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Purification and identification of an estrogen binding protein from rat brain: oligomycin sensitivity-conferring protein (OSCP), a subunit of mitochondrial F0F1-ATP synthase/ATPase

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Abstract

Early studies have suggested the presence in the central nervous system of possible estrogen binding sites/proteins other than classical nuclear estrogen receptors (nER). We report here the isolation and identification of a 23 kDa membrane protein from digitonin-solubilized rat brain mitochondrial fractions that binds 17β-estradiol conjugated to bovine serum albumin at C-6 position (17β-E-6-BSA), a ligand that also specifically binds nER. This protein was partially purified using affinity columns coupled with 17β-E-6-BSA and was recognized by the iodinated 17β-E-6-BSA (17β-E-6-[^{125}I]BSA) in a ligand blotting assay. The binding of 17β-E-6-BSA to this protein was specific for the 17β-estradiol portion of the conjugate, not BSA. Using N-terminal sequencing and immunoblotting, this 23 kDa protein was identified as the oligomycin-sensitivity conferring protein (OSCP). This protein is a subunit of the F0F1 (F-type) mitochondrial ATP synthase/ATPase required for the coupling of a proton gradient across the F0 sector of the enzyme in the mitochondrial membrane to ATP synthesis in the F1 sector of the enzyme. Studies using recombinant bovine OSCP (rbOSCP) in ligand blotting revealed that rbOSCP bound 17β-E-6-[^{125}I]BSA with the same specificity as the purified 23 kDa protein. Further, in a ligand binding assay, 17β-E-6-[^{125}I]BSA also bound rbOSCP and it was displaced by both 17β-E-6-BSA and 17α-E-6-BSA as well as partially by 17β-estradiol and diethylstilbestrol (DES), but not by BSA. This finding opens up the possibility that estradiol, and probably other compounds with similar structures, in addition to their classical genomic mechanism, may interact with ATP synthase/ATPase by binding to OSCP, and thereby modulating cellular energy metabolism. Current experiments are addressing such an issue.

1. Introduction

Rapid effects of estrogens, especially in tissues lacking nuclear estrogen receptors (nER), are incompatible with the classical genomic mechanism [1] and nongenomic mechanisms have been postulated [2–5]. Early studies from our laboratory indicate that the crude mitochondrial–synaptosomal membrane fraction (P2) of the female rat brain contains estrogen binding sites by using an estradiol-bovine serum albumin conjugate linked at C-6 position, 17β-E-6-[^{125}I]BSA, as a ligand [6,7]. Using 17β-E-6-[^{125}I]BSA, we have also identified three major estrogen binding proteins in brain P2 membrane preparations by ligand blotting technique with M_r of about 23, 28 and 32 kDa [8]. One of these proteins, the 23 kDa protein, is enriched in mitochondrial fraction (mP2) and preliminary ligand blotting

Abbreviations: nER, nuclear estrogen receptors, DES, diethylstilbestrol, 17β-E-6-CMO, 17β-estradiol 6-(O-carboxymethyl)oxime, BSA, bovine serum albumin, 17β-E-6-BSA, 17β-estradiol 6-(O-carboxymethyl)oxime-BSA, 17β-E-6-[^{125}I]BSA, [^{125}I]-labeled 17β-E-6-BSA, 17α-E-6-BSA, 17α-estradiol 6-(O-carboxymethyl)oxime-BSA, 17β-E-17-HS, 17β-estradiol 17-hemisuccinate, 17β-E-17-BSA, 17β-estradiol 17-hemisuccinate-BSA, T-3-BSA, testosterone 3-(O-carboxymethyl)oxime-BSA, CB, cerebellum, B, whole brain without cerebellum and olfactory bulb, P2, mitochondrial-synaptosomal membrane fraction, mP2, mitochondrial fraction, AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride, PVDF, polyvinylidene difluoride, OSCP, oligomycin sensitivity-conferring protein, rbOSCP, recombinant bovine OSCP, PBS, phosphate buffer solution, PEI, polyethylenimine, DCC, dextran-coated charcoal, K_d, dissociation constant, K_i, inhibition constant, IC_{50}, half-inhibition concentration, B_{max}, binding sites, SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, M_r, molecular weight.

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data using purified fractions from P2 by 17β-E-6-BSA linked affinity columns revealed significant purification [8].

The use of estradiol-BSA conjugates to identify estrogen-binding proteins originated more than twenty years ago [9,10]. However, as suggested in 1980s by many researchers, the estradiol-BSA does have problems, especially when employed to identify nER [11–13]. The suspected problems are mostly likely due to binding of estradiol-BSA conjugates to other lower affinity estrogen binders. Therefore, the value of estradiol-BSA ligands to detect nER diminished. However, since this ligand has estradiol linked to BSA, a large, hydrophilic protein, it has become a useful ligand in recent years for demonstrating the rapid, nongenomic actions of estrogens on cell membranes. The estradiol-BSA conjugate is biologically active and mimics the effects of free, unconjugated 17β-estradiol [4]. For instance, like free 17β-estradiol, the 17β-estradiol-BSA conjugate increased cytosolic [Ca^{2+}], IP_3 and DAG in osteoblasts [14], reduced the Ca^{2+} current in striatal cells [15] and potentiated the kainate-induced current in hippocampal CA1 neurons [5]. All of the aforementioned effects are believed to be mediated by receptors on the plasma membrane, since the BSA conjugates should be impermeant to intact cells. 17β-E-6-BSA linked to fluorescein isothiocyanate has also been used to identify membrane estrogen binding sites in breast cancer cells [16] and a pituitary cell line [17].

Similarly, progesterone linked to [^{125}I]-labeled BSA through C-11 position [18] or C-3 position [6] has been used to demonstrate specific steroid membrane binding sites. In addition, the kainate subtype of glutamate receptor can be identified by conjugating a kainate derivative, kainylaminooxyacetylglycine to BSA to obtain a multivalent ligand with very high binding affinity [19].

Because the estradiol-BSA conjugates had much higher affinity than free estrogens to lower affinity estrogen binders, it could be very useful in qualitatively identifying other estrogen binding proteins. However, the relative binding affinity will be difficult to determine, especially when the conjugates contain large molecules such as BSA and each conjugate will be also linked to different number of estrogens with an average ratio of about 32 estradiol/BSA. Also, due to the multivalent property of the ligand, the free estradiol will be much less efficient in competing off the ligand. Nevertheless, once the binding protein is identified, its functional importance can be examined by using free estrogens instead.

In this paper, we demonstrate the usefullness of estradiol-BSA conjugates in identifying an estradiol binding protein. First, using classical [^{3}H]estradiol binding assays of uterine nER we showed that the conjugation of estradiol to BSA did not alter its general specificity although its affinity varied depending upon the position on the steroid through which BSA was coupled. Second, we report data for the isolation and characterization of a 23 kDa protein from crude brain mitochondrial fractions, earlier identified by ligand blotting as an estradiol-binding protein [8]. Third, this protein was identified by both N-terminal sequencing and immunoblotting as OSCP. Finally, the recombinant bovine OSCP bound the 17β-E-6-[^{125}I]BSA conjugate and the binding was partially competed off by estradiol and DES.

Some of the preliminary data were reported earlier in a review paper [4].

