Uterine Estrogen-Induced Protein: Physical and Immunological Comparison with Ovalbumin

(estrogenic regulation/uterus-oviduct/gel electrophoresis/isoelectric focusing/immunoprecipitation)

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ABSTRACT The physical and immunological properties of the estrogen-induced protein from rat uterus are compared with those of ovalbumin from chick oviduct. Both proteins are induced by estrogen and are under estrogenic regulation. Electrophoretic mobility on sodium dodecyl sulfate-polyacrylamide gels and elution behavior on Sephadex G-100 column chromatography indicate that the uterine protein has a molecular weight of about 42,000, similar to that of ovalbumin, and likewise is composed of only one subunit. On isoelectric focusing in polyacrylamide gels, both proteins focus at a pH of 4.6. The uterine protein, in addition, shows a component with an isoelectric point of 5.1. Despite considerable similarity in the physical properties of the uterine protein and ovalbumin, there is no significant crossreactivity of anti-ovalbumin gamma globulin with the uterine protein, implying that these two estrogen-regulated proteins are immunologically distinct.

The induction of the synthesis of a specific uterine protein called "induced protein" (IP) is the earliest known biosynthetic tissue response after estrogen binding. IP synthesis, which is detectable by 40 min (1), is preceded by synthesis of RNA required for IP synthesis [detectable by 10 min (2)]; this synthesis can be induced by physiological concentrations of estrogens *in vitro* (3-6) and can be inhibited by some anti-estrogens (7). However, the function of IP is unknown and little has been reported about its physical properties.

The similarity we observed in the migration of IP and ovalbumin in several gel electrophoretic and protein separation procedures was intriguing because ovalbumin, from chick oviduct, is also a protein whose synthesis is induced by estrogen (8, 9). If IP and ovalbumin were closely related, it might be suggestive of the function of IP and have evolutionary implications. We, therefore, compared the physical and immunological properties of IP and ovalbumin.

This report shows that IP and ovalbumin have similar electrophoretic properties on polyacrylamide gels and have nearly identical molecular weights and similar isoelectric points as judged by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, Sephadex column chromatography, and isoelectric focusing. However, despite considerable similarity in their physical properties, the two proteins show no significant immunological crossreactivity, as monitored by immunoprecipitation techniques.

MATERIALS AND METHODS

Incubation of Uteri In Vitro and Measurement of IP Synthesis. Uteri excised from 22- to 24-day-old Holtzman rats

were incubated *in vitro* for 1 hr at 37° [3 uteri per 2 ml of Eagle's HeLa medium (Difco)] with 50 nM 17 β -estradiol (Mann Research) or an equal volume of solvent control (0.5% ethanol) and then allowed to incorporate radioactive amino acid [either 20 μ Ci/ml of L-[4,5-³H]leucine (2.0 Ci/mmol) for experimental uteri or 5 μ Ci/ml of L-[¹⁴C]leucine (316 mCi/mmol) for controls, Schwarz BioResearch] for 2 hr at 37° for determination of IP synthesis. The methods used for double-isotope labeling of uterine proteins and for preparation of uterine soluble proteins were as described (5).

Polyacrylamide Gel Electrophoresis. Uterine supernatant fractions were separated on 6% polyacrylamide gels (9 $\times$ 0.7 cm) that were prepared and run in TBE buffer (66 mM Tris-20 mM boric acid-3 mM Na₂ EDTA, pH 8.6 at 25°) and processed as described (5).

