Electrophoretic Analysis of the Estrogen Receptor

MOLYBDATE STABILIZATION AND IDENTIFICATION OF THE CLASSICAL ESTROGEN RECEPTOR*

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Conditions are defined which permit analysis of estrogen receptors from the mammalian uterus by polyacrylamide gel electrophoresis, thereby solving a long-standing problem encountered in previous attempts at such analysis, namely the failure of a large portion of the receptor population to enter such gels. A paramount requirement for entry of the estrogen-receptor complex into polyacrylamide gels is its maintenance in an untransformed state which does not form aggregates that are excluded from these gels. Of the multiple estrogen-binding proteins separated, only one (relative mobility of 0.5–0.6) possessed the definitive characteristics of the classical estrogen receptor. The inclusion of molybdate in extraction buffers selectively enhanced receptor recovery and facilitated its separation. Moreover, the estrogen-receptor complex so resolved is separated from other types of estrogen-binding proteins present in the uterine cytosol. These findings show that the molybdate-stabilized estrogen receptor exists in a single discrete form, but otherwise exhibits multiple forms that are probably artifactual. Electrophoresis in discontinuous buffers, but not in a continuous buffer system, promoted aggregate formation. This finding has implications concerning the subunit structure of the untransformed receptor.

Although much evidence supports this outline, many uncertainties and questions remain (4). One area of uncertainty concerns the structure of the native or untransformed receptor. Available evidence indicates that it is an oligomeric protein; yet its size and quaternary structure remain largely unknown or controversial, as do other basic features of its structure. Furthermore, it is not certain that all subunits of the native receptor contain estrogen-binding sites.

A major problem that has impeded progress in defining the structure of the native receptor has been its instability, which may be due in part to its susceptibility to proteolysis, to subunit dissociation, and to transformation (6). These events reduce the amount of native receptor and increase the heterogeneity of its forms. Another major problem stems from the presence in crude preparations of nonreceptor estrogen-binding proteins such as serum albumin, α-fetoprotein (7), and the type II binding proteins (8). Their presence complicates identification and quantification of the E-R complex unless they are physically separated from the receptor (9).

These considerations point to two important needs: (a) a way to stabilize the untransformed receptor; and (b) a procedure for separating the receptor from other types of estrogen-binding proteins while preserving its native structure. The discovery that molybdate and similar oxyanions stabilize the untransformed glucocorticoid receptor (10) has been extended to other steroid receptors including estrogen receptors (11, 12). These findings may represent a solution to the instability problem. In general, molybdate-stabilized receptors are less heterogeneous and substantially larger than previously described forms (5, 13), but their biological significance is controversial (3). At issue is whether these larger forms are the true native receptor or aggregates formed by molybdate-induced binding of receptor to extraneous materials.

An outline of the major events underlying the mechanism of action of 17β-estradiol has emerged within the past 20 years, and its prominent feature is the crucial role of specific receptor proteins (for reviews, see Refs. 1–5). The first detectable interaction of estrogen within its target cells is its binding to its specific receptor with high affinity (Kd, 1 nM) in a stereospecific manner. This initial estrogen-receptor (E-R') complex appears to undergo rapid, but ill-defined, structural changes through a process called transformation or "activation." This process is thought to impart new properties and capabilities to at least some constituent(s) of the initial complex, including the ability to bind to DNA, presumably at specific regulatory sequences. This in turn would selectively alter gene expression.

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1 The abbreviations used are: E-R, estrogen-receptor; PAGE, polyacrylamide gel electrophoresis; disc-PAGE, polyacrylamide gel electrophoresis in a discontinuous buffer; disc-AGE, agarose gel electrophoresis in a discontinuous buffer; RF, relative mobility; pl, isoelectric point; TES, N-tris(hydroxymethyl)methyl-2-amino-ethanesulfonic acid.

EXPERIMENTAL PROCEDURES

Materials—Immature female rats of the Sprague-Dawley strain were purchased from Timco Breeding Laboratories, Houston, TX.
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Results

Electrophoretic Distribution of Estrogen-binding Proteins—The failure of steroid receptor to enter the polyacrylamide-resolving gels of disc-PAGE systems has been attributed to the small pore size of such gels relative to receptor size (15). Increasing the pore size of the resolving gels appears to be a major factor in permitting entry of the progesterone receptor in such gels (15), but this remedy has not been successful when applied to other receptors (12, 16), indicating that additional factors may be involved.