2. Experimental procedures

2.1. Chemical reagents and materials

BSA (fraction V), 17β-estradiol, 17α-estradiol, DES, oligomycin, 17β-E-6-CMO, 17β-E-6-BSA (32 E/BSA), 17β-E-17-BSA (35 E/BSA), T-3-BSA (35 T/BSA), bacitracin, digitonin, FAST DAB tablets, Coomassie Brilliant Blue R-250, PEI (50% aqueous solution, average M.W. 60,000) were purchased from Sigma Chemical Co. (St. Louis, MO). 17α-E-6-BSA (32 E/BSA) was from Steraloid, Inc. (Wilton, NH). AEBSF was from Boehringer Mannheim (Indianapolis, IN). Carrier-free sodium iodine-125 ($Na^{125}I$, 1 mCi/10 μl) was obtained from Amersham Co. (Arlington Heights, IL). 17β-E-6-[^{125}I]BSA (200–800 Ci/mmol) was prepared as described [8]. [^{3}H]17β-estradiol (85 Ci/mmol) was from NEN (Boston, MA). Bio-Safe II was from Research Products International Corp. (Mount Prospect, IL). AminoLink columns were purchased from Pierce Chemical Co. (Rockford, IL). Prepacked PD-10 columns were purchased from Pharmacia Biotech Inc. (Piscataway, NJ). Precasted 4–20% gradient gels (1 mm thick) and the protein standard markers were obtained from Bio-Rad (Hercules, CA). Fast stain was from Zoion Research, Inc. (Allston, MA). Nitrocellulose membranes (0.45 μm pore size, type HA) were from Millipore Co. (Bedford, MA). PVDF membranes were from Applied Biosystems (Foster City, CA). The polyclonal antibody against bovine OSCP was kindly provided by Dr. G.I. Belogrudov, Dr. A. Matsuno-Yagi and Dr. Y. Hatefi of the Scripps Research Institute, La Jolla, CA. The rbOSCP was kindly provided by Dr. Y. Hatefi (originally from Dr. S. Joshi) initially and later by Dr. S. Joshi of Boston Biomedical Research Institute and Harvard Medical School. The rbOSCP was expressed as the mature sequence in *E. coli* and recovered from inclusion bodies [20].

The concentrations of steroid-BSA conjugates are based on the total molecular weight (M_r) for the con-

jugates, not the steroid moieties. Therefore, the concentrations of the conjugates in terms of estradiol equivalents are 32–35 times higher.

2.2. Animals

Immature (30 days old) and adult female Sprague-Dawley rats (60–120 days old, unknown estrous cycle) were maintained on a 14:10 h light/dark cycle (lights on at 07.00) with food and water available ad lib. Animals were taken care of in accordance with federal and institutional guidelines and sacrificed by decapitation.

2.3. Subcellular fractionation

Crude mitochondrial–synaptosomal membrane fractions (P2) or mitochondrial fractions (mP2) from cerebellum (CB) and brain (B, whole brain without CB and olfactory bulb) of adult female rats were prepared in P2-Tris buffer (50 mM Tris·HCl/120 mM NaCl/5 mM KCl/1 mM $MgSO_4$/1 mM $CaCl_2$/10% glycerol, pH 7.4 at 4°C) plus 0.5 mM AEBSF and 0.1 mM bacitracin as described previously [8]. The uterine cytosol (rich in nER) was prepared from immature female rats in TE buffer (10 mM Tris, 2 mM EDTA, pH 7.5) according to a modified procedure of Bruns et al. [21]. Briefly, immature female rats were sacrificed and the uteri were removed quickly into cold TE buffer. The uteri were minced and homogenized in the same Teflon glass homogenizer at 1 uterus/ml TE for 2 min. The homogenate was then ultracentrifuged for 60 min at 125,000*g* and the supernatant obtained was the uterine cytosol fraction. All the fractions were stored at −80°C and assayed for protein by the Bradford method [22] using BSA as a reference.

2.4. Solubilization

Frozen P2 or mP2 fractions from CB or B were thawed and solubilized in P2-Tris buffer containing 1% digitonin at 4°C as reported [8]. The recovery of proteins was usually 40–50%.

2.5. Affinity purification of estrogen binding proteins

Estrogen affinity columns were prepared by linking the primary amine groups on the BSA part of 17β-E-6-BSA or 17α-E-6-BSA (2 mg) to agarose activated aldehydes in AminoLink columns according to manufacturer's instructions. A control column coupled with BSA was prepared in the same way. Coupling efficiency was between 30% to 40% for 17β-E-6-BSA, 60% for 17α-E-6-BSA and 82% for BSA. The purification was done as described before [8] with slight modifications. Briefly, one ml of a solubilized P2 or mP2 fraction (about 1–1.8 mg proteins) was applied to the column and incubated for 2–3 h at 4°C. The same procedure was repeated for additional samples. The column was then washed with 15 ml P2-Tris buffer to removed the unbound protein, followed by washing with 5×1 ml 5 mM Tris buffer plus 1% glycerol. The bound proteins were eluted with 12×1 ml 0.1 N acetic acid (pH 2.7). Fifty μl from each of the one-milliliter eluant fractions were removed and assayed for protein concentration by the Bradford method [22]. The protein-containing eluant fractions were pooled and lyophilized prior to gradient SDS-PAGE. In some experiments, the eluted protein fractions were neutralized right away with 50 μl 1 M Tris·HCl at pH 9.4 and then applied to the same column for repurification.

2.6. SDS-PAGE

The samples were treated at 95°C for 3 min in the presence of 1× reducing sample buffer. The samples were cooled and then applied to a 4–20% precast polyacrylamide gradient minigel, and SDS-PAGE was performed at 200 V for 35 min according to Laemmli [23]. The gels were either stained with Fast stain, or electrotransferred to nitrocellulose membranes or PVDF membranes for further analysis.

2.7. N-terminal sequencing

Partially purified proteins were separated by SDS-PAGE in the presence of 2 mM reduced glutathione and were electrotransferred to PVDF membranes at 50 V for 45 min using Bio-Rad mini Trans-Blot cell according to Matsudaira [24]. The membranes were stained with 0.1% Coomassie Brilliant Blue R-250. The bands of interest were excised and N-terminal amino acids were sequenced on an Applied Biosystems model 577A sequencer coupled to a model 120A on-line PTH analyzer using Edman chemistry.

2.8. Protein blotting

Proteins separated by SDS-PAGE were electrotransferred to nitrocellulose membranes at room temperature with cooling unit for 2 hours at constant 50 V according to Towbin et al. [25] or transferred to PVDF membranes as described above. The blots were blocked with 2% (nitrocellulose membranes) or 3% (PVDF membranes) BSA in PBS at 37°C for 1.5 h and washed with PBS three times, each for 5 min.

For ligand blotting, the blots were then incubated with 0.2–1.6 nM 17β-E-6-[^{125}I]BSA ($1.8–10 \times 10^5$ cpm/ml, 70% counting efficiency) in the absence or in the presence of various concentrations of competitors at 4°C, washed, dried and exposed to Kodak SB film

as described earlier [8]. The incubation buffer was P2-Tris buffer plus 0.08% BSA except as noted. In some cases, the blots were also exposed to a storage phosphor screen overnight and the relative radioactivity bound to the specific protein bands was quantitated using an image analysis software, ImageQuant, by a PhosphorImager 445 SI system (Molecular Dynamics). The relative radioactivity specifically bound to a protein band was obtained by subtracting the background radioactivity from the total radioactivity bound to that protein.

The blots as blocked above or the blots after ligand blotting were also used for detection of OSCP by immunoblotting. The blots were incubated at room temperature with rabbit anti-bovine OSCP antibody serum (1:1000 to 1:5000 dilution) in 1% BSA in PBS for 1.5 h, washed 3 times with PBS, each 5 min. The blots were then incubated with goat anti-rabbit IgG second antibody conjugated to horse-radish peroxidase (Sigma, 1:1000 dilution) for 1 h at room temperature and washed again 3 times, each 5 min. The blots were developed using FAST DAB enzyme substrate to visualize the protein band.