SDS-Polyacrylamide Gel Electrophoresis. Samples were run on 10% polyacrylamide-0.1% SDS, pH 7.2 gels (9 cm $\times$ 0.7 cm) by the method of Weber and Osborn (10). For preparation of the IP sample, gel slices are cut from TBE-polyacrylamide gels and mashed into small pieces. The proteins in the gel fragments from two (2.3 mm) gel slices are eluted by incubation with 0.8 ml of sample buffer [10 mM sodium phosphate, pH 7.2, 1% SDS, 1.5% 2-mercaptoethanol, 10% (v/v) glycerol, 0.002% Bromophenol Blue] for 40 hr on a rotary shaker at room temperature. This procedure gives over 95% elution of counts. Part of this eluate is then heated to 60-65° for 10 min before application to an SDS gel. Protein molecular weight standards are mixed with sample buffer, heated at 60-65° for 10 min, and 100 μ l (50 μ g) are applied per gel. [¹⁴C]Ovalbumin was prepared (11) with [¹⁴C]formaldehyde and sodium borohydride and had a specific activity of 2.06 $\times$ 10⁵ dpm/mg. Ovine ¹⁴C-labeled luteinizing hormone, kindly supplied by Dr. Jane Liu, was labeled by the same procedure and purified by Sephadex G-75 column chromatography. Unpurified whole uterine supernatant (in 1.3 mM EDTA) was made 0.01 M in sodium phosphate, pH 7.2, 1.3% in 2-mercaptoethanol, and 0.26% in SDS by addition of small aliquots of concentrated solutions, and then heated as above before application to the gel. The methods used for staining of proteins with Coomassie brilliant blue and for determination of radioactivity in gels have been reported (12).

Sephadex Column Chromatography. Preparations of whole uterine soluble proteins, and of TBE gel-purified IP and ovalbumin were analyzed on a Sephadex G-100 (Pharmacia) column (14 $\times$ 0.8 cm) run at 4° in 50 mM Tris-HCl buffer, pH 8.1, (4°) and standardized with blue dextran, chlorophenol red, bovine-serum albumin, ovalbumin, pepsin, and

Abbreviations: IP, induced protein; TBE, Tris-borate-ethylene-diamine tetracetate; SDS, sodium dodecyl sulfate.

FIG. 1. Electrophoretic distribution on polyacrylamide gel of uterine soluble proteins synthesized *in vitro* after a 1-hr incubation *in vitro* with 50 nM 17β -estradiol. Uteri (4 per group) were first incubated with either (50 nM) 17β -estradiol or ethanol only (0.5%) in 2.0 ml of Eagle's HeLa medium for 60 min at 37° and then allowed to incorporate labeled leucine (^3H for estradiol-treated and ^{14}C for control uteri) into protein for 2 hr at 37° . Control and estradiol-treated uteri were homogenized together. Part of the supernatant fraction of centrifuged homogenates (300 μl ; from 0.75 experimental and 0.75 control uteri) was separated by polyacrylamide gel electrophoresis.

chymotrypsinogen. Fractions of 0.25 ml were collected and analyzed for absorbance at 278 nm and radioactivity content.

Isoelectric Focusing in Polyacrylamide Gel. Gels (8 $\times$ 0.7 cm) were composed of 4.95% acrylamide, 0.13% N,N' -methylenebisacrylamide, 2% Ampholine carrier ampholytes pH 3-6 or 3-10 (LKB Produkter AB), 0.2% N,N,N',N' -tetramethylethylenediamine, and 0.14% ammonium persulfate. Samples, or gel slices (from TBE-polyacrylamide gels) cut into quarters, were applied to the top of the gel and were overlaid with either 20% sucrose, or with 10% sucrose under a layer of 5% sucrose-1% ampholytes, before addition of the cathodic buffer. Anodic and cathodic baths were filled with 0.2% sulfuric acid and 0.4% monoethanolamine, respectively. Electrofocusing was performed at 4° at a constant current of 1 mA per gel until the voltage increased to 150 V. Thereafter, the voltage was maintained at 150 V until the amperage had dropped and then remained stable for at least 1 hr. Total time of the run was usually 10-11 hr.

After focusing, gels to be prepared for radioactivity measurement were frozen and sliced transversely into 2.3-mm discs; radioactivity was eluted in 0.8 ml of 1% SDS and counted in toluene-10% Biosolv (Beckman) scintillation fluid. Gels to be stained with Coomassie blue were first fixed in 12.5% trichloroacetic acid.

The course of the pH gradient in gels was determined by slicing unfixed gels transversely into 2.3-mm slices, soaking each slice in 1 ml of distilled water for 3-4 hr at room tem-

FIG. 2. Coelectrophoresis on polyacrylamide gel of ^3H -labeled uterine soluble proteins (300 μl ; from 1.5 experimental uteri), synthesized *in vitro* after a 1-hr estradiol stimulation *in vitro*, and ^{14}C -labeled ovalbumin.

perature, and then measuring the pH of the water-ampholyte solution with a pH meter.

