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Characterization of Two Uterine Proteases and Their Actions on the Estrogen Receptor†

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ABSTRACT: We have characterized two previously undetected proteases from the calf uterine cytosol and measured their actions on the estrogen receptor. One is an exopeptidase, purified 60-fold, that hydrolyzed amino acid (lysine-, and alanine-, or leucine-) *p*-nitroanilide substrates and leucylglycylglycine, did not hydrolyze [^{14}C]methemoglobin, was completely inhibited by 1 mM bestatin or puromycin (specific inhibitors of leucine aminopeptidase like enzymes), and was unable to influence the sedimentation of the 8S form of the estrogen receptor in sucrose gradients containing dilute Tris buffer. A commercial porcine leucine aminopeptidase, like the calf uterine aminopeptidase, did not convert the 8S estrogen receptor to a 4S form. Evidently, removal of the N-terminal amino acid(s) from the estrogen receptor by exopeptidase action cannot alter the sedimentation of the 8S form of the receptor, or the N-terminal amino acid(s) of the receptor is (are) unaccessible or resistant to exopeptidase activity. The second, a receptor-active protease, is an endopeptidase that did not hydrolyze any of the synthetic amide or peptide substrates tested but did possess [^{14}C]methemoglobin-degrading activity and the ability to convert the 8S estrogen receptor to a modified 4S form in sucrose gradients containing dilute Tris buffer. The modified 4S receptor was separable from the native receptor by DEAE-cellulose chromatography. The endopeptidase did not require Ca^{2+} for activity, and its chromatographic properties were distinctly different from a previously isolated Ca^{2+}-activated protease. It was inhibited by leupeptin or dipyridyl disulfide, suggesting the presence of a thiol group that is essential for its activity. These data indicate that a decrease in the sedimentation rate of the estrogen receptor in sucrose gradients with low salt or a change in the receptor's elution on DEAE-cellulose chromatography is not related to receptor activation but is produced by the receptor-active protease or other proteases.

Resolution of the molecular characteristics of the estrogen receptor and other steroid hormone receptors is a key step in relating structure to function for genomic regulatory proteins. Multiple forms of the estrogen receptor have been reported (Notides, 1978; Stancel et al., 1973). Several laboratories including our own have demonstrated two major forms of the receptor. The cytoplasmic or nonactivated estrogen receptor sediments into sucrose gradients containing 0.15 or 0.4 M KCl as a 4S protein, while the nuclear or activated estrogen receptor sediments at 5S (Jensen & DeSombre, 1973; Notides & Nielsen, 1974). The activated 5S receptor is a dimer composed of two 4S estrogen receptor molecules (Notides et al., 1975, 1981). The cytoplasmic estrogen receptor can also be transformed into the activated 5S receptor by salting out during ammonium sulfate fractionation (Weichman & Notides, 1979). Furthermore, analysis of the estrogen receptor in sucrose gradients containing low salt concentrations (only dilute Tris buffer) aggregates the estrogen receptor to a third form—the 8S species. The 8S estrogen receptor aggregate is observed either before or after purification by ammonium sulfate precipitation.

In studying the molecular properties of the steroid hormone receptors, it is important to discern those properties of the receptor that are inherent in its molecular structure from those that are inadvertently produced by the action of extraneous factors such as endogenous proteases. The endogenous proteases are also of concern when isolation and purification of the native receptor are undertaken. Previous reports have indicated that the steroid hormone receptors are hydrolyzed by endogenous proteases to species having decreased sedimentation coefficients in sucrose gradients containing low salt concentrations (Notides et al., 1972, 1973; Puca et al., 1972, 1977; Sherman et al., 1978; Wilson & French, 1979). Proteolysis of the receptor frequently may lead to a loss of the DNA-binding domain of the receptor (Notides et al., 1976); however, a very limited proteolysis of the receptor may result in retention of part or the complete DNA-binding domain of the receptor (Wrange & Gustafsson, 1978).

In this report we characterize two calf uterine proteases, previously undetected, and describe their effects on the estrogen receptor.

Materials and Methods

Materials. 17β-[2,4,6,7-$^{3}H_4$]Estradiol (90 or 102 Ci/mmol) and [*methyl*-^{14}C]methylated methemoglobin (24.4 μCi/mg) were obtained from New England Nuclear. Leucine aminopeptidase was purchased from Worthington Biochemical Corp. Alkaline phosphatase (*Escherichia coli*), diisopropyl fluorophosphate (DFP), puromycin hydrochloride, 4,4′-dipyridyl disulfide, and lysine-, leucine-, and alanine-*p*-nitroanilide were obtained from Sigma Chemical Company. Leupeptin was purchased from Peninsula Laboratories. Sucrose and am-

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monium sulfate are ultrapure grades obtained from Schwarz/Mann. DEAE-cellulose and Sephadex G-200 were purchased from Whatmann and Pharmacia, respectively.

Buffers. The following buffer solutions were used: TD buffer containing 40 mM Tris-HCl–0.5 mM dithiothreitol (DTT), pH 7.4; TDK buffer containing 40 mM Tris-HCl–0.5 mM DTT–150 mM KCl, pH 7.4; TPD buffer containing 40 mM Tris–phosphate–0.5 mM DTT, pH 7.6. All pH values are at 22 °C.