2.9. [^{3}H]-Estradiol binding assays of uterine cytosol

The saturation assay and the competition assay of the binding of [^{3}H]17β-estradiol to classical intracellular nER in uterine cytosol of immature rats were performed in duplicates at 4°C in TE or TE plus 0.08% BSA (TEB). [^{3}H]17β-estradiol (0.1–10 nM) was incubated for 1.5–3 h with uterine cytosol (80–440 μg proteins) and competing compounds in 250 μl total volume. The concentration of the ethanol used to dissolve the ligand and steroids was in the range of 0.004–0.4% in the final solution and had no effect on the binding. 200–500-fold excess 17β-estradiol or DES was used to determine the nonspecific binding. The bound [^{3}H]17β-estradiol was separated from free using a rapid filtration method through PEI-treated Whatman GF/B filters according to Bruns et al. [21]. Briefly, the glass filters were soaked in 0.3% PEI (v/v) for 1–3 h at room temperature and placed on a model 1225 Sampling Manifold. The manifold was then transferred to the cold room (4°C) for about 1 h before use. After the incubation, the reaction solution was poured onto PEI-treated filters in the manifolds under reduced pressure in the cold room. The tubes and filters were washed twice with 5 ml incubation buffer (TE or TEB) and the radioactivity retained by the filters was counted using aqueous scintillation cocktail, Bio-Safe II. The K_d and B_{max} of saturation assay were determined by 'LIGAND' program [26] and K_i for different competitors was calculated according to Cheng and Prusoff [27].

2.10. 17β-E-6-[^{125}I]BSA binding assays of recombinant bovine oligomycin-sensitivity conferring protein (rbOSCP)

The ligand binding assay was based on the nonspecific absorption of basic OSCP protein to negatively-charged glass filters [28]. The assay was performed in duplicates at 4°C in 0.5 ml P2-Tris plus 0.08% BSA (binding buffer) containing 0.1 μg rbOSCP and 45,000 cpm (about 0.14 nM) of total 17β-E-6-[^{125}I]BSA and incubated for 30 min at 4°C. The bound ligand was separated from free by rapid filtration through a GF/C glass filter paper in a mannifold and washed with 5 ml binding buffer twice. The retained radioactivity in the filter paper was counted using a gamma counter (70% efficiency).

3. Results

Saturation assays of [^{3}H]17β-estradiol (0.1–10 nM) binding to nER in the uterine cytosol in the TE and TEB (TE + 0.08% BSA) buffers were performed using a rapid filtration method as described in Section 2. Consistent with early reports [29], 17β-estradiol bound the rat uterine nER with a K_d of about 0.2 nM and a B_{max} of 0.98 pmol/mg protein in TE (not shown). Inclusion of 0.08% BSA (12 μM) did not significantly affect both K_d and B_{max} (0.19 nM and 1.01 pmol/mg protein, respectively, not shown). Therefore, the competition assays of uterine cytosol were performed in the presence of 0.08% BSA. Consistent with saturation data, unlabeled 17β-estradiol displaced the binding with a K_i of 0.16 nM (Fig. 1). 17β-E-6-BSA displaced the binding with about 10 times less affinity than 17β-estradiol (K_i = 1.5 nM), whereas 17α-E-6-BSA had one half the affinity as 17β-E-6-BSA (Fig. 1), consistent with the early finding that 17α-estradiol had one-half of the affinity of 17β-estradiol for nER [30]. 17β-E-17-BSA was shown to have very high affinity (K_i = 0.024 nM) (Fig. 1). This high affinity of 17β-E-17-BSA was also demonstrated using dextran-coated charcoal (DCC) absorption or gel filtration (Sephadex G-25 fine) to separated bound from free (not shown). The difference in affinity between 17β-E-6-BSA and 17β-E-17-BSA is probably due to its conjugation at different C-position in the estradiol molecule, and is consistent with the early studies that 17β-E-6-CMO had about 180 times lower affinity than 17β-E-17-HS [31]. In one separate experiment, we had used 1 μM 17β-E-6-CMO to displace the [^{3}H]17β-estradiol binding to uterine cytosol, resulting in 37% decrease of the specific binding (i.e., with IC50 larger than 1 μM), an affinity more than 700 times less than 17β-E-6-BSA. The apparent higher affinities of estradiol-BSA conjugates than their estradiol-derivatives are most likely

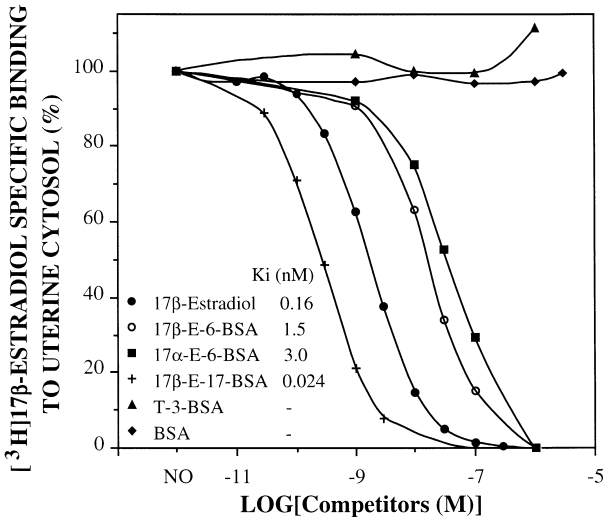

Fig. 1. Competition curve of specific [^{3}H]17β-estradiol (2 nM) binding to uterine cytosol (80 μg) using various unlabeled competitors in duplicates in TEB. The Kd (0.20 nM) obtained from saturation assay in TEB was used to calculate the Ki values for the different competitors. 'NO' indicates the absence of competitors.

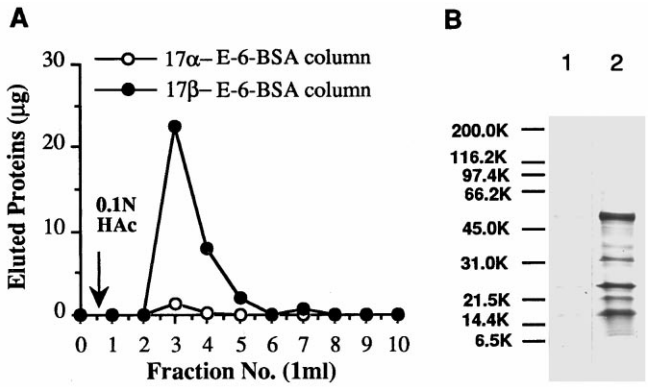

Fig. 2. (A) Elution profile of 17α-E-6-BSA column versus 17β-E-6-BSA column. 1.74 mg solubilized CB-P2 was applied to a 17α-E-6-BSA column. The initial washed fractions from this column (4 ml, representing more than 90% of total proteins applied) were collected and applied to a 17β-E-6-BSA affinity column. The retained proteins from both columns were eluted. (B) Proteins retained (30% of eluted fractions from each of above columns) were separated by SDS-PAGE. Lane 1, retained proteins by 17α-E-6-BSA column (about 0.5 μg). Lane 2, retained proteins by 17β-E-6-BSA affinity column (10 μg). The broad range M_r protein markers (Bio-Rad, in kDa) used in (A) and (B) were myosin, 200; β-galactosidase, 116.2; phosphorylase B, 97.4; BSA, 66.2; ovalbumin, 45.0; bovine carbonic anhydrase, 31.0; soybean trypsin inhibitor, 21.5; lysozyme, 14.4 and aprotinin, 6.5.

due to the multivalent property of the conjugates. Consistent with the properties of classical nER, T-3-BSA and BSA had little effect up to 1 μM (Fig. 1). Therefore, steroid-BSA conjugates maintain their relative specificity.