To further examine the influence of pore size in resolving gels on entry and resolution of E-R, we used two disc-gel systems (designated disc-PAGE and disc-AGE) which were identical except for the polymer used and the pore sizes obtained (see Fig. 1). The same discontinuous buffer system was used in both gel systems. The resolving gels of disc-PAGE consisted of highly cross-linked polyacrylamide (5% T; 15% C) which produces gels of relatively large pore size compared to conventional gels (15). Even larger pore sizes were obtained by using agarose gels (1% w/v) in the disc-AGE system where in molecular sieving does not occur for proteins having mobilities between 0.1 uterine equivalent and 0.2 uterine equivalent.

Incubation with Hormone—Hormone-protein complexes were formed using two separate incubation conditions: (a) in the presence of 17β-[3H]estradiol alone at 13 nM, and (b) in the presence of 17β-[3H]estradiol at 13 nM with a 100-fold molar excess of competitor, either diethylstilbestrol or estradiol. These incubations, which were 1- to 24-h duration at 0-4 °C, were terminated by mixing with dextran-coated charcoal (17) which removes unbound ligand from the samples by adsorption. The charcoal was in turn removed by centrifugation at 8000 × g for 1 to 2 min and aliquots of the resulting supernatant containing the hormone-protein complexes were immediately used for binding assays, protein assays, and analysis by gel electrophoresis.

Binding Assays for Cytosol Samples—From each of these samples, replicate aliquots (100 μl = 0.1 uterine equivalent) were added to 5 ml of scintillation fluid (Ready-Solv HP, Beckman) and subjected to liquid scintillation spectrometry for determinations of protein-bound hormone. The counting efficiency, determined by internal standardization, was 32%.

Analysis of Estrogen-binding Proteins by Gel Electrophoresis—Aliquots of labeled uterine cytosol (200 μl = 0.2 uterine equivalent) were loaded onto gels and subjected to electrophoresis using conditions similar to those described by Miller et al. (15). Current was regulated at 2.0 mA/gel, and the temperature of the lower buffer was regulated at 0-2 °C. Electrophoresis was terminated when the tracking dye had migrated a predetermined distance, usually 5.5 cm, into the resolving gel. After electrophoresis, polyacrylamide gels were sliced transversely (2-mm segments) or frozen at -80 °C for subsequent processing. Agarose gels were sliced immediately. Gel segments were incubated in 5 ml of scintillation fluid at room temperature for 4 h or more and then subjected to liquid scintillation spectrometry. The counting efficiency in both gel types was 32% by internal standardization.

Electrophoretic resolution of estrogen-binding proteins—Rat uterine cytosol was incubated for 2 h at 0 °C with [3H]estradiol (13 nM) in the presence of a 100-fold molar excess of competitor ligand to determine nonspecific (NS) binding (Δ) or with [3H]estradiol alone for total binding (●). Immediately after removal of unbound ligand, aliquots (200 μl = 0.2 uterine equivalent) of each cytosol were subjected to electrophoretic analysis in two gel systems, disc-AGE (A) and disc-PAGE (B). Gels were then sliced (2 mm/slice) and radioactivity/slice was determined. Nonspecific binding per slice Δ was subtracted from total binding per corresponding slice (●) to determine specific binding (○) in both the disc-AGE system (C) and in the disc-PAGE system (D). The position of the tracking dye is marked by T.D.
Comparisons of electrophoretic profiles of estrogen-binding proteins in disc-AGE and disc-PAGE systems are shown in Fig. 1 for uterine cytoplasmic proteins prepared in buffer A. In terms of either total (Fig. 1, A and B) or specific (Fig. 1, C and D) binding, the uterine proteins were separated within each gel system into two major peaks, one having fast mobility ("F" peak) and the other having slow mobility ("S" peak).

