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PROPERTIES OF THE MINERALOCORTICOID RECEPTOR IMMUNOPURIFIED FROM BOVINE KIDNEY

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Abstract—The mineralocorticoid receptor (MCR) from bovine kidney was purified on an affinity column containing covalently linked polyclonal IgG raised in the rabbit against rat kidney protein purified in the presence of RU 26752 that is specific to the MCR. The immuno-affinity eluate was excluded as a single peak during gel permeation chromatography and could be resolved as a single band of approximately 98 kDa by western blot and gel electrophoresis. Immunohistochemistry revealed MCR-specific staining in both the cortical and glomerular regions of bovine kidney. Interestingly, the purified MCR could not be activated in the presence of the specific ligand RU 26752 whereas binding to DNA-cellulose increased by 100% when crude cytosol was left at room temperature for 45 min. The binding of calcium to the MCR resulted in an increase in the fluorescence signal that could be partially reversed by EDTA. By a calcium-specific fluorescence dye technique, 1.13 nM of ionized Ca^{2+} was bound per 0.01 nM MCR. The binding of ATP^{32} to the immunopurified receptor was observed following chromatography on P-10 columns. The fluorescence signal of etheno-ATP was maximally attenuated by the receptor at 1/1 stoichiometry of the ATP–MCR complex. Asparagine-linked complex chain N-glycosylation of the purified MCR was also observed. Analysis by far-UV circular dichroism spectra showed that MCR contains 33% α helices and 30% β sheets, compatible with a relatively flat conformation of the native protein. These data provide experimental proof for the predicted computer simulation regarding the structural features of the steroid receptor superfamily and suggest crosstalk between several protein families.

Key words: mineralocorticoids; receptor; immunopurification; post-translational modifications; kidney

Steroid hormones bind to specific cellular receptors that act as *trans*-activating signals in the promotor region of DNA in appropriate target organs [1, 2]. Although most members of this superfamily of proteins have been cloned and analysed by molecular biology techniques *in vitro* [3, 4], the regulation of receptor function within the mammalian tissue *in vivo* has remained speculative [5, 6].

Mineralocorticoids regulate the sodium–water balance in vertebrates, and are implicated in the pathology of hypertension-related syndromes [7, 8]. Progress in MCR¶ research has been particularly slow in the past due to its instability and the unavailability of suitable ligands. More recently, the MCR-specific spirolactone derivative RU 26752 [9] permitted biochemical purification of this receptor, resolved as a single band by SDS–PAGE [10, 11]. This homogeneous antigen was used to generate a polyclonal antiserum in the rabbit whose specificity for the MCR was assessed by a number of criteria (precipitation, macroaggregation, ELISA, western blot) and it was possible to photolabel this receptor for the very first time [12, 13].

The present investigations were undertaken to understand the physicochemical properties of the native, endogenous MCR. Since an identical MCR gene permits the expression of widely different organ-specific responses [5], the question was asked whether post-translational modifications could conceivably be responsible for the divergent phenotypes. The antibody characterized above [12, 13] permitted large scale purification of the native MCR for the very first time. The immunopurified protein appears functionally distinct from the receptor in crude cytosol, as well as from the cloned product. The results provide experimental proof for a number of structural predictions obtained from computer simulation and are compatible with the increasing body of evidence suggesting crosstalk between several protein families for the expression of the pharmacological action of steroid hormones via the steroid receptor superfamily [1, 2].

MATERIALS AND METHODS

Receptor purification and activation. Bovine kidney was obtained from the local slaughterhouse and frozen within minutes after ablation from the animal. Tissue was homogenized (g/mL) in a Waring blender in 10 mM PBS containing (per L) 0.25 g KH_2PO_4, 1.38 g Na_2PO_4, 0.25 g KCl, 9 g NaCl and 0.01% sodium azide, supplemented with 20 mM diiso-

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¶ Abbreviations: MCR, mineralocorticoid receptor; ELISA, enzyme-linked immunoabsorbent assay; $[\theta]_r$, Mean residual molar ellipticity; DBD, DNA binding domain.

propylfluorophosphate. The homogenate was centrifuged at 30,000 *g* for 30 min, followed by 45 min at 105,000 *g*, all at 4°, and finally passed through an immunoaffinity column as follows.

Whole serum obtained from rabbits, immunized with the MCR purified from rat kidneys in the presence of RU 26752 that binds specifically to the MCR [10, 11], was fractionated on a DEAE–trisacryl column to obtain the IgG fraction [12, 13]. Immune IgG was affinity-linked to CNBr-activated Sepharose 4B (Pharmacia lot No. QH 12518) according to the standard procedure [14]. The resin was packed in a C10/20 column (Pharmacia, Uppsala, Sweden) and washed with an excess of PBS containing 0.5 M NaCl. A 150–200 mL volume of renal cytosol was brought to 0.3 M KCl and passed through the 10 mL resin bed at 50–60 mL/hr at 4°. The resin was washed with PBS until absorbance at 280 nm fell to 0; the MCR retained by the IgG was thereafter eluted with 0.1 M glycine buffer, pH 2.4, containing 0.15 M NaCl and 0.01% sodium azide. Pooled fractions (*ca.* 15 mL) were neutralized with solid Tris, dialysed overnight against the desired buffer, and concentrated in vaccum, all at 4°.