Assay of Enzymatic Activity. Column fractions or samples (≤0.1 mL) were assayed for hydrolytic activity toward 3 mL of 1 mM lysine-, leucine-, or alanine-*p*-nitroanilide (Sigma Chemical Co.) in 40 mM Tris-HCl, pH 7.4 at 22 °C. Solutions of 1 mM leucine-*p*-nitroanilide also contained 1% dimethyl sulfoxide. One unit of enzyme activity is equal to the hydrolysis of 1 μmol of substrate (E at 405 mm is 9620 for *p*-nitroaniline; Wachsmuth et al., 1966).

The hydrolytic activity toward [*methyl*-^{14}C]methylated methemoglobin was measured as soluble counts following the addition of trichloroacetic acid to reaction mixtures containing this substrate and the appropriate enzyme. Each protein or buffer sample (50 μL) was incubated with 100 μg (100 μL) of labeled methemoglobin (0.02–0.03 μCi) for 20 h at 22 °C and cooled to 0 °C, and then 40 μL of a 2% solution of cold methemoglobin carrier and 140 μL of a 10% trichloroacetic acid solution were added. The reaction mixture was centrifuged and 0.1 mL of the supernatant was removed for scintillation counting.

Exopeptidase activity was assayed by the incubation of 1 mg of leucylglycylglycine in 0.4 mL of enzyme solution or TKD buffer for 2.5 h at 37 °C. Digestion was monitored by thin-layer chromatography on silica gel sheets (Eastman Kodak Co.) in 1-butanol–acetic acid–water (70:15:15). Following chromatography, the sheets were sprayed with a 2% solution of ninhydrin in acetone and heated for 5 min at 80 °C. Glycylglycine, glycine, leucylglycylglycine, leucylglycine, and leucine had R_f values of 0.11, 0.17, 0.41, 0.52, and 0.61, respectively. Protein determinations were performed by using Bio-Rad Laboratories protein assay dye reagent (Bradford, 1976); the protein standard was bovine serum albumin.

Preparation of the Estrogen Receptor. Calf uteri were homogenized at 0–4 °C in 5 volumes of TD buffer with a Polytron P-10 (Brinkman). The homogenate was centrifuged at 20000*g* for 10 min, and then the supernatant was removed and centrifuged for 1 h at 220000*g*. The supernatant from the 220000*g* centrifugation is referred to as the "cytosol". Cytosol was incubated with 10 nM [^{3}H]estradiol for 1 h at 0 °C. Solid ammonium sulfate was slowly added until 25% saturation was reached, stirred for 1 h at 0 °C, and then centrifuged for 20 min at 20000*g*. The resulting pellet was washed with a small volume of TDK buffer containing ammonium sulfate at 25% saturation, then dissolved in TDK (half to two-thirds the original volume of cytosol), and dialyzed against 500 volumes of TDK buffer for 3–5 h at 0–4 °C.

Ammonium Sulfate Fractionation of Cytosol for Receptor-Active Protease. Cytosol was prepared as described above except that 40 mM Tris-HCl, pH 7.4, was substituted for the TD buffer. A pellet from each ammonium sulfate fraction of the cytosol (35–55%, 45–55%, 45–65%, and 45–75%) was dissolved in TKD ($^1/_{10}$–$^1/_{20}$ the original volume of cytosol) and dialyzed against 500 volumes of TDK buffer for 3 h at 0–4 °C. The dialyzate was clarified by centrifugation, the supernatant was removed, and unlabeled estradiol was added (1 μM) to block all nonspecific and specific estradiol-binding sites. The addition of unlabeled estradiol to the protease fractions avoids artifacts caused by the dissociation of [^{3}H]-estradiol from the receptor and subsequent rebinding by unoccupied sites, nonspecific or specific, in the protease fractions during mixing experiments. In addition, the receptor–protease mixture is incubated at 0 °C rather than 28 °C, since the protease is active at 0 °C and the dissociation of [^{3}H]estradiol is very slow at 0 °C as compared with that at 28 °C. Each ammonium sulfate fraction of the cytosol was assayed for its ability to convert the receptor from the 8S form to a modified 4S form. To 3 mL of the partially purified [^{3}H]estradiol–receptor (25% ammonium sulfate fraction) in TDK buffer, 0.5 mL of enzyme fraction or TDK buffer containing 1 μM estradiol was added. The reaction was stopped after 20–22 h at 0 °C by adding ammonium sulfate to 25% saturation. The precipitate was removed and dissolved in 0.5 mL of TD buffer and 0.2-mL aliquots (containing alkaline phosphatase as a sedimentation marker, 6.3S) were applied to a 10–30% linear gradient of sucrose in TD buffer. Following centrifugation for 19 h at 1 °C in a Beckman SW 56 rotor, the gradients were fractionated (0.1 mL) and the radioactivity was measured. The activity of the receptor–active protease was measured by calculating the ratio of the peak heights of the 4S and 8S peaks.

Sephadex G-200 Chromatography of the 45–65 Percent Ammonium Sulfate Fraction of Cytosol. The 45–65% ammonium sulfate fraction of cytosol (32 mL) dissolved in 5 mL of 0.1 M Tris-HCl buffer, pH 7.4, was dialyzed for 3 h against 200 volumes of the same buffer. The dialyzate was centrifuged and then applied to a column (1.6 × 65 cm) of Sephadex G-200 equilibrated with 0.1 M Tris-HCl, pH 7.4. Fractions (1.4 mL) were monitored for absorbance at 278 nm and for hydrolytic activity toward lysine-*p*-nitroanilide, [*methyl*-^{14}C]methylated methemoglobin, leucylglycylglycine, and estrogen receptor.