Preparation of Anti-ovalbumin Antibodies. Anti-ovalbumin serum was kindly provided by Dr. Edward Voss, Department of Microbiology, University of Illinois. Rabbits were immunized by injecting 5 mg of ovalbumin (5 $\times$ crystallized; Worthington) in complete Freund's adjuvant (Difco) into multiple intradermal and foot-pad sites. A booster injection was given at 6 weeks, and bleedings were begun 1 week thereafter. The serum used in this study, collected from the marginal ear vein, was a late hyperimmune serum (several months after antigen administration). The crude gamma globulin fraction isolated from the antiserum by precipitation with 50% saturated ammonium sulfate followed by 40% saturated ammonium sulfate (8) contained 34 A_{278} units/ml with an $A_{250}:A_{278}$ ratio of 0.49. One A_{278} unit of this gamma globulin would precipitate between 10 and 15 μg of ovalbumin at the equivalence point. The crude gamma globulin fraction from preimmune rabbit serum, prepared by ammonium sulfate precipitation, contained 55 A_{278} units/ml with an $A_{250}:A_{278}$ ratio of 0.41. One A_{278} unit of this "control" rabbit-serum gamma globulin precipitated 7% as much ovalbumin as did one A_{278} unit of anti-ovalbumin gamma globulin. Anti-rabbit gamma globulin (sheep antiserum to rabbit gamma globulin, kindly provided by Dr. Gary L. Jackson, Dept. of Veterinary Medicine, University of Illinois) was prepared by precipitation successively with 50% followed by 40% saturated ammonium sulfate, and contained 84 A_{278} units/ml with an $A_{250}:A_{278}$ ratio of 0.41.

^{125}I Labeling of Ovalbumin. Ovalbumin (10 μg ; Worthington, 5 $\times$ crystallized) was labeled with 1 mCi (2.5 μl ; 4.6×10^{-10} mol) of Na^{125}I (Isolab Inc., Akron, Ohio) in the presence of 25 μg of Chloramine T for 30 sec at room temperature, in 0.4 M phosphate buffer, pH 7.6 (reaction volume 58 μl) (13). Sodium metabisulfite (68 μg in 25 μl) was then added, and the reaction mixture was immediately passed over a Biogel P-60 (BioRad) column (15 $\times$ 0.8 cm) that had been coated with 0.1% gelatin in 10 mM phosphate-150 mM NaCl, pH 7.6. Fractions (0.4 ml; in 10 mM phosphate-150 mM NaCl, pH 7.6) were collected into tubes containing 0.5 ml of 0.1% gelatin. Gamma counting revealed the ovalbumin counts in fractions 6-9, cleanly separated from the free ^{125}I (tubes 15-25). The most immunoreactive ovalbumin was found in fractions 6-8. Fraction 8, which was used in all

studies, was diluted 1:10 with 0.1% gelatin in phosphate-NaCl buffer to contain about 14,000 dpm of ^{125}I -labeled ovalbumin per 10 μl for storage. SDS-gel analysis of this ^{125}I -labeled ovalbumin after incubation with anti-ovalbumin gamma globulin, followed by anti-rabbit gamma globulin showed that all of the ovalbumin was present in the pellet. Similar incubations with normal rabbit-serum gamma globulin, followed by SDS-gel analysis, revealed 27% of the immunoreactive ovalbumin in the pellet and 73% in the supernatant.

Antibody Precipitation Reaction. The precipitin reaction with increasing amounts of nonradioactive ovalbumin and a constant amount (1.7 A_{278} units) of either anti-ovalbumin gamma globulin or normal rabbit-serum gamma globulin ("control") was performed as described (8), except that precipitates were dissolved in 0.5 ml of 1 N NaOH and absorbances were read at 278 nm.