Since CB-P2, B-P2 or B-mP2 contained the 23 kDa estrogen binding protein which is enriched in mitochondrial fractions [8], we have used these fractions for the purification of the estrogen binding proteins by affinity columns coupled to estradiol-BSA, which had been used earlier for purification of bovine nER [31]. Membrane proteins were solubilized by 1% digitonin and the solubilized proteins were applied to 17α-E-6-BSA columns or 17β-E-6-BSA affinity columns. As shown in Fig. 2A, using the solubilized CB-P2 as the starting material, the 17β-E-6-BSA column (1.6% recovery of solubilized proteins) retained about 20-fold more of total proteins than 17α-E-6-BSA affinity column (0.08% recovery) as measured by Bradford assay. SDS-PAGE under reducing condition revealed that both columns retained similar sizes of proteins (M_r of about 53, 33, 23, 21 and 18 kDa mainly) but significantly less amount by 17α-E-6-BSA column (Fig. 2B). From fourteen experiments, an average of 0.83% of the total protein loaded into the 17β-E-6-BSA columns was retained, whereas about 0.05% total recovery was obtained by the 17α-E-6-BSA column in three separate experiments.

Similar purification results were obtained with solubilized B-P2 and B-mP2 fractions as determined from Bradford assay and SDS-PAGE analysis (not shown). Therefore all CB-P2, B-P2 or B-mP2 contained same proteins that could be retained by 17β-E-6-BSA affinity columns. Repassing the affinity purified B-P2 fraction through the same affinity column showed that the major retained band is the 23 kDa protein (not shown), indicating that this protein is most likely an estrogen binding protein. The other proteins were either copurified with this protein or, less likely, contaminants.

The retention of these proteins by 17β-E-6-BSA affinity columns is not due to BSA or the agarose matrix in the columns, since a control BSA column constructed similarly (and also with little leakage) retained none of the 23 kDa protein with little total recovery of proteins, whereas in a parallel experiment a 17β-E-6-BSA column retained the 23 kDa protein as well as the other four major proteins (53, 33, 21 and 18 kDa) (not shown). This result indicates that the 23 kDa protein retained in the 17β-E-6-BSA column was specific for 17β-estradiol.

That the 23 kDa protein is an estradiol binding protein is further supported by ligand blotting studies using 17β-E-6-[^{125}I]BSA. Out of at least five major protein bands, only the 23 kDa protein of the purified B-P2 fraction was specifically and strongly labeled with 17β-E-6-[^{125}I]BSA, which corresponds to the 23 kDa protein in the same purified fraction as stained by Amido Black (not shown). The binding was displaced mostly by 50–500 nM unlabeled 17β-E-6-BSA, and partially by 500 nM 17α-E-6-BSA. The radioactivity retained by the 23 kDa protein was quantitated and the specific binding was decreased to 12% of the total

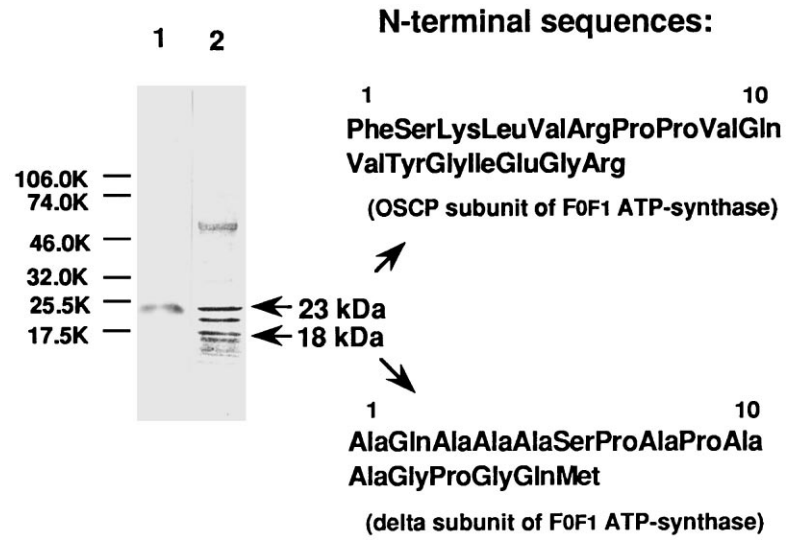

Fig. 3. Detection and N-terminal sequencing of affinity purified proteins. The purified CB-P2 proteins were applied to the gel in two lanes and were separated by SDS-PAGE (5 μg each lane). The proteins then were electrotransferred to PVDF membrane. Lane 1 was used for ligand blot analysis with 0.25 nM 17β-E-6-[^{125}I]-BSA (2.6×10^5 cpm/ml). Lane 2 was stained with Coomassie Brilliant Blue and the N-terminal sequences of the 23 and 18 kDa proteins are shown. The 53 and 21 kDa proteins appeared to be N-blocked and no sequence information was obtained. The prestained low range M_r protein markers (Bio-Rad, in kDa) were phosphorylase B, 106.0; BSA, 74.0; ovalbumin, 46.0; carbonic anhydrase, 32.0; soybean trypsin inhibitor, 25.5 and lysozyme, 17.5.

specific binding by 50 nM unlabeled 17β-E-6-BSA and to 3% by 500 nM ligand. However, 500 nM 17α-E-6-BSA only displaced 46% of the specific binding (half-inhibition concentration, IC50 of about 500 nM). The binding is not due to BSA since there was 12 μM BSA present in the incubation buffer and further addition of BSA had no effect on the binding (not shown, but see Fig. 5C). The binding of 17β-E-6-[^{125}I]BSA to this 23 kDa protein is consistent with the results of 17α-E-6-BSA column experiment (Fig. 2) and the repurification experiment of the affinity purified B-P2 fraction mentioned earlier.

We have identified this 23 kDa 17β-estradiol binding protein (Fig. 3, lane 1) by N-terminal sequencing in 3 different experiments. The N-terminal sequence (17 amino acids) of this 23 kDa protein was identical to the N-terminal sequence of mature rat oligomycin sensitivity-conferring protein (OSCP) [32,33], a subunit of mitochondrial F0F1 (F-type) proton ATP synthase/ATPase. In addition, microsequencing of the 18 kDa protein revealed identical N-terminal sequence (16 amino acids) with the delta subunit [34] of the same ATP synthase/ATPase, indicating that this protein, together with most other proteins of the affinity purified fraction, were probably the different subunits of the ATP synthase and copurified with OSCP.

Further evidence that 23 kDa protein is an estradiol binding protein and is OSCP comes from competition studies with unconjugated, free 17β-estradiol and immunoblotting studies using a polyclonal antibody against bovine OSCP [35,36]. As shown in Fig. 4 (lane 1), affinity purified B-mP2 contained the 23 kDa protein together with other copurified proteins. The 23 kDa was specifically labeled with 17β-E-6-[^{125}I]BSA (lane 2). The binding of this protein was displaced significantly by 10 μM 17β-estradiol (lane 3). After ligand blotting the same blots used in lanes 2 and 3 were also tested with the antibody against mature bovine OSCP. The polyclonal antibody recognized the same protein band detected by 17β-E-6-[^{125}I]BSA as shown in lanes 4 and 5. Absence of this first antibody resulted in no protein band (not shown).