Purification of Activity toward Lysine-p-nitroanilide. The protein precipitating between 45% and 65% saturation (ammonium sulfate) was dissolved in 70 mL of TPD buffer and dialyzed against 25 volumes of the same buffer overnight at 0 °C. The dialyzate was centrifuged and the supernatant applied to a 2.5 × 9 cm column of DEAE-cellulose equilibrated with TPD buffer. The column was washed with TPD buffer (100 mL) before elution with TPD buffer containing 0.2 M KCl. Fractions (4 mL) showing activity toward lysine-*p*-nitroanilide were pooled and ammonium sulfate was added to 70% of saturation. The ammonium sulfate pellet was dissolved in 0.1 M Tris–phosphate, pH 7.6, dialyzed against 500 volumes of the same buffer, and then applied to a 1.6 × 61 cm column of Sephadex G-200 equilibrated with 0.1 M Tris–phosphate–0.5 mM DTT, pH 7.5. Fractions (1.4 mL) active toward lysine-*p*-nitroanilide were pooled and concentrated by ultrafiltration using an Amicon Diaflo YM 10 or YM 30 membrane to a final volume of 1.8 mL. The concentrate was dialyzed against TPD buffer and then applied to a 1.1 × 27 cm column of DEAE-cellulose equilibrated with the same buffer. The column was washed with 20–30 mL of TPD buffer and then eluted with 140 mL of TPD buffer containing a KCl gradient from 0 to 0.4 M KCl. Fractions active toward lysine-*p*-nitroanilide were pooled and concentrated by ultrafiltration to a final volume of 1.9 mL. This solution was dialyzed against 500 volumes of TPD buffer.

Purification of Receptor-Active Protease. Cytosol (165 mL) was prepared with 40 mM Tris-HCl, pH 7.4. The ammonium sulfate precipitate (45–65% fraction) was dissolved in 15 mL of 0.1 M Tris-HCl, pH 7.4, and dialyzed for 3 h against the same buffer. The dialyzate was centrifuged and applied to

Table I: Distribution of Estrogen Receptor, Receptor-Active Protease, and Activity toward Lysine-*p*-nitroanilide in Ammonium Sulfate Fractions

ammonium sulfate saturn (%)	receptor pptd[a] (%)	receptor-active protease pptd[b] (% 4S formed)	act. toward lysine-*p*-nitroanilide[c] (milliunits/min)
0–25	55	2	0.06
35–55	19	27	2.57
45–55	6	33	6.50
45–65	4	41	7.00
45–75	6	44	6.86

[a] Calf uterine cytosol prepared in buffer TD was equilibrated with 10 nM [^{3}H]estradiol (total binding) or 10 nM [^{3}H]estradiol and 1 μM estradiol (nonspecific binding). The percent receptor precipitated is the concentration of receptor recovered (specific binding) from the redissolved ammonium sulfate pellet in relation to the receptor concentration in the uterine cytosol. [b] The ammonium sulfate fractions were dissolved in buffer TDK and equilibrated with 1 μM estradiol for 1 h at 0 °C and then incubated for 20 h at 0 °C with the [^{3}H]estradiol receptor. The [^{3}H]-estradiol receptor was prepared from [^{3}H]estradiol-labeled cytosol that was precipitated at 25% ammonium sulfate saturation. The percent of the receptor sedimenting at 4S in sucrose gradients containing low salt concentrations was measured following centrifugation. [c] One milliliter of cytosol was precipitated with the ammonium sulfate concentration cited and then redissolved in 1 mL of buffer TD, and the activity of each fraction toward lysine-*p*-nitroanilide was assayed at 22 °C.

a 2.5 × 25 cm column of Sephadex G-200 equilibrated with 0.1 M Tris-HCl, pH 7.4. Fractions (3.2 mL) were monitored for absorbance at 278 nm, and those showing hydrolytic activity toward the estrogen receptor were pooled. Ammonium sulfate was added to 70% of saturation at 0 °C, and the ammonium sulfate pellet was dissolved in 15 mL of 40 mM Tris-HCl, pH 7.6, and dialyzed overnight against 100–200 volumes of the same buffer. The dialyzate was applied to a 1.1 × 27 cm column of DEAE-cellulose equilibrated with 40 mM Tris-HCl, pH 7.6 (20 mL/h). The column was washed with 20–30 mL of buffer/h and then eluted with 140 mL of a 0–0.4 M KCl gradient in 40 mM Tris-HCl, pH 7.6, to elute the protease. Fractions (1.4 mL) were monitored for absorbance at 278 nm and hydrolytic activity toward [*methyl*-^{14}C]methylated methemoglobin and estrogen receptor.

Results

Proteolysis of Estrogen Receptor in Cytosol. This investigation of the calf uterine proteases and their actions on the estrogen receptor has revealed two additional proteases whose properties and activities are different from a previously described Ca^{2+}-activated protease (Puca et al., 1977). Calf uterine cytosol in buffer TD and equilibrated with [^{3}H]estradiol was incubated for 2–5 h at 0 or 28 °C in the absence or presence of 1 mM leupeptin, 0.1 mM EDTA, or 10 mM sodium molybdate. The estrogen receptor incubated at 0 °C sedimented as the 8S form of the receptor in low-salt sucrose gradients (Figure 1). Cytosol incubated for 2.5 h at 28 °C