Immunoprecipitations of ^{125}I -labeled ovalbumin, or double-labeled (^3H and ^{14}C) whole uterine supernatant, or labeled IP (extracted from TBE-polyacrylamide gel slices), were performed in polycarbonate tubes (Sorvall no. 270) that were coated with 0.1% gelatin. In a typical precipitation, 20 μl of anti-ovalbumin gamma globulin (0.68 A_{278} unit) or 20 μl of control normal rabbit-serum gamma globulin (0.68 A_{278} unit) and 20 μl of either 10% sodium deoxycholate or 250 mM EDTA were added to the tubes first, followed by 10–250 μl of sample in 10 mM phosphate–150 mM NaCl, pH 7.6, or water. Phosphate–NaCl buffer was then added to make incubations that were 320 μl and 10 mM in phosphate, 150 mM in NaCl, pH 7.6. In a few cases, to enable better SDS-gel analysis of reaction supernatants, the identical procedure was followed except that salt was omitted and incubations were made 20 mM in sodium phosphate buffer, pH 7.6. The contents of the tubes were mixed and incubated at 37° for 1 hr and then at 4° for 18 hr. Sixty microliters of undiluted anti-rabbit gamma globulin, which give full precipitation of the antibody–antigen complex, was then added to the tubes. Tubes were mixed, incubated at 4° for 18 hr, and then centrifuged for 5 min at $17,300 \times g$. The supernatant was carefully withdrawn, and 350 μl of either phosphate–NaCl buffer or 20 mM phosphate buffer was added. The precipitates were broken up with a glass rod, and the tubes were centrifuged again. After an additional wash and centrifugation, the precipitate was either counted directly in a gamma counter or solubilized by boiling with a solution containing dithiothreitol, Tris, and SDS (8) before scintillation or gamma counting and analysis by SDS–polyacrylamide gel electrophoresis. The amount of radioactivity precipitated by control gamma globulin and by anti-ovalbumin gamma globulin was calculated. In each experiment, multiple immunoprecipitations were performed, with different amounts of antigen to ensure antibody excess.

RESULTS

Polyacrylamide Gel Electrophoresis. Fig. 1 shows a typical polyacrylamide gel electrophoretic separation of newly labeled uterine soluble proteins after estrogen stimulation *in vitro*. The radioactivity count profile, and even more clearly the count ratio ($^3\text{H}/^{14}\text{C}$, experimental to control), show that estrogen has increased the rate of incorporation of radioactive amino acid into one protein band relative to control (slices 16–18), designated the induced protein (abbreviated "IP"), as has been shown previously (5). If a similar preparation of ^3H -labeled (estrogen-treated) uterine soluble proteins

FIG. 3. SDS–polyacrylamide gel electrophoresis of double-labeled protein eluted from polyacrylamide gel slices encompassing the IP peak. Proteins contained in these gel slices, i.e., 16 and 17 of Fig. 1, were eluted with 0.8 ml of SDS–sample buffer and 0.6 ml was subjected to electrophoresis.

is electrophoresed with ^{14}C -labeled ovalbumin (Fig. 2), both IP and ovalbumin migrate at very similar rates, with IP about one gel slice ahead of ovalbumin.

SDS–Polyacrylamide Gel Electrophoresis and Sephadex Column Chromatography. Proteins contained in the TBE–polyacrylamide gel slices (from gels identical to that seen in Fig. 1) encompassing the IP peak were eluted with SDS–sample buffer and then subjected to electrophoresis in SDS–polyacrylamide gels. Fig. 3 demonstrates that IP migrates as a single species of molecular weight 42,000 by comparison with protein references run in parallel gels (Fig. 4, *inset*) or as internal radioactive markers (^{14}C ovalbumin and ^{14}C luteinizing hormone) (Fig. 4). Only one predominant macromolecular species is present, and the counts in this component represent 54% (Fig. 3) or 45% (Fig. 4) of the total ^3H counts on the SDS gel. The $^3\text{H}/^{14}\text{C}$ ratio in the IP peak (Fig. 3) increases nearly six-fold (compared with a 2-fold increase in the $^3\text{H}/^{14}\text{C}$ ratio in the original TBE–polyacrylamide IP peak; Fig. 1), indicating that we have obtained significant purification of the IP by this SDS–gel procedure. If the identical, but single-labeled, ^3H IP peak from TBE gels is electrophoresed with ^{14}C ovalbumin and ^{14}C luteinizing hormone, IP migrates nearly identically with, although a bit more broadly than, ^{14}C ovalbumin (Fig. 4).