One confounding factor is the presence of protein subunits of ATP synthase with similar molecular weights as OSCP. It was found that in regular SDS-PAGE, subunits d and 6 of the ATP synthase migrated at the similar position as OSCP [32,36]. This raises the possibility that these two proteins could be responsible for the binding, but could not be detected by microsequencing due to N-terminal blockage. Therefore, we had used recombinant bovine OSCP (rbOSCP) [20] to clarify this issue. The rbOSCP migrated to the same position as the 23 kDa protein in the purified B-P2 fraction (Fig. 5A). Like the 23 kDa protein, the rbOSCP was labeled by 17β-E-6-[^{125}I]BSA and displaced by unlabeled ligand (Fig. 5B), but not by BSA and only partially by 17α-E-6-BSA (Fig. 5C). In addition, in a ligand binding assay, 17β-E-6-[^{125}I]BSA bound rbOSCP, and the binding was displaced by unlabeled 17β-E-6-BSA (IC50 = 1 nM), 17α-E-6-BSA (IC50 = 50 nM) and partially by 17β-estradiol and DES (IC50 of about 10 μM), but not by BSA (Fig. 6). Recall that 10 μM 17β-estradiol also displaced the labeled ligand significantly in the ligand blotting

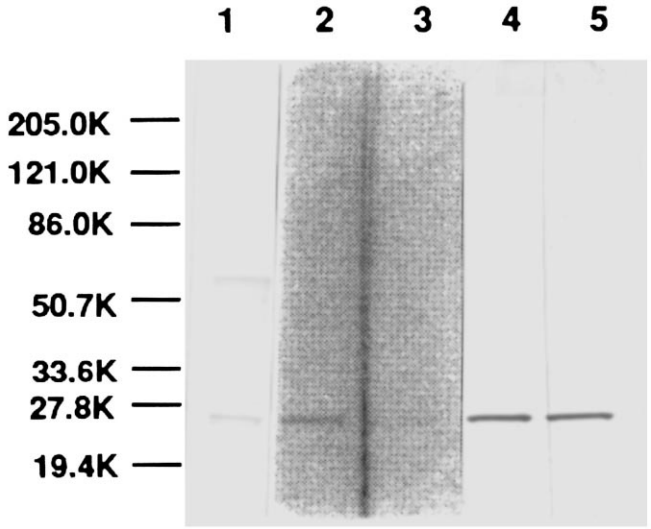

Fig. 4. Protein blotting of affinity purified mP2. Purified brain mP2 proteins (2 μg in each lane) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Lane 1 was the Coomassie Brilliant Blue staining of the transferred proteins on nitrocellulose membrane. Lanes 2 and 3 were incubated with 17β-E-6-[^{125}I]BSA (2.7×10^5 cpm/ml, 0.28 nM) in the absence (lane 2) or in the presence of 10 μM free 17β-estradiol (lane 3) and the computerized image from Phosphoimaging scanning is shown. Note that in this preparation, only the 23 kDa protein was labeled by the ligand. Ethanol (0.1%) was included in lanes 2 and 3, and 0.08% BSA was omitted in the incubation buffer to facilitate the displacement by estradiol. After autoradiography and phosphorimaging, the same strips in lanes 2 and 3 were probed with an antibody against bovine OSCP as shown in lanes 4 and 5, respectively. Note that the antibody recognizes the same band detected by the ligand. The prestained broad range M_r markers were myosin, 205.0; β-galactosidase, 121.0; BSA, 86.0; ovalbumin, 50.7; bovine carbonic anhydrase, 33.6; soybean trypsin inhibitor, 27.8; lysozyme, 19.4 and aprotinin, 7.4 (not shown).

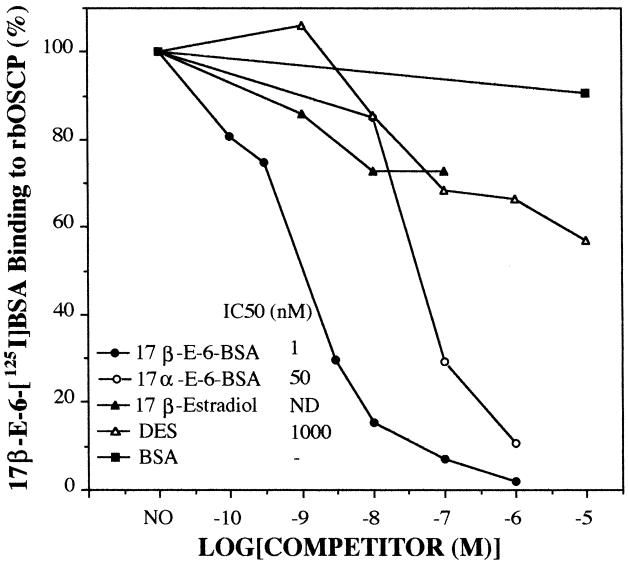

Fig. 6. Competition curves of specific 17β-E-6-[^{125}I]BSA (45000 cpm/0.5 ml, 0.14 nM) binding to recombinant bovine OSCP (rbOSCP, 0.1 μg in 0.5 ml, 6.5 nM) using various unlabeled competitors in duplicates as shown. 'NO' indicates the absence of competitor. ND, not determined. The total binding to rbOSCP was 9–12% of total ligand added. The apparent lower affinity of free estradiol and DES in displacing the 17β-E-6-[^{125}I]BSA binding is most likely due to the multivalency of the labeled ligand. In addition, at μM concentrations of estradiol there is a solubility problem, so the effective concentration is difficult to estimate. For details see text.

shown in Fig. 4 (lane 3). It is worthwhile to emphasize here that the IC50 for BSA conjugates are based on the estradiol-BSA molecules. Therefore, in terms of estradiol equivalents, the estimated IC50 for 17β-E-6-