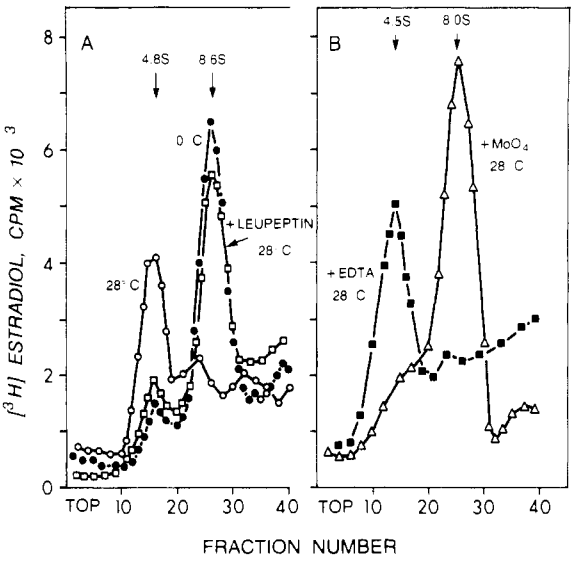

FIGURE 1: Sucrose gradient analysis of the calf uterine cytosol in TD buffer that was incubated 2.5 h at 0 (●), 28 (○), or 28 °C with the addition of 1 mM leupeptin (□) (A) or in TD buffer containing 0.1 mM EDTA incubated 2.5 h at 28 °C (■) or in TD buffer containing 10 mM Na_2MoO_4 incubated for 2.5 h at 28 °C (△) (B).

in the absence or presence of 0.1 mM EDTA was converted to a 4S sedimenting form in low-salt sucrose gradients, which indicated that although proteolysis of the receptor occurred in the absence of Ca^{2+}, molybdate and leupeptin inhibited the proteolysis of the receptor (Figure 1). The sedimentation coefficient of the receptor following proteolysis was 3.6–5.2 S; the intact receptor varied from 7.6 to 8.6 S in sucrose gradients containing low salt concentrations. The higher the receptor concentration the higher the sedimentation coefficient.

The ability of the calf uterine cytosol to hydrolyze low molecular weight peptides and amides was investigated with the following synthetic substrates: N^{α}-benzoyl-DL-arginine-*p*-nitroanilide, *N*-benzoyl-L-tyrosine-*p*-nitroanilide, *N*-benzoyl-L-tyrosine ethyl ester, hippuryl-L-phenylalanine, *N*-benzoyl-L-prolyl-L-phenylalanyl-L-arginine-*p*-nitroanilide, and *N*-benzoyl-L-valyl-L-glycyl-L-arginine-*p*-nitroanilide. No detectable cleavage of any of these substrates was observed at 22 °C in buffer TD; however, marked hydrolysis of L-lysine-, L-leucine-, and L-alanine-*p*-nitroanilide substrates and of [^{14}C]methemoglobin was observed.

Ammonium Sulfate Precipitation of Uterine Protease Activity. Investigation of the ammonium sulfate fractionation of the uterine cytosol indicated that the activity that converted the estrogen receptor from an 8S to 4S sedimenting form was predominatly in the 45–65% ammonium sulfate fraction (Table I). The maximal hydrolytic activity toward lysine-*p*-nitroanilide was also found in the 45–65% ammonium sulfate fraction of the uterine cytosol (Table I); ammonium sulfate fractionation produced an increase in the enzyme's specific activity 2–3-fold over that of the cytosol (Table II). The hydrolysis of the [^{14}C]methemoglobin was more pronounced

Table II: Purification of Exopeptidase Activity toward Lysine-*p*-nitroanilide

step	total protein (mg)	total units[a] (milliunits)	sp act. (milliunits/mg)	purifcn (*x*-fold)	recovery (%)
cytosol	1124	10790	10	1	100
45–65% NH_4SO_4 fraction	240	7200	30	3	67
DEAE-cellulose (stepwise elution)	70	3780	54	5	35
Sephadex G-200	28	2632	94	9	24
DEAE-cellulose (gradient elution)	1.7	1042	613	61	10

[a] One unit of enzyme activity is the amount of enzyme which hydrolyzes 1 μmol of lysine-*p*-nitroanilide/min at 22 °C.

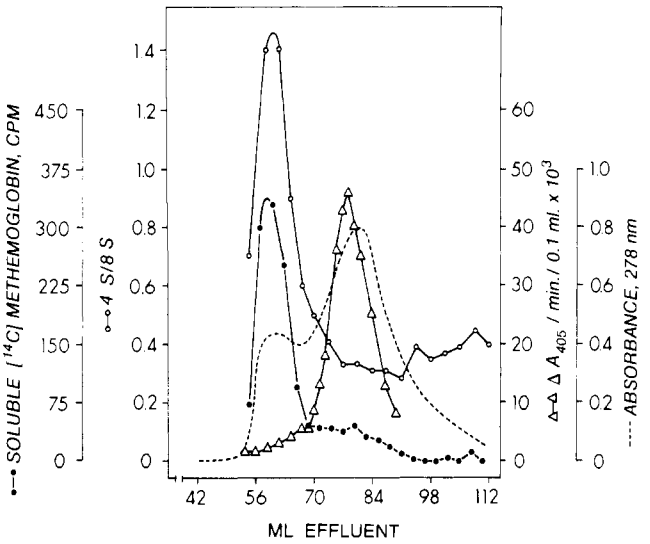

FIGURE 2: Sephadex G-200 gel chromatography of the 45–65% ammonium sulfate fraction of uterine cytosol. The 1.6 × 65 cm column was equilibrated with 0.1 M Tris-HCl, pH 7.4. The fractions were monitored for absorbance at 278 mm (---), hydrolytic activity toward lysine-*p*-nitroanilide (△) and [*methyl*-^{14}C]methylated methemoglobin (●), and hydrolytic activity toward the estrogen receptor (○).

in the 40–70% ammonium sulfate fraction than in the 0–25% fraction (data not shown).