This preparation was permeated through a 2000 SW (30 × 0.75 cm) column (Tosoh Corp., Tokyo), using the "System gold" HPLC apparatus (Beckman, Japan) at 30 mL/hr at room temperature and 10 mM phosphate, pH 7.5. ELISA, western blots, and gel electrophoresis were performed at different stages to monitor elution pattern and purity [10–13].

The DBD was tested with the MCR-specific ligand [^{3}H]RU 26752, as described previously [9–13]. Very briefly, the crude cytosol or the purified MCR was equilibrated with the radioligand at 4° for 60 min and divided into two lots. One half was left at 4° and the other activated for 45 min at 25°. The quantity retained by DNA-cellulose was then counted in 10 mL Picofluor (Packard, Paris, France).

Analysis of post-translational modifications. Asparagine-linked N-glycosylation was assayed by gas chromatography of 800 mg of the acid-digested receptor, along with known sugar markers, and the extent of glycosylation was calculated using mannitol as reference [15].

Tryptophan fluorescence spectra of MCR in 10 mM phosphate pH 7.5 were recorded at 22° in a Kontron SFM 25 fluorometer, after excitation with 450 V at 270 nm along a 1 cm light path. MCR (4 mM) was assessed in the presence of 1 mM EDTA and/or 1 mM $CaCl_2$.

Ionized calcium bound to MCR was quantitated by the decrease in the fluorescence signal of Fura-2, recorded between 250 and 400 nm. The buffer solution containing (mM) NaCl (115), KCl (3), $MgSO_4$ (1), KH_2PO_4 (0.2), K_2HPO_4 (0.8), HEPES (10), $NaHCO_3$ (25) was supplemented with protein, $CaCl_2$, or EGTA (17).

MCR in 10 mM phosphate pH 7.5, 100 mM NaCl and 1 mM $MgCl_2$ was incubated with 5 mCi ATP^{32} and passed through a P-10 (Biorad, California, USA) column (1.5 cm × 40 cm), equilibrated and eluted with this buffer at 30 mL/hr at 4°. Fractions were quantitated for protein and radioactivity was estimated in 10 mL Picofluor 40.

An excitation wavelength of 310 nm at 450 V was used for the fluorescence spectra of 2.5 mM MCR, supplemented with increasing quantities of etheno-ATP (Sigma, lot 111H 7841) in the buffer used for the P-10 column. Spectra were recorded at room temperature following 5 min equilibration after each addition of etheno-ATP [16].

Circular dichroism measurements were recorded with a Jobin-Yvon Mark IV dichograph linked to a Minc digital 11 miniprocessor. Ten mM protein (*ca.* 1 mg/mL) in 1 mM phosphate buffer pH 7.5 was scanned along a 0.01 cm path at 20° [18]. Results are expressed in mean residual molar ellipticity $[\theta]_r$ (degrees $cm^2 \cdot dmol^{-1}$).

Immunohistochemistry was performed according to procedures developed in our laboratory and