The F peak had a higher relative mobility in disc-AGE ($R_F = 0.79$) than in disc-PAGE ($R_F = 0.5-0.6$), as expected on the basis of the difference in pore size. Since several lines of evidence identifying the F peak as the classical estrogen receptor will be presented, this peak will be referred to as the E-R peak. Co-migrating with the tracking dye in both gel systems was a relatively small peak of nonspecific binding material, which was not separated from the receptor peak in the disc-AGE system but was located at its leading edge (Fig. 1A). In the disc-PAGE system, however, the E-R peak was completely separated from the tracking dye peak (Fig. 1B), which may be due to serum albumin and perhaps $\alpha$-fetoprotein also. Both proteins co-migrated with the tracking dye, and both are known to bind estrogens (29, 21).

The S peak was more heterodisperse in disc-AGE than in disc-PAGE, indicating much charge heterogeneity. Nonspecific, total, and specific binding of estrogen in this peak varied substantially among different preparations. Also, most of the nonspecific binding material co-migrated with the S peak.

An additional peak of estrogen-specific binding, designated "A," (Fig. 1D), is sometimes present at the interface of the stacking gel and the polyacrylamide resolving gel in the disc-PAGE system but is always absent in the disc-AGE system. Conditions will be described that inhibit its formation and suggest that it is a large aggregated form of the receptor.

Cytosol equivalent to 0.2 uteri (= 360 $\mu$g of protein) was the standard sample load. This was about 66% of the maximum permissible load for crude cytosol; recovery of specifically bound [$^{3}H$]estradiol in the E-R peak increased linearly up to 0.3 uterine equivalent of cytosol loaded. The recovery of estrogen-specific binding sites within these gels averaged 87% and ranged from 68 to 110%. Free estradiol ($R_F = 0.08-0.12$) did not co-migrate with any of the estrogen-binding peaks, and, moreover, essentially all of the free hormone is removed from the samples by incubation with charcoal just before electrophoresis.

Molybdate Selectively Protects the Receptor—To test the effects of molybdate on receptor properties entails comparisons of samples prepared in the presence and absence of molybdate. However, qualitative and quantitative differences exist in different receptor preparations, which could compromise such comparisons. To minimize this source of variation, both the control and experimental samples originated from aliquots of the same preparation.

Adding molybdate to homogenates or to 900 $\times$ g supernatants significantly enhanced recovery of specific estrogen-binding sites measured in the cytosol. Recovery of these sites increased progressively with increases in molybdate from 0.2 to 10 mM and remained essentially constant from 10 to 30 mM. A similar result has been reported for estrogen receptors of human uterus and human breast cancer cells (22).

To determine whether or not stabilization of estrogen-binding proteins by molybdate also affects entry, mobility, resolution, and recovery of these proteins in the gels, aliquots (200 $\mu$l) of control and molybdate-stabilized cytosols were analyzed by disc-AGE and disc-PAGE. Representative results are shown in Fig. 2. For clarity, only the profiles of specific binding are shown. Inclusion of molybdate enhanced the recovery of the F peak in both gel systems but had no significant effect on recovery of binding sites in the slow peak of either gel system. Furthermore, the molybdate-enhanced recovery in the F peak (shaded area in Fig. 2) was equal to the increased recovery of estrogen-specific binding sites measured in the cytosol of the same samples (3000 cpm in this example). This observation held at all concentrations of molybdate tested (6 experiments; 128 gels).

As shown in Fig. 3, recovery of specific binding sites in the receptor peak increased with increases in molybdate from 0.2
to 10 mM and remained essentially constant from 10 to 30 mM. Binding in the slow peak, however, remained independent of molybdate at all concentrations tested. Also, the mobility of the fast peak was independent of molybdate. Relat ed addition of molybdate to control cytosols after preparation did not increase the amount of fast component above the control values when analyzed within 1 to 2 h, but such additions did inhibit further conversion of the E-R to the A form that otherwise occurs during more prolonged incubations (data not shown).

Kinetics of Estrogen Binding—Specific estrogen binding was maximum by 1 h, and addition of molybdate (20 nM) to control cytosol immediately after preparation did not alter its estrogen binding rate or capacity. Moreover, there was no significant destruction of estrogen-specific binding sites during 24-h incubations for either control or molybdate-stabilized samples.

Effect of Stacking on Receptor Mobility—Although the estrogen receptor entered the resolving gels without exclusion in both disc-gel systems, neither appeared to achieve stacking under our initial conditions, i.e. using samples prepared in buffer A. Concentration and stacking of tracking dye from the upper buffer did not occur, and the front was more diffuse than normal. However, both disc gel systems did achieve stacking when using protein standards dissolved in deionized water. These observations suggested that the ionic strength of samples prepared in buffer A may have been too high for proper stacking.