Sephadex G-200 Chromatography of the 45–65 Percent Ammonium Sulfate Fraction of Cytosol. Cytosolic proteins precipitating between 45% and 65% ammonium sulfate saturation were dissolved in 0.1 M Tris-HCl, pH 7.4, and chromatographed on a 1.6 × 65 cm Sephadex G-200 column. The column fractions were monitored for activity toward lysine-*p*-nitroanilide, solubilization of [^{14}C]methemoglobin, and conversion of the 8S receptor to the 4S form (Figure 2).

The proteolytic activity that converted the 8S receptor to the 4S form and demonstrated [^{14}C]methemoglobin-degrading activity coeluted in the void volume of the column. These two activities were separable from the major peak of hydrolytic activity toward lysine-*p*-nitroanilide, although a small shoulder of this activity was included in the void volume (Figure 2). To determine whether the hydrolytic activity toward lysine-, and leucine-, and alanine-*p*-nitroanilide resided in the same enzyme, we assayed all column fractions for their relative activities toward these three substrates. The elution profiles toward these three substrates were the same; however, the hydrolytic activities toward leucine-*p*-nitroanilide and alanine-*p*-nitroanilide were 40% and 30%, respectively, of that toward the lysine-*p*-nitroanilide. The fractions from the Sephadex G-200 column were also analyzed for activity toward L-leucylglycylglycine. The activity that produced hydrolysis of L-leucylglycylglycine to leucine and glycylglycine (data not shown) coeluted with fractions having the maximal activity toward lysine-*p*-nitroanilide and minimal [^{14}C] methemoglobin-degrading activity, which demonstrated a substrate specificity of an exopeptidase activity (Figure 2).

Characterization of the Activity toward Lysine-p-nitroanilide. The exopeptidase activity assayed with lysine-*p*-nitroanilide was purified with a five-step procedure (Table II) that resulted in a 61-fold purification and a 10% yield. The final DEAE-cellulose chromatography with a linear salt gradient eluted the exopeptidase at a KCl concentration of 0.17 M. The relative activity toward the three *p*-nitroanilide substrates varied slightly between the steps of the purification procedure. However, after the final purification step the relative activities toward leucine-*p*-nitroanilide and alanine-*p*-nitroanilide were 40% and 30%, respectively, of that toward lysine-*p*-nitroanilide. The addition of Ca^{2+} or Mg^{2+} did not significantly increase the rate of leucine, alanine-, or lysine-*p*-nitroanilide hydrolysis by the purified exopeptidase. Attempts to purify the exopeptidase activity in the presence of buffers containing EDTA resulted in a loss of activity, which suggests the influence of some cation. Puromycin and bestatin, specific inhibitors of leucine aminopeptidase like enzymes (Bury & Pennington, 1973; Umezawa & Aoyogi, 1977), inhibited the hydrolysis of the lysine-*p*-nitroanilide by the purified enzyme, whereas leupeptin and DFP showed no or little inhibition (Table III).

Table III: Action of Inhibitors on Uterine Exopeptidase Activity

inhibitor	concn (mM)	rel act. of exopeptidase[a] (%)
none		100
DFP	1	90
leupeptin	0.1	95
	1	81
puromycin	0.1	4
	1	0
bestatin	0.1	6
	1	0

[a] The hydrolysis of lysine-*p*-nitroanilide by the purified uterine exopeptidase was assayed in the presence of the indicated concentrations of inhibitors at 22 °C. The activity equal to 100% for the control was 68.7 milliunits/min.

The purified uterine exopeptidase was not able to modify the aggregational properties of the estrogen receptor that occurred in sucrose gradients containing low salt concentrations. The purified [^{3}H]estradiol receptor (the 25% ammonium sulfate fraction) was incubated with 466 milliunits of the purified exopeptidase (containing 1 μM estradiol) for 22 h at 0 °C. The reaction was terminated by reprecipitation of the receptor with 25% ammonium sulfate. Both the buffer control and the enzyme-treated receptor sedimented as the 8S form of the receptor. The 8S form of the receptor was also observed after the addition of 0.5 mM $CaCl_2$ to the reaction mixture (data not shown).

Commercially available, hog renal leucine aminopeptidase was tested for its ability to modify the aggregation properties of the estrogen receptor. Leucine aminopeptidase was pretreated with 1 mM DFP for 30 min at 0 °C to inhibit a DFP-sensitive contaminant that would have modified the receptor. The DFP-treated leucine aminopeptidase was 89% as active as the control toward leucine-*p*-nitroanilide. The [^{3}H]estradiol receptor incubated (20 h at 0 °C or 3 h at 10 °C) with DFP-treated leucine aminopeptidase sedimented as the 8S receptor (data not shown).

Characterization of the Receptor-Active Protease. Following Sephadex G-200 chromatography of the 45–65% ammonium sulfate fraction of uterine cytosol, the void volume fractions that were active toward methemoglobin and the estrogen receptor (Figure 2) were concentrated, dialyzed against 40 mM Tris-HCl, pH 7.6, and then applied to a DEAE-cellulose column. The receptor-active protease that converted the 8S receptor to the 4S form and had methemoglobin-degrading activity coeluted at 0.2 M KCl from the DEAE-cellulose column (Figure 3).