Proteins migrating ahead of the IP on TBE gels (i.e., slices 14 and 15 of Fig. 1) were resolved on SDS gels as a major band at 25,000 molecular weight, with minor labeled components at 32,000 and 42,000 (the latter corresponding to IP; enhanced $^3\text{H}/^{14}\text{C}$ ratio). Proteins running behind IP on TBE gels (i.e., slices 18 and 19 of Fig. 1) migrated as five labeled species with molecular weights between 14,000 and 50,000. Except for the 42,000-dalton IP protein, none of these other species was present in elevated amounts in estrogen-stimulated uteri.

FIG. 4. Electrophoresis on SDS-polyacrylamide gel of TBE gel-purified ^3H IP with ^{14}C ovalbumin and ^{14}C luteinizing hormone (^{14}C LH). ^3H -Labeled protein was eluted with SDS-sample buffer from TBE-polyacrylamide gel slices (i.e., slices 16 and 17 of Fig. 1) encompassing the IP peak. Inset is a semilogarithmic plot of molecular weight against distance of migration. A line of best fit has been drawn through the data points (protein references). Arrows designate the positions of IP and coelectrophoresed ^{14}C -labeled reference proteins.

TBE gel-purified IP migrated as a single species on Sephadex G-100 column chromatography and eluted very slightly (1 fraction) ahead of ovalbumin. This is consistent with the molecular weight determined on SDS gels and implies that IP consists of only one subunit. Although an IP sample purified on TBE gels migrates as a well-resolved species on SDS gels and Sephadex column chromatography, we have been unable to resolve IP in double-labeled preparations of whole uterine soluble proteins by either SDS-gel electrophoresis or Sephadex G-100 column chromatography, even though IP is well resolved when such preparations are separated by TBE-polyacrylamide gel electrophoresis (Fig. 1).

Isoelectric Focusing in Polyacrylamide Gels. When TBE gel slices (e.g., Fig. 1, slices 16–18) containing the ^3H IP peak are subjected, along with ^{14}C ovalbumin, to isoelectric focusing in polyacrylamide gels in pH gradients of 3–6 or 3–10, the ^3H -labeled protein migrates to positions coincident with isoelectric points of pH 4.6 and 5.1, with a minor component near pH 5.4 (Fig. 5). ^{14}C Ovalbumin appears as a single sharp band at pH 4.6, coincident with one of the ^3H -labeled species. Staining of the ovalbumin samples, either ^{14}C -labeled or unlabeled, reveals one major and two minor bands; these probably correspond to ovalbumins A₁, A₂, and A₃, with isoelectric points of 4.58, 4.65, and 4.74, respectively (14). In order to determine whether all of the ^3H -labeled species resolved upon isoelectric focusing represent proteins whose synthesis is induced by estrogen, TBE gel slices con-

FIG. 5. Isoelectric focusing on polyacrylamide gel (pH 3–6 ampholytes) of ^3H -labeled protein contained in the IP peak of TBE-polyacrylamide gels (i.e., 3/4 of slices 16 and 17 of Fig. 1) and ^{14}C -labeled ovalbumin.

taining the double-labeled IP peak were subjected to isoelectric focusing. Fig. 6 shows the results of such a focusing on pH 3–6 gels and demonstrates that both the pI 4.6 and 5.1 species are associated with elevated $^3\text{H}/^{14}\text{C}$ or experimental/control ratios of incorporation (ratio 5.0–5.4 compared to baseline ratio of 3.4), implying that these species are both estrogen-induced proteins.

Isoelectric focusing of double-labeled whole uterine soluble proteins (without prior purification) at pH 3–6 or 3–10 reveals the bulk of the counts between pH 5.2 and 6.2, with a peak at pH 5.5 and a minor shoulder near pH 4.7. We always find a small but highly reproducible increase in the $^3\text{H}/^{14}\text{C}$ ratio in such preparations at pH 4.6, consistent with the results obtained with the TBE gel-purified material. The ratio change at pI 5.1 is presumably obscured by the large quantity of control proteins focusing in this region.

Immunological Comparison of IP and Ovalbumin. The potency and specificity of the gamma globulin fractions of anti-ovalbumin and normal rabbit sera were determined with unlabeled and ^{125}I -labeled ovalbumin.