To investigate this possibility, cytosol was prepared in a buffer of lower ionic strength, buffer B. For comparisons of electrophoresis under stacking and nonstacking conditions, the same disc-PAGE system was used with and without stacking gels. In addition, a continuous buffer system was formed by using resolving gel buffer in the “stacking gel” as well as in the resolving gel. The resulting profiles of estrogen-binding proteins are shown in Fig. 4. Rapid stacking of the tracking dye was observed in the disc gels with cytosol prepared in buffer B. When stacking gels were present, a large portion of the binding components of the fast peak was converted to the A form, presumably a large aggregate, that would not enter the polyacrylamide resolving gels of the disc-PAGE system (Fig. 4A). When stacking gels were omitted, however, essentially all the fast peak entered the polyacrylamide resolving gel (Fig. 4B), and there was little or no aggregate retained at the top of the resolving gel. Complete entry of the fast peak was also obtained in the continuous buffer system as shown in Fig. 4C. We emphasize that the polyacrylamide resolving gels of these three gel systems were identical. Hence, limiting pore size alone cannot account for the exclusion of native receptor in the disc-PAGE system. These results clearly show that the events associated with stacking in the disc-PAGE system cause the conversion of the receptor toforms that do not enter the polyacrylamide resolving gels. That it is the size of these excluded forms rather than a loss of negative charge which prevented their movement into these gels is evident since these forms did enter the “nonsieving” agarose-resolving gels in the disc-AGE system.

Having established satisfactory if not optimal conditions for preparation and electrophoretic analysis of the estrogen-binding proteins, we turned to the problem of identifying the peak or peaks that possess characteristics of the classical E-R.

The Fast Peak Displays Saturable High-affinity Binding of Estradiol—To test for saturability of estrogen binding among the uterine proteins resolved within polyacrylamide gels, aliquots of uterine cytoplasm were incubated for 21 h at 0–4 °C in increasing concentrations of [3H]estradiol with or without a corresponding 100-fold molar excess of diethylstilbestrol. After removal of unbound ligand, aliquots (200 μl = 0.2 uterine equivalent) of each sample were analyzed by PAGE. The resulting profiles (Fig. 5) show that the fast peak displays saturation of estrogen binding at concentrations near 10 nM, whereas estrogen binding to material in the slow peak and in the tracking dye peak shows no evidence of saturation at concentrations of estradiol up to 40 nM.

When these data for the fast peak were plotted as a function of the [3H]estradiol concentration (not shown), specific binding had the form of a rectangular hyperbola which is characteristic of saturable binding of a ligand to a finite or limited number of binding sites. Nonspecific binding under the fast peak remained a very low percentage of the total binding over the entire range of estradiol concentrations, indicating that the fast component is well separated from most of the nonspecific binding material.

Scatchard plots (23) of these data indicated a single class of high-affinity binding site in the fast peak with a Kd estimated to be 2.2 and 1.1 nM in replicate experiments. However, saturation analysis by gel electrophoresis does not provide adequate data, from a statistical viewpoint, for a high degree of confidence in the absolute value of these estimates. Hence, we regard our estimates of Kd as tentative relative measures of the binding affinity. Nevertheless, these estimates do indicate that the fast peak is composed of a single class of high-affinity binding site, and they do fall within the range of values reported for the classical estrogen receptor (1, 13).

Estrogen binding in the slow peak decreased substantially with increases in the duration of exposure to dextran-coated charcoal at 0–4 °C, whereas estrogen binding in the fast peak remained essentially constant, at least up to 1 h. These findings imply that the rate of dissociation of [3H]estradiol from the binding sites in the P peak is very slow, whereas the rate of dissociation of hormone from the S peak is appreciable. At 0–4 °C, 17β-estradiol dissociates from low and intermediate
would deplete one or more of the gel peaks comprising the uterine cytosol (control). However, such injections may "de-normal" the complement of estrogen-binding proteins of untreated rats from an operational point of view. The receptor is found in the cytosol. Thus, the often "locked" in the nucleus, and it is no longer extracted by repeated "translocation" phenomenon remains valid from an operational point of view. Recent studies support an alternative view in which the receptor is initially localized in the nucleus, not the cytoplasm to nucleus as a function of time after exposure to hormone, 200-μl aliquots of each sample were subjected to PAGE analysis. The resulting distributions of total binding are shown for cytosol incubated with the following concentrations of [3H]estradiol: 40 nM (●); 20 nM (——); 10 nM (○); 5 nM (----); 2.5 nM (-----); 1.25 nM (-----); and 0.5 nM (-----).