Pretreatment of the receptor-active protease with 0.5 mM DFP or the presence of 0.1 mM EDTA or 10 mM sodium molybdate had no effect on the protease's ability to modify the aggregational properties of the estrogen receptor (Figure 4). However, 1 mM leupeptin completely inhibited the purified protease's ability to convert the 8S receptor to the 4S

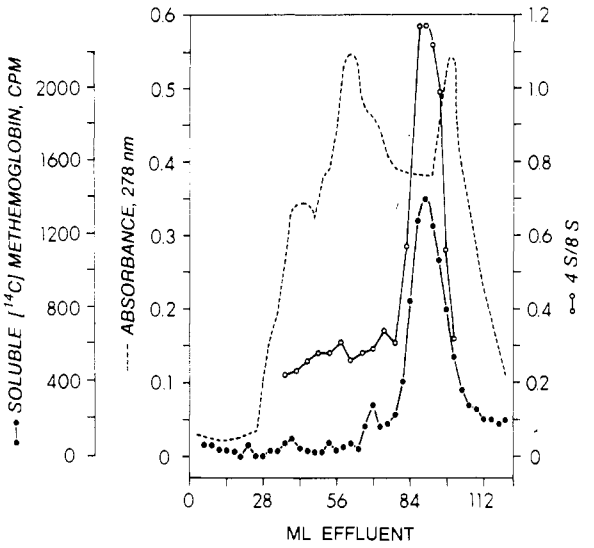

FIGURE 3: DEAE-cellulose chromatography of the receptor-active protease pooled from a Sephadex G-200 chromatography. The DEAE-cellulose column (1.1 × 2.7 cm) was equilibrated with 40 mM Tris-HCl, pH 7.6, and eluted with a 140 mL of a 0–0.4 M KCl gradient in Tris-HCl buffer, pH 7.6. The fractions were analyzed for [*methyl*-^{14}C]methylated methemoglobin degrading activity (●), activity toward the estrogen receptor (○), and absorbance at 278 nm (---).

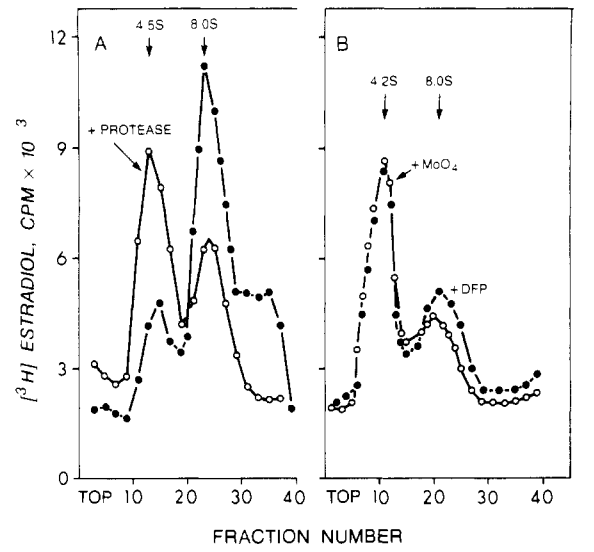

FIGURE 4: Sucrose gradient analysis of the estrogen receptor following incubation with the receptor-active protease. The [^{3}H]estradiol receptor in the 25% ammonium sulfate fraction, dissolved in buffer TDK, was incubated 20 h at 0 °C with buffer TDK containing 1 μM estradiol (●) or purified receptor-active protease in TDK containing 1 μM estradiol (○) (A). The [^{3}H]estradiol receptor was incubated 20 h at 0 °C with purified receptor-active protease containing 10 mM Na_2MoO_4 and 1 μM estradiol (○) or 0.5 mM DFP and 1 μM estradiol (●) (B). The reaction was stopped by a 25% ammonium sulfate precipitation of the receptor; the precipitate was dissolved and subjected to gradient analysis.

form (data not shown), as was observed in the unfractionated cytosol (Figure 1). The activity of the receptor-active protease added to the [^{3}H]estradiol receptor fractions was 3–10-fold greater than that found in the unfractionated cytosol. This is an approximation of the protease concentration, since the methemoglobin-degrading activity can only be taken as a semiquantitative assay.

The presence of Ca^{2+}, Mg^{2+}, EDTA, molybdate, or DFP had little or no effect on the methemoglobin-degrading activity of the receptor-active protease (the void volume fraction of the Sephadex G-200 chromatography), whereas 1 mM leupeptin or 1 mM 4,4′-dipyridyl disulfide (an inhibitor of thiol proteases; Gohda & Pitot, 1981) was very effective in inhibiting solubilization of the methemoglobin. In contrast, dithiothreitol did not enhance the protease's activity (Table IV).

Table IV: Action of Effectors on Receptor-Active Protease[a]

effector	concn (mM)	rel act. of the protease (%)
none		100
$CaCl_2$	5	108
$MgCl_2$	5	93
Na_2MoO_4	10	91
EDTA	0.1	98
	2	93
DTT	1	91
leupeptin	1	10
DFP	0.5	101
4,4′-dipyridyl disulfide	0.1	28
	1	9

[a] The Sephadex G-200 fraction of the receptor-active protease, dialyzed overnight against 40 mM Tris-HCl and 150 mM KCl, pH 7.4, was preincubated for 30 min at 0 °C with the concentration of effector cited. Activity toward [*methyl*-^{14}C]methylated methemoglobin was measured as described under Materials and Methods. Each value is the mean of triplicate determinations; the soluble counts equal to 100% for the control (no addition) were 674 ± 71 cpm.