Immunological studies on crude uterine supernatant were hampered by a high degree of nonspecific precipitation. Incubation of whole uterine supernatant (double-labeled uterine soluble proteins containing IP) with either anti-ovalbumin gamma globulin or normal rabbit-serum gamma globulin and then anti-rabbit gamma globulin resulted in precipitation of 29% and 21% of the trichloroacetic acid-precipitable counts, respectively. Redissolved pellets from both precipitations showed some counts at 21,000 and 42,000 molecular weight on SDS gels. However, neither peak was associated with any $^3\text{H}/^{14}\text{C}$ ratio change. Further, the fact that normal rabbit-serum gamma globulin precipitated 72% as many counts as did the anti-ovalbumin gamma globulin suggests significant nonspecific precipitation.

However, incubation of different amounts of TBE gel-purified IP (from 0.5 or 0.25 uterus) with anti-ovalbumin gamma globulin indicated clearly the absence of cross-reactivity, as monitored by double-immunoprecipitation and

SDS-gel analysis of supernatants and redissolved precipitates. In three assays, with gel-purified IP, 5-6% of the IP counts (total counts = 2880 ^3H cpm) were precipitated by anti-ovalbumin gamma globulin; an equal number of counts was precipitated by normal rabbit-serum gamma globulin. In both cases, the remainder of the IP counts was present in the supernatants as a 42,000-dalton species, as monitored by SDS-polyacrylamide gel electrophoresis.

DISCUSSION

Polyacrylamide gel electrophoresis, SDS-polyacrylamide gel electrophoresis, Sephadex column chromatography, and isoelectric focusing demonstrate that the uterine estrogen-induced protein has physical properties similar to those of ovalbumin, another estrogen-induced and -regulated protein. Both have molecular weights near 42,000 and isoelectric points near 4.6 (14). However, IP shows an additional component with pI 5.1. Despite these similarities in the physical properties of IP and ovalbumin, we have been unable to detect any significant crossreactivity of anti-ovalbumin gamma globulin with IP, as monitored by immunoprecipitation, implying that IP and ovalbumin are immunologically distinct proteins.

The fact that we see two regions of elevated $^3\text{H}/^{14}\text{C}$ ratio upon isoelectric focusing of TBE gel-purified induced protein seems to indicate that IP is not a single species. Although this is consistent with the report by Mayol and Thayer (15) that isoelectric focusing of their gel-purified material showed multiple components with elevated ratios, their pI values (between 3.5 and 4.0) are clearly lower than those we find (4.6 and 5.1). Our pI values are consistent with that suggested by Notides (16) on the basis of mobility of IP in whole uterine supernatant in acrylamide gel systems of different pH.

During the preparation of this manuscript, Iacobelli *et al.* (17) reported a large-scale partial purification of IP by techniques somewhat different from those reported here. Their molecular weight and isoelectric point determinations are similar to those we have found, although their sequence of purification steps is different, and their final characterization is by staining of protein bands with only limited radioactivity determinations.

Several of our findings are relevant to any purification studies of IP. As a first step, IP (in whole uterine supernatant) is best resolved on TBE-polyacrylamide gels, when compared with techniques using separation either on the basis of size alone (SDS gels or Sephadex) or charge alone (isoelectric focusing). Considerable additional resolution of the first TBE gel-purified IP material can be obtained on SDS gels, or on isoelectric focusing.

It is interesting that IP and ovalbumin have similar physical properties because both proteins are induced by estrogen and are clearly under estrogenic regulation (1-6, 8, 9). However, the lack of any significant immunological crossreactivity of anti-ovalbumin gamma globulin with IP shows that these two proteins are different. In addition, while ovalbumin is synthesized by only the tubular gland cells of the chick oviduct magnum and is a major secretory product (composing about 50% of the total soluble protein synthesized by the oviduct magnum), IP is synthesized in both endometrium and myometrium of the rat uterus (Katzenellenbogen, B. S. and Leake, R. E., manuscript submitted for publication) and represents a much smaller percent (about 2%) of the total newly synthesized uterine soluble protein.

FIG. 6. Isoelectric focusing on polyacrylamide gel (pH 3-6 ampholytes) of ^3H - and ^{14}C -labeled protein contained in the IP peak of TBE-polyacrylamide gels (i.e., slices 16 and 17 of Fig. 1). Ratios are not reported for gel slices having ^3H values below 20 cpm.

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