affinity binding sites 20-200 times faster than from the E-R complex, which has a half-life of 20 to 34 h at 4 °C (1, 9). Hence, the extraordinary stability of the estrogen-receptor complex is a property shared exclusively with the F peak. At 100-fold molar excess, neither progesterone nor testosterone competed for estrogen-binding sites in the fast peak, but diethylstilbestrol displaced from 95 to 98% of the bound [3H]estradiol in this peak as did unlabeled estradiol. On the other hand, all of these ligands showed significant competition for the estrogen-binding sites in the slow peak. Hence, only the fast peak exhibited ligand-binding specificity like that of the classical E-R.

**Estrogen Selectively Translocates the Fast Component**—The concept of "translocation" or movement of the E-R from cytoplasm to nucleus as a function of time after exposure to estrogen is based on evidence from radioautography and from cell fractionation studies (24, 25), but it may not actually occur in vivo. Recent studies support an alternative view in which the receptor is initially localized in the nucleus, not the cytoplasm, thereby obviating the need for translocation (26, 27). In either case the net effect of estrogen treatment is the same; at 1 h after an intraperitoneal injection of a saturating dose of 17β-estradiol, at least 90% of the receptor appears to be "locked" in the nucleus, and it is no longer extracted by hypotonic buffers; but in the absence of estrogen, most of the estrogen receptor is found in the cytosol. Thus, the often repeated "translocation" phenomenon remains valid from an operational point of view.

Hence, it was of interest to determine if like treatment would deplete one or more of the gel peaks comprising the normal complement of estrogen-binding proteins of untreated uterine cytosol (control). However, such injections may "de-posit" loosely bound estradiol within the tissue (28, 29) which may then bind to any residual "cytoplasmic" E-R thereby masking its subsequent detection. This potential masking may be circumvented by the use of two separate procedures: "pre-stripping" of unlabeled hormone by adsorption to charcoal (30) and by the use of exchange assays (31-33).

As shown in Fig. 6, only the fast peak was depleted (95%) by estrogen treatment. Control profiles displayed the normal complement of estrogen-binding proteins. These data were obtained from "prestripped" samples, but virtually identical results were obtained using samples that were incubated under exchange conditions (not shown). The elevated temperature (20 °C) and exposure to charcoal in prestripping did not alter the normal profiles when molybdate was present in the samples at 20 mM. Hence, the fast peak mimics the translocation behavior of the classical E-R in vivo.

**Tissue Specificity**—The relative abundance of the F peak among various tissues was as follows: (a) it was most abundant in the major target tissues such as the uterus and pituitary; (b) it was far less abundant in the so called nontarget tissues such as the liver and kidney; and (c) it was completely absent in serum. Hence, the F peak displays a tissue specificity very similar to that of the classical E-R. Shown in Fig. 7 are typical gel profiles obtained by PAGE analysis of cytosols from uteri, liver, kidney, and adrenal at comparable protein loads. The comparatively high levels of estrogen-binding sites in the tracking dye peak of liver and kidney samples is probably due to high vascularity of these tissues and hence high levels of serum albumin.
which are too large to move into polyacrylamide-resolving state which then binds tightly to some extraneous component they are different proteins which are separated into individual subunits of disc-PAGE; and tissue specificity. Therefore, we conclude that the fast peak is the classical estrogen receptor.

To account for the effect of stacking on the E-R, we put forth a hypothesis that is based on the events occurring during the stacking process and on three assumptions concerning the structure and transformation of the native receptor. These assumptions are as follows: (a) the fast peak is the untransformed receptor; (b) at least some of its subunits are different proteins which are bound together by noncovalent forces; and (c) dissociation of these subunits is sufficient for transformation. During the stacking process, each protein in the sample is concentrated into individual ultrathin zones which are arranged contiguously in an order reflecting their individual surface charge. Also, stacking promotes the dissociation of multimeric proteins that are composed of dissimilar subunits.