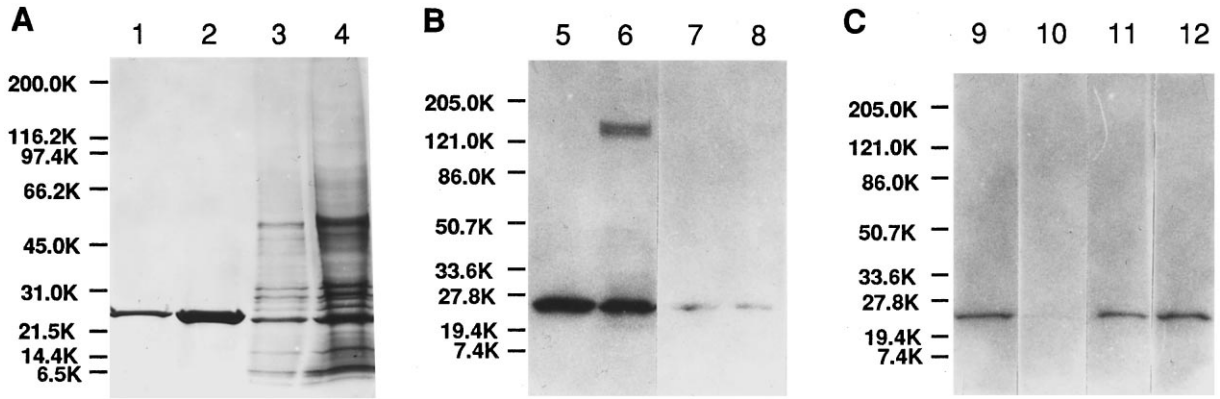

Fig. 5. (A) SDS-PAGE of recombinant bovine OSCP (rbOSCP, lanes 1 and 2, 1 and 5 μg, respectively) and 17β-E-6-BSA affinity column-retained proteins from digitonin-solubilized B-P2 (lanes 3 and 4, 2 μg and 10 μg, respectively). (B) RbOSCP (5 μg, lanes 5 and 7) and purified B-P2 (10 μg, lanes 6 and 8) were separated in SDS-PAGE and transferred to a nitrocellulose membrane. The binding proteins were detected by incubating the membrane with 17β-E-6-[^{125}I]BSA (1×10^6 cpm/ml, 1.6 nM) in the absence (lanes 5 and 6) and presence (lanes 7 and 8) of 1 μM unlabeled 17β-E-6-BSA. Interestingly, in lane 6 not only the OSCP band was specifically identified, the ligand also labeled a 130 kDa protein. This may represent a binding protein from plasma membrane. (C) Ligand blotting of rbOSCP (1 μg in each lane) using 17β-E-6-[^{125}I]BSA (1×10^6 cpm/ml, 1.6 nM) in the absence of competitors (lane 9), and in the presence of 0.5 μM 17β-E-6-BSA (lane 10), 0.5 μM 17α-E-6-BSA (11), and 12 μM BSA (lane 12). The broad range M_r markers used in (A) and prestained broad range M_r markers used in (B) and (C) are the same as in Figs. 2 and 4.

BSA and 17α-E-6-BSA would be about 50 and 500 nM, respectively.

4. Discussion

We found, using a classical [^{3}H]estradiol binding assay of rat uterine cytosol, that conjugation of estradiol or testosterone to BSA did not alter their specificity to nER. However, the conjugation at different positions in estradiol did result in different affinities. Modification of estradiol by addition of a small group such as carboxylmethyloxime (CMO) or hemisuccinate (HS) had been shown to decrease its affinity for nER and the affinity remaining would depend on the position of the conjugation [31]. By conjugating many modified estradiol molecules to a single BSA molecule, the affinity of the whole complex increased dramatically, since 17β-E-6-BSA (32 mol estradiol/mol BSA) had more than 700 times higher affinity than that of 17β-E-6-CMO. Similar increase in affinity was also true for 17β-E-17-BSA. This high affinity of estradiol-BSA conjugates is most likely due to the multivalent property of the conjugates [19], although the enhancing effect of BSA by changing the solubility of the steroid can not be excluded. However, BSA itself or T-3-BSA had no affinity for nER. We have determined the affinity of estradiol-BSA conjugates for nER to be significantly higher than the conjugates used earlier by De Goeij et al. [37]. This difference in affinity could arise by several reasons. First, the 17β-E-6-BSA and 17β-E-17-BSA conjugates prepared by De Goeij et al. [37] had less average molar ratio of estradiol to BSA (7–22, instead of 32–35). Second, BSA was not included in their assay to decrease nonspecific binding of BSA conjugates. Finally, their steroid-BSA conjugates contained significant nonprotein-linked steroids (65–70%), which did not seem the case for the steroid-BSA conjugates we purchased for use in our studies. Nevertheless, our data agreed well with them in the specificity and relative binding affinity of the conjugates. T-3-BSA was also shown by them to have no binding affinity for nER.

We chose 17β-E-6-BSA to identify possible membrane estrogen binding proteins since 17β-E-6-BSA had relatively lower affinity than 17β-E-17-BSA for classical nER while still it had high affinity for membrane estrogen binding sites [6,7]. By using 17β-E-6-BSA-linked affinity columns that were similar to the ones used by Sica et al. [31] for purification of bovine nER, we had purified an estradiol binding protein with an apparent M_r of about 23 kDa from P2 or mP2 fractions of female rat brains. This protein was recognized by 17β-E-6-[^{125}I]BSA, and displaced by 17β-E-6-BSA, 17α-E-6-BSA and 17β-estradiol, but not by BSA. Therefore, the estradiol part of the conjugate, not BSA, was responsible for the interaction between 17β-E-6-[^{125}I]BSA and the 23 kDa protein. Note that in Fig. 4, we have omitted BSA from the incubation medium to facilitate the displacement of the binding by free estradiol. The N-terminal microsequencing of this protein indicated complete identity to mature rat OSCP [32,33], a component of the F0F1 proton ATP synthase/ATPase (F-type H^+-ATPase, the complex V of the oxidative phosphorylation pathway) of mitochondria required for coupling of proton gradient to ATP synthesis [38–40]. The M_r of this protein estimated by SDS-PAGE was close to the calculated M_r from OSCP cDNA sequence (21 kDa) [33]. Furthermore, the N-terminal sequence of the 18 kDa copurified protein was the same as the delta subunit of rat ATP synthase/ATPase, and the number of major proteins retained by the 17β-E-6-BSA affinity column corresponded well with the expected number and molecular sizes of subunits of the F0F1 ATP synthase/ATPase, which consists of more than a dozen subunits [32,33,38,39]. In addition, this 23 kDa protein was specifically recognized by an antibody against bovine OSCP [35,36]. Further, the rbOSCP [41,42] had the same mobility as this 23 kDa protein and also bound 17β-E-6-[^{125}I]BSA. The binding was displaced by 17β-E-6-BSA, 17α-E-6-BSA and partially by 17β-estradiol and DES, but not BSA. All these data indicate that this 23 kDa protein is OSCP and it is an estrogen binding protein, though with much weaker affinity than nER. The fact that the estradiol-BSA conjugates binds with higher affinity for the OSCP than free estradiol can be explained by the multivalency of the former and the the differences in solubility since estradiol at high concentrations do not dissolve well in the medium. So the effective concentrations are difficult to estimate. However, inspite of the difference between the two ligands, the data indicate that estradiol and DES at submicromolar or micromolar concentrations can displace the binding. Interestingly, similar pharmacological concentrations (μM) of estrogens had been used in many previous studies on the effects of estrogens, particularly the rapid, nongenomic actions of estrogens on vascular tissues [e.g., [43]], neuroprotection [e.g., [44], and antioxidant property [e.g., [45]]. It is conceivable that an interaction of estrogen and mitochondrial ATP synthase could be the underlying mechanism for some of these actions, since ATP is the universal energy provider in the cells and mitochondria are an essential part in excitotoxicity, oxidative damage, and neurodegeneration [46].

The finding of OSCP as a binding protein for estradiol and probably DES is consistent with several previous observations. Early experiments carried out with isolated mitochondria exposed in vitro to 17β-estradiol and DES at μM concentrations inhibited the electron transport chain, and decreased oxygen uptake

and consumption [47]. In addition, DES at 10–20 μM inhibited the ATP-dependent ^{45}Ca uptake by isolated myometrial mitochondria, but not microsomal Ca^{2+} uptake [48]. The inhibition is more sensitive in human myometrial mitochondria (IC50 of about 10 μM) than in rat cardiac mitochondria. The direct interaction of DES with rat liver mitochondrial F0F1 ATP synthase/ATPase was demonstrated by McEnery and Pedersen [49] and McEnery et al. [50]. Their studies showed that DES at low μM concentrations inhibited the ATP hydrolysis in purified mitochodrial F0F1 ATP synthase/ATPase complex by interacting with F0 sector [49]. In a subsequent paper the same laboratory using a highly purified F0 preparation from rat liver mitochondria showed that DES inhibited passive proton transport through F0 whereas in intact mitochondria, DES stimulated ATP hydrolysis at low μM concentrations while inhibiting it at high μM concentrations [50]. The authors postulated that DES might preferentially target F0 and form proton channels near the F0-membrane lipid interface to affect the F0 sector of the ATPase, but the exact site in F0 is not known. From our studies, it is likely that DES specifically modulates the ATPase activity by targeting the OSCP subunit of the F0 sector. Current studies using the digitonin-solublized brain mP2 indicate that both DES and estradiol inhibited the F0F1-ATPase activity at low μM concentrations [51]. Estradiol at physiological concentrations has also been shown to decrease ATP levels in the uterus [52–55], but this effect is believed to be mainly due to 17β-estradiol-induced nER-mediated RNA transcription [52].