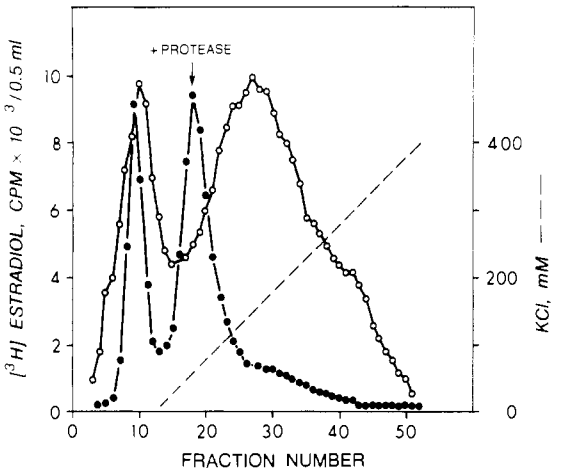

FIGURE 5: DEAE-cellulose chromatography of the estrogen receptor following incubation with the receptor-active protease. The DEAE-cellulose column (1.2 × 4 cm) was equilibrated with buffer TD and eluted with a 0–0.4 M KCl gradient in 120 mL of buffer TD at a flow rate of 45 mL/h. The [^{3}H]estradiol–receptor in the 25% ammonium sulfate fraction was dialyzed against buffer TD and subjected to DEAE-cellulose chromatography (○), or the receptor was incubated for 20 h at 0 °C with the purified, receptor-active protease (●). The protease was preincubated with 1 μM estradiol. The radioactivity of fractions 1–14 are the breakthrough volume containing unbound [^{3}H]estradiol and unadsorbed proteins.

The action of the receptor-active protease on the estrogen receptor resulted in a modified form of the receptor that was eluted from a DEAE-cellulose column with a lower salt concentration than that used to elute the partially purified estrogen receptor. The [^{3}H]estradiol–receptor (the 25% ammonium sulfate fraction of the uterine cytosol) was eluted from a DEAE-cellulose column with a KCl gradient at approximately 0.16–0.2 M KCl (Figure 5). This is the identical elution position of the estrogen receptor following its in vitro transformation to the activated 5S receptor (Notides et al., 1981). Preincubation of the [^{3}H]estradiol receptor with the receptor-active protease for 20 h at 0 °C resulted in a marked shift in its DEAE-cellulose elution position. The protease-modified receptor was eluted with 0.06–0.09 M KCl or approximately

the same elution position as the nonactivated form of the receptor (Notides et al., 1981).

Discussion

This report characterizes two neutral proteases from the calf uterine cytosol, previously undetected, and describes their actions on the estrogen receptor. The receptor-active protease was not active toward a number of common chromogenic synthetic amides or peptides but possessed methemoglobin-degrading activity. It converted the aggregative state of the estrogen receptor, by a limited proteolysis, from an 8S to 4S sedimenting form in sucrose gradients with low salt concentrations (TD buffer). The receptor-active protease did not require Ca^{2+} to convert the estrogen receptor to the modified 4S form but was more facile when the receptor-active protease and receptor were incubated in TKD buffer rather than in TD buffer. In contrast to the human uterine protease (Notides et al., 1973) the calf receptor-active protease was not inhibited by DFP but was effectively inhibited by dipyridyl disulfide, which indicates that a sulfhydryl group is essential for maintaining activity. Although dithiothreitol did not increase the receptor-active protease's activity, it remains to be demonstrated that it is a thiol protease. The receptor-active protease is distinctly different from a previously described Ca^{2+}-activated protease of calf uterus (Puca et al., 1972, 1977) and other tissues (Sherman et al., 1978; Vedeckis et al., 1980; Dayton et al., 1976).

In contrast to the Ca^{2+}-activated protease, the receptor-active protease does not require Ca^{2+} for proteolytic activity, can degrade methemoglobin, is precipitated by ammonium sulfate at 45–65% saturation (25–45% saturation for the Ca^{2+}-activated protease), and is eluted in the void volume of the Sephadex G-200 column (elution of the Ca^{2+}-activated protease is in the included volume) under similar buffer conditions.

Sodium molybdate protected the estrogen receptor in the crude cytosol from limited proteolysis (Figure 1). After partial purification of the receptor by ammonium sulfate, the addition of molybdate could no longer inhibit conversion (i.e., limited proteolysis) of the receptor to the 4S sedimenting form in sucrose gradients with low salt (Figure 4). Neither could molybdate inhibit the purified receptor-active protease's ability to degrade the methemoglobin (Table IV). This would suggest that molybdate interacts only with the nonactivated form of the receptor or some component associated with the receptor but not with the activated receptor that is induced by ammonium sulfate precipitation (Weichman & Notides, 1979) and consequently cannot protect the receptor from the receptor-active protease. Exclusive binding of molybdate by the nonactivated conformation of the estrogen receptor has been previously demonstrated (Mauck et al., 1982). The molybdate by ionic bonding with free Ca^{2+} in the crude cytosol may inhibit the Ca^{2+}-activated protease. The Ca^{2+}-activated protease is able to induce an 8S to 4S conversion of the receptor (Puca et al., 1977). Sherman et al. (1980) have previously demonstrated that molybdate increases the resistance of the steroid hormone receptors to proteolysis.