The hypothesis is stated as follows: (a) the oligomeric subunits of the receptor are dissociated by the stacking forces of disc-PAGE; (b) the subunits do not reassociate because they are different proteins which are separated into individual zones of the “stack”; (c) from the third assumption (dissociation = transformation), the preceding steps result in transformation of the estrogen-binding subunit to a DNA-binding state which then binds tightly to some extraneous component (perhaps RNA) in the crude cytosol. Thus aggregates form which are too large to move into polyacrylamide-resolving gels (A peak). Ben-Or and Chrambach also concluded that the glucocorticoid receptor is converted to similar aggregates in disc-PAGE analysis.

Sherman and co-workers (6, 12) have presented clear evidence that molybdate stabilizes a discrete oligomeric form of the E-R complex (sedimentation coefficient, 9–10 S; M, 310,000–340,000) and that it also inhibits conversion of this form of the receptor to giant aggregates. This molybdate-stabilized receptor, which is substantially larger and less heterogeneous than previously described forms, may represent: (a) a single functional unit of the native untransformed receptor with all of its natural components intact; (b) a complex of two or more such functional units, i.e. dimer, trimer, etc. (5); or (c) an artificial 5–10 S complex consisting of a smaller native receptor (e.g. 6–8 S) bound randomly to extraneous materials by molybdate (3). This last possibility now seems very unlikely (5).

Many of the diverse proposals put forth to explain molybdate stabilization of steroid receptors (3, 6) imply that the loss of receptor-binding sites is through the deleterious action of enzymes, namely proteases, nucleases, or phosphatases. If molybdate preserves steroid-binding sites of receptors by inhibiting the action of such enzymes, then: (a) for crude preparations made without molybdate, significant and progressive loss of the binding sites would occur during incubations; and (b) such losses would occur at a greater rate in control samples compared to molybdate-stabilized preparations. Our results from kinetic studies are contrary to these predictions. Others have also noted long-term (60–80 h) quantitative survival of estrogen-binding sites in crude cytosol from rat uteri (9).

To account for the loss of E-R when prepared in the absence of molybdate and increased recovery when molybdate is added, we propose the following working hypothesis. Molybdate acts directly on the receptor to stabilize its native oligomeric state; this is synonymous to inhibition of transformation. The molybdate-stabilized untransformed receptor will not undergo aggregation to extraneous material that leads ultimately to its loss from the cytosol during centrifugation. Molybdate has been shown to inhibit transformation of the E-R (11). In the absence of molybdate, however, some of the E-R undergoes subunit dissociation which we have assumed is tantamount to transformation. This manifests itself in the binding of the receptor to polyanions of variable size in the crude homogenate, e.g. RNA and ribonucleoprotein particles (34), thereby producing heterogeneous aggregates, some of which are of sufficient size to sediment from the preparations during subsequent centrifugation. Hence, some of the receptor would be lost from the 105,000 g supernatant without necessarily involving destruction of its binding sites. This may account for reports of a microsomal form of this receptor (35) and also for reports suggesting that RNA is a receptor component (12, 36). This hypothesis also offers an explanation for the occurrence of receptor in a discrete form when extracted with hypotonic buffers containing molybdate but in highly variable heterogeneous states when extracted in the absence of molybdate.

If aggregate formation depends on the presence of extraneous polyanions in cytosol, then separation of the receptor from these polyanions would eliminate formation of aggregates. This has been tested using partially purified 3.5 S receptor with the result that no excluded aggregates formed upon disc-PAGE analysis.9 The high mobility of the E-R in nonsieving agarose gels implies that this complex has a high negative charge density. This high negative surface charge need not be uniformly distributed but may reside primarily on a dissociable subunit that does not contain estrogen-binding sites. If this is true, loss of this highly charged component from the complex might account for the shift to a more basic pI that receptors undergo upon transformation (37). By virtue of its high resolution, speed, capacity for simultaneous analysis of multiple samples, simplicity, and economy, PAGE may eventually supplant other procedures for routine analysis of steroid receptor. Because the basis of separation of proteins by PAGE analysis is different from those of other methods commonly used to study steroid receptor, new and complementary information about its structure and properties may be gained.

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