Aside from the functional and structural role of OSCP in ATP synthesis, fragments of bovine OSCP have been identified as a potential allergen [56]. OSCP gene was overexpressed in a metastatic stomach cell line, but not in nonmetastatic tumor cell line [57], indicating a potential role of OSCP in metastasis of cancer cells. OSCP gene was expressed at low levels in normal tissues, and was considered as one of the limiting subunits in the assembly of F0F1 ATP synthase/ATPase [58]. In Alzheimer's disease patients, the ATP synthase is significantly decreased [59]. Therefore, the expression of the OSCP gene is coupled to the functional states of cells. Estrogen could play an important role in these processes by binding to OSCP.

Our results and the aforementioned data suggest a new mechanism with possibly broad functional implications by which estrogens can affect the function of diverse cells through binding to the F0F1-ATP synthase/ATPase, a key enzyme of the cell energy machinery. The ATP synthase/ATPase could be also the target site for estrogen metabolites as well as environmental estrogens, or phytoestrogens in food sources. Current work is under way to test the effects of these compounds on the ATPase activity.

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References

[1] R.M. Evans, The steroid and thyroid hormone receptor superfamily, Science 240 (1988) 889–895.
[2] C.M. Szego, R.J. Pietras, Lysosomal functions in cellular activation: propagation of the actions of hormones and other effectors, Int. Rev. Cytol. 88 (1984) 1–302.
[3] B.S. McEwen, Nongenomic and genomic effects of steroids on neural activity, Trends Pharmacol. Sci. 12 (1991) 141–147.
[4] V.D. Ramirez, J. Zheng, Membrane sex-steroid receptors in the brain, Frontiers Neuroendocrinol. 17 (1996) 402–439.
[5] R.L. Moss, Q. Gu, M. Wong, Estrogen: nontranscriptional signaling pathway, Rec. Progress Hormone Res. 52 (1997) 33–69.
[6] J. Zheng, A. Ali, V.D. Ramirez, Steroids conjugated to bovine serum albumin as tools to demonstrate specific steroid neuronal membrane binding sites, J. Psychiat. Neurosci. 21 (1996) 187–197.
[7] V.D. Ramirez, J. Zheng, K.M. Siddique, Membrane receptors for estrogen, progesterone and testosterone in the rat brain: fantasy or reality, Cell. Mol. Neurobiol. 16 (1996) 175–198.
[8] J. Zheng, V.D. Ramirez, Demonstration of membrane estrogen binding proteins in rat brain by ligand blotting using a 17β-estradiol-[^{125}I]bovine serum albumin conjugate, J. Steroid Biochem. Mol. Biol. 62 (1997) 327–336.
[9] L.P. Pertschuk, E.H. Tobin, P. Tanapat, E. Gaetjens, A.C. Carter, N.D. Bloom, R.J. Macchia, K.B. Eisenberg, Histochemical analyses of steroid hormone receptors in breast and prostatic carcinoma, J. Histochem. Cytochem. 28 (1980) 799–810.
[10] S.H. Lee, Validity of a histochemical estrogen receptor assay: supported by the observation of a cellular response to steroid manipulation, J. Histochem. Cytochem. 32 (1984) 305–310.
[11] G.C. Chamness, W.D. Mercer, W.L. McGuire, Are histochemical methods for estrogen receptor valid? J. Histochem. Cytochem. 28 (1980) 792–798.
[12] K.S. McCarty Jr., D.S. Reintgen, H.F. Seigler, K.S. McCarty Sr., Cytochemistry of sex steroid receptors: a critique, Breast Cancer Res. Treatment 1 (1982) 315–325.
[13] L.P. Pertschuk, E.H. Tobin, A.C. Carter, K.B. Eisenberg, V.C. Leo, E Gaetjens, N.D. Bloom, Immunohistologic and histochemical methods for detection of steroid binding in breast cancer: a reappraisal, Breast Cancer Res. Treatment 1 (1982) 297–314.
[14] M. Lieberherr, B. Grosse, M. Kachkache, S. Balsan, Cell sig-