The second protease isolated from the calf uterine cytosol was an exopeptidase, having leucine aminopeptidase like activity, which readily hydrolyzed the *p*-nitroanilide of an unblocked amino acid (lysine-, alanine-, or leucine-*p*-nitroanilide). The purified exopeptidase had no methemoglobin-degrading activity and even at concentrations higher than found in the cytosol the exopeptidase was not able to convert the 8S receptor to the 4S sedimenting form in sucrose gradients with a low salt concentration. These data suggest that removal of the N-terminal amino acids from the receptor is not sufficient cause for the receptor to lose its aggregative property or that the N-terminal amino acid of the estrogen receptor is unaccessible, blocked, or resistant to exopeptidase action. The precipitation of the exopeptidase, the receptor-active protease, and the Ca^{+}-activated protease (Puca et al., 1977) from the calf uterine cytosol occurs at a greater than 25% ammonium sulfate saturation. This would indicate that ammonium sulfate fractionation is a suitable first step for removal of the estrogen receptor (precipitated at 25% ammonium sulfate saturation) from these proteases.

Reports that steroid hormone receptors show a conversion (after briefly heating or exposing the receptors to high KCl concentrations) from 8S to 4S in sucrose gradients containing a low salt concentration and that receptors show new ionic species separable by DEAE-cellulose chromatography have been taken to indicate a relationship between these molecular changes and receptor activation (Sakaue & Thompson, 1977; Hutchens et al., 1981). In addition, glucocorticoid receptor (Wrange & Gustafsson, 1978) has been shown to retain its DNA–cellulose binding property following partial proteolysis, which suggests that proteolysis is a stepwise process and that the retention of the DNA-binding domain of the receptor cannot be taken to indicate either the absence of proteolysis or that proteolysis is a necessary step of receptor activation. Our studies (Notides & Nielsen, 1974; Weichman & Notides, 1979; Notides et al., 1981) indicate that estrogen receptor activation is a 4S receptor monomer–5S receptor dimer equilibrium modulated by estradiol binding toward the activated 5S receptor and is independent of a proteolytic step. Thus, the action of nonspecific proteases on the steroid hormone receptors should be considered a potential cause of some changes in sedimentation or ionic properties of the receptor, prior to assuming that such changes are caused by conformation transitions, dissociation of subunits, or phosphorylation–dephosphorylation of the receptor and related to receptor activation.

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Effects of Limited Tryptic Proteolysis of Bovine Neurophysins on Molecular Properties of Hormone Binding, Self-Association, and Antigenicity†

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ABSTRACT: Limited tryptic fragmentation of disulfide-intact bovine neurophysins I and II (NP-I and -II, respectively) has been found to cause selective disruption of both hormone binding and neurophysin self-association. Loss of binding interactions, measured as a loss of ability to stimulate retardation of ^{125}I-labeled neurophysin on Met-Tyr-Phe-aminobutylaminoagarose, is complete within 3 h at 37 °C. Reverse-phase high-performance liquid chromatography (HPLC) analysis of tryptic digests of neurophysin I allows detection of two major protein products and the peptide fragment 1-8. Release of the latter N-terminal piece occurs at about the same rate as loss of binding interactions. Reverse-phase HPLC elution behavior before and after performic acid oxidation and amino acid composition of the protein products led to their identification as NP-I-(9-93) (the 9-93 sequence) and [des-19,20]NP-I-(9-93) (the 9-93 sequence with the dipeptide 19-20 missing) for the more rapidly and more slowly formed species, respectively. NP-I-(9-93), unlike intact neurophysin I, is not retarded strongly by either Met-Tyr-Phe-aminobutylaminoagarose or neurophysin II-Sepharose. In contrast, both NP-I-(9-93) and [des-19,20]NP-I-(9-93) are equally as effective as intact NP-I in binding neurophysin I antibodies. The role of amino-terminal residues in promoting hormone binding, self-association, and antigenic recognition interactions is considered.

The neurophysins and their associated neuropeptide hormones oxytocin and vasopressin embody a class of protein-peptide interacting complexes which originate from single-chain, hypothalamic precursors, and, within neurosecretory granules, are transported to and stored in the neurohypophysis before release of proteins and peptides (Livett, 1978; Pickering, 1978; Acher, 1979; Breslow, 1979). As mature proteins, neurophysins exhibit not only hormone binding but also hormone-mediated self-association (Breslow et al., 1971; Chaiken et al., 1975; Nicolas et al., 1976, 1980; Pearlmutter & Dalton, 1980; Tellam & Winzor, 1980; Angal & Chaiken, 1982). Further, given concentrations of neurophysins and hormones in neurosecretory granules that are relatively much higher than K_d values for hormone binding and self-association (Dreifuss, 1975), both the peptide-protein and protein-protein interactions of the mature species likely play a role in stabilizing these molecules during neuronal transport and storage.

The molecular structures responsible for these neurophysin interactions have been defined as yet to only a limited degree. A partially cohesive view of the hormone binding surface has been deduced by a combination of studies such as examination of hormone and peptide ligand binding specificities (Breslow & Weis, 1972; Breslow et al., 1973; Camier et al., 1973; Glasel et al., 1976; Nicolas et al., 1976), spectroscopic changes induced by binding of unlabeled ligands (Balaram et al., 1973; Griffin et al., 1973; Sur et al., 1979) and spin-labeled ligands (Lord & Breslow, 1980), effects of chemical modifications (Walter & Hoffman, 1973; Schlesinger et al., 1979), and photoaffinity labeling (Abercrombie et al., 1982b). The proximity to the hormone binding site of both a carboxylate anion and Tyr-49 has been discussed (Breslow & Garguilo, 1977; Nicolas et al., 1978; Abercrombie et al., 1982b). In

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