uterine cytoplasmic extracts [M_r 115 000; 6.4 S; R_s = 4.5 nm; K_m for estrogen receptor = 1.25×10^{-8} M (Puca et al., 1977)]. Whether enzyme activities similar to these are present in other steroid receptor systems requires a more detailed analysis of these systems. A recent report (Wrange & Gustaffson, 1978) demonstrated a proteolytic activity which presumably separates the hormone- and DNA-binding domains of the glucocorticoid receptor. Additionally, Sherman et al. (1978) have shown the existence of meroreceptors in a number of different steroid hormone systems.

We have performed some preliminary experiments to determine the relationship of the two enzyme activities (peaks I and II) observed upon DEAE-Sepharose chromatography. Peak I hydrolyzes [^{3}H]B receptor protein to the same hormone-binding fragment (meroreceptor) as peak II, and both enzyme forms elute at the same position upon Sephadex G-200 chromatography (data not shown). Thus, peaks I and II have the same enzymatic activity and apparent Stokes radius. We cannot yet tell if peak I consists of subunits of peak II or is a partial proteolytic digest. Rechromatography of peak II yields some peak I material, but peak I does not convert to peak II, even after salt removal and concentration. In addition, low levels of Ca^{2+} do not cause a quantitative conversion of peak II material to peak I. Therefore, we are at present still uncertain about the relationships between these two forms of the enzyme. We chose to use peak II material for subsequent studies because it was the more pure of the two species.

We plan to use the purified protease in subsequent studies on the molecular structure of the chick oviduct progesterone receptor, specifically with respect to the biologically active binding domains for progesterone and nuclear constituents. The companion paper describes experiments using the protease to hydrolyze the progesterone receptor.

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

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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

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[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.

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