naling and estrogens in female rat osteoblasts: a possible involvement of unconventional nonnuclear receptors, J. Bone Mineral Res. 8 (1993) 1365–1376.
[15] P.G. Mermelstein, J.B. Becker, D.J. Surmeier, Estradiol reduces calcium currents in rat neostriatal neurons via a membrane receptor, J. Neurosci. 16 (1996) 595–604.
[16] T. Nenci, E. Marchetti, A. Marzola, G. Fabris, Affinity cytochemistry visualizes specific estrogen binding sites on the plasma membrane of breast cancer cells, J. Steroid Biochem. 14 (1981) 1139–1146.
[17] T.C. Pappas, B. Gametchu, C.S. Watson, Membrane estrogen receptors identified by multiple antibody labeling and impeded-ligand binding, FASEB J. 9 (1995) 404–410.
[18] F.C. Ke, V.D. Ramirez, Binding of progesterone to nerve cell membranes of rat brain using progesterone conjugated to I125-bovine serum albumin as a ligand, J. Neurochem. 54 (1990) 467–472.
[19] N. Eshhar, G. Lederkremer, M. Beaujean, O. Goldberg, P. Gregor, A. Ortega, A. Triller, V.I. Teichberg, Kainyl-bovine serum albumin: a novel ligand of the kainate subtype of glutamate receptor with a very high binding affinity, Brain Res. 476 (1989) 57–70.
[20] S. Joshi, A.A. Javed, L.C. Gibbs, Oligomycin sensitivity-conferring protein (OSCP) of mitochondrial ATP synthase, J. Biol. Chem. 267 (1992) 12860–12867.
[21] R.F. Bruns, K. Lawson-Wendling, T.A. Pugsley, A rapid filtration assay for soluble receptors using polyethylenimine-treated filters, Anal. Biochem. 132 (1983) 74–81.
[22] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[23] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[24] P. Matsudaira, Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes, J. Biol. Chem. 261 (1987) 10035–10038.
[25] H. Towbin, R. Staehelin, J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets, Proc. Natl. Acad. Sci. USA 76 (1979) 4350–4354.
[26] P.J. Munson, D. Rodbard, LIGAND: a versatile computerized approach for characterization of ligand-binding systems, Anal. Biochem. 107 (1980) 220–239.
[27] Y.-C. Cheng, W.H. Prusoff, Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (IC_{50}) of an enzymatic reaction, Biochem. Pharm. 22 (1973) 3099–3108.
[28] A. Dupuis, J.-P. Issartel, J. Lunardi, M. Satre, P.V. Vignais, Interactions between the oligomycin sensitivity conferring protein (OSCP) and beef heart mitochondrial F1-ATPase. 1. Study of the binding parameters with a chemically radiolabeled OSCP, Biochemistry 24 (1985) 728–733.
[29] R.L. Sutherland, C.K.W. Watts, L.C. Murphy, Binding properties and ligand specificity of an intracellular binding site with specificity for synthetic oestrogen antagonists of the triphenylethylene series, in: M.K. Agarwal (Ed.). Hormone Antagonists, Walter de Gruyter & Co, Berlin, 1982, pp. 147–161.
[30] S.G. Korenman, Comparative binding affinity of estrogens and its relation to estrogenic potency, Steroids 13 (1969) 163–177.
[31] V. Sica, I. Parikh, E. Nola, G.A. Puca, P. Cuatrecasas, Affinity chromatography and the purification of estrogen receptors, J. Biol. Chem. 248 (1973) 6543–6558.
[32] F. Cretin, L.G. Baggetto, L. Denoroy, C. Godinot, Identification of F0 subunits in the rat liver mitochondrial F0F1-ATP synthase, Biochim. Biophys. Acta 1058 (1991) 141–146.
[33] T. Higuti, K. Kuroiwa, Y. Kawamura, K. Morimoto, H. Tsujita, Molecular cloning and sequence of cDNAs for the import precursors of oligomycin sensitivity conferring protein, ATPase inhibitor protein and subunit c of H^+-ATP synthase in rat mitochondria, Biochim. Biophys. Acta 1172 (1993) 311–314.
[34] D.M. Medeiros, L. Whiry, A.J. Lincoln, L.J. Prochaska, Cardiac nonmyofibrillar proteins in copper-deficient rats: amino acid sequencing and Western blotting of altered proteins, Biol. Trace Element Res. 36 (1993) 271–282.
[35] G.I. Belogrudov, J.M. Tomich, Y. Hatefi, ATP synthase complex-proximities of subunits in bovine submitochondrial particles, J. Biol. Chem. 270 (1995) 2053–2060.
[36] C. Hekman, Y. Hatefi, The F0 subunits of bovine mitochondrial ATP synthase complex: purification, antibody production and interspecies cross-immunoreactivity, Arch. Biochem. Biophys. 284 (1991) 90–97.
[37] A.F.P.M. De Goeij, J.K. van Zeeland, C.J.L. Beek, F.T. Bosman, Steroid-bovine serum albumin conjugates: molecular characterization and their interaction with androgen and estrogen receptors, J. Steroid Biochem. 24 (1986) 1017–1031.
[38] P.L. Pedersen, L.M. Amzel, ATP synthases, J. Biol. Chem. 268 (1993) 9937–9940.
[39] J.E. Walker, I.R. Collinson, The role of the stalk in the coupling mechanism of F1F0-ATPases, FEBS Lett. 346 (1994) 39–43.
[40] S. Engelbrecht, W. Junge, Subunit delta of H^+-ATPase: at the interface between proton flow and ATP synthesis, Biochim. Biophys. Acta 1015 (1990) 379–390.
[41] Y.A. Ovchinnikov, N.N. Modyanov, V.A. Grinkevich, N.A. Aldanova, O.E. Trubetskaya, I.V. Nazimov, T. Hundal, L. Ernster, Amino acid sequence of the oligomycin sensitivity-conferring protein (OSCP) of beef-heart mitochondria and its homology with the delta-subunit of the F1-ATPase of *Escherichia coli*, FEBS Lett. 166 (1984) 19–22.
[42] J.E. Walker, N.J. Gay, S.J. Powell, M. Kostina, M.R. Dyer, ATP synthase from bovine mitochondria: sequences of imported precursors of oligomycin sensitivity conferral protein, factor 6 and adenosinetriphosphatase inhibitor protein, Biochemistry 26 (1987) 8613–8619.
[43] M.Y. Farhat, M.C. Lavigne, P.W. Ramwell, The vascular protective effects of estrogen, FASEB J. 10 (1996) 615–624.
[44] C. Behl, M. Widmann, T. Trapp, F. Holsboer, 17β-estradiol protects neurons from oxidative stress-induced cell death in vitro, Biochem. Biophys. Res. Commun. 216 (1995) 473–482.
[45] T. Miura, S. Muraoka, T. Ogiso, Inhibition of lipid peroxidation by estradiol and 2-hydroxyestradiol, Steroids 61 (1996) 379–383.
[46] M.F. Beal, Mitochondria, free radicals and neurodegeneration, Curr. Opinion Neurobiol. 6 (1996) 661–666.
[47] G.M. Tomkins, E.S. Maxwell, Some aspects of steroid hormone action, Ann. Rev. Biochem. 32 (1963) 677–708.
[48] S.C. Batra, L.P. Bengtsson, Inhibition by diethyl stilbestrol of calcium uptake by human myometrial mitochondria, Eur. J. Pharmacol. 18 (1972) 281–283.
[49] M.W. McEnery, P.L. Pedersen, Diethylstilbestrol F0 'proton channel' of rat liver mitochondria: rapid purification of a functional complex and a study of its interaction with the unique probe diethylstilbestrol, J. Biol. Chem. 261 (1986) 1745–1752.
[50] M.W. McEnery, J. Hullihen, P.L. Pedersen, F0 'proton channel' of rat liver mitochondria: rapid purification of a functional complex and a study of its interaction with the unique probe diethylstilbestrol, J. Biol. Chem. 264 (1989) 12029–12036.
[51] J. Zheng, V.D. Ramirez, Wine phytoestrogens as inhibitors of rat mitochondrial F0F1-ATPase: is red wine better than white? The 4th International Congress of Neuroendocrinology, Kitakyushu, Japan, 1998.

[52] S.A. Aaronson, Y. Natori, H. Tarver, Effect of estrogen on uterine ATP levels, Proc. Soc. Exp. Biol. Med. 120 (1965) 9–10.
[53] J.M. Oliver, A.E. Kellie, The effects of oestradiol on the acid-soluble nucleotides of rat uterus, Biochem. J. 119 (1970) 187–191.
[54] H. Degani, A. Shaer, T.A. Victor, A.M. Kaye, Estrogen-induced changes in high-energy phosphate metabolism in rat uterus: ^{31}P NMR studies, Biochemistry 23 (1984) 2572–2577.
[55] H. Degani, T.A. Victor, A.M. Kaye, Effects of 17β-estradiol on high energy phosphate concentrations and the flux catalyzed by creatine kinase in immature rat uteri: ^{31}P nuclear magnetic resonance studies, Endocrinology 122 (1984) 1631–1638.
[56] S. Parkkinen, M. Rytkonen, J. Pentikainen, T. Virtanen, R. Mantyjarvi, Homology of a bovine allergen and the oligomycin sensitivity-conferring protein of the mitochondrial adenosine triphosphate synthase complex, J. Allergy Clin. Immunol. 95 (1995) 1255–1260.
[57] A.N. Salesiotis, C.K. Wang, C.D. Wang, A. Burger, H. Li, A. Seth, Identification of novel genes from stomach cancer cell lines by differential display, Cancer Lett. 91 (1995) 47–54.
[58] H. Sangawa, T. Himeda, H. Shibata, T. Higuti, Gene expression of subunit c (P1), subunit c (P2) and oligomycin sensitivity-conferring protein may play a key role in biogenesis of H + -ATP synthase in various rat tissues, J. Biol. Chem. 272 (1997) 6034–6037.
[59] H. Schagger, T.G. Ohm, Human diseases with defects in oxidative phosphorylation 2. F1F0 ATP-synthase defects in Alzheimer disease revealed by blue native polyacrylamide gel electrophoresis, Eur. J. Biochem. 227 (1995) 916–921.