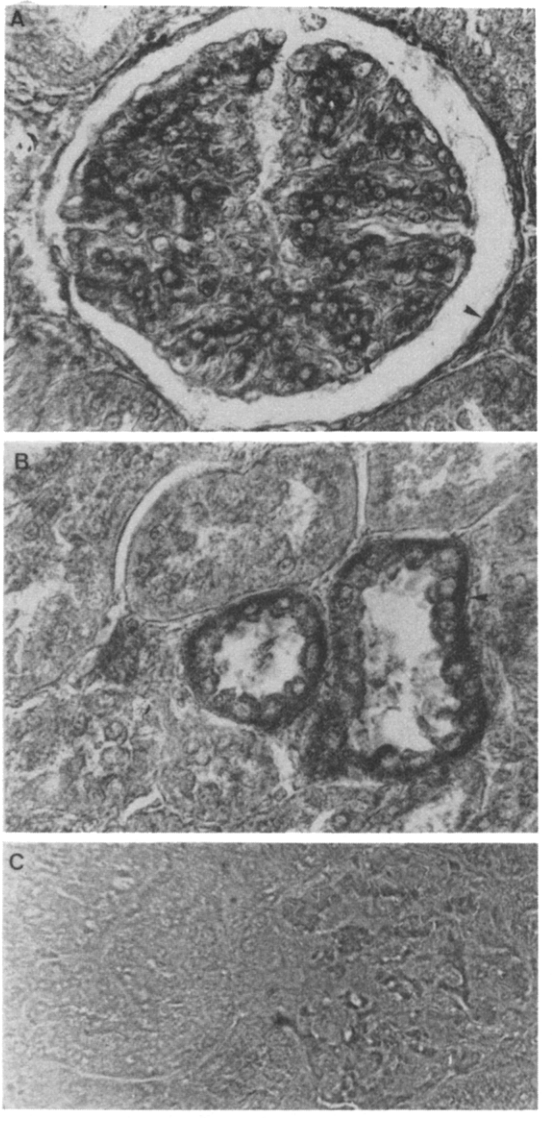

Fig. 1. Mineralocorticoid receptor specific immunostaining in bovine kidney. Arrows point to MCR-specific staining in the glomerular (A) and cortical (B) regions with the polyclonal antiserum whereas the preimmune control (C) is negative; ×400.

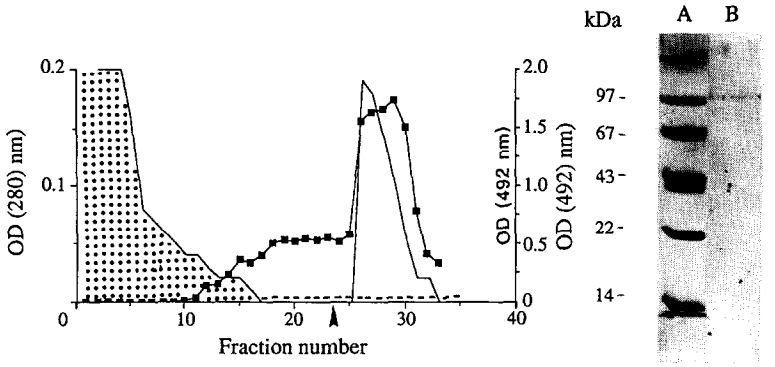

Fig. 2. Immunopurification of the mineralocorticoid receptor. The shaded area (• • •) represents bovine renal cytosol loaded onto the column; the protein retained was eluted by the glycine buffer (——). ELISA was performed on all fractions (■) and suggests column saturation since the MCR could no longer be retained by the antibody (fractions 10–20). The anti-MCR antibody was omitted from the reaction mixtures in mock ELISA runs (– – –). Pool of fractions 26–28 was concentrated and processed for western blot analysis (B), along with molecular weight markers (A).

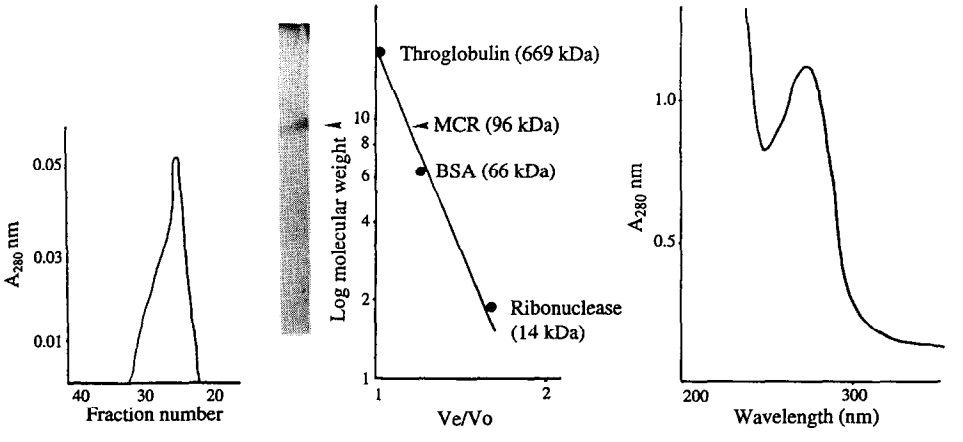

Fig. 3. Gel permeation chromatography of the mineralocorticoid receptor. Immunopurified MCR (Fig. 2), was run through the HPLC column (left panel) and resolved as a single band on polyacrylamide gels (as for Fig. 2) stained with the Coomassie reagent that had previously been calibrated (middle panel). The absorbance spectrum of the eluate is shown in the right panel.

described in detail previously [13, 19]. Photomicrographs were recorded on an Agfa-ortho 25 ASA film.

[^{3}H]RU 26752 (50 Ci/mM, lot X 3025 A) and the radioinert ligand were kindly provided by Roussel-Uclaf, Romainville, France. Adenosine-5′-^{32}P-triphosphate (3000 Ci/mM, batch B9312) was purchased from Amersham, U.K.

RESULTS

Receptor purification and activation

The histochemical study in Fig. 1 shows that the basal cells of the cortical collecting duct, Bowman's capsule, and glomerular parenchyma, stained positive for the MCR with the polyclonal antiserum raised in the rabbit against rat renal antigen. Thus, bovine kidney appeared to be far richer in MCR than rat kidney [13], and therefore suitable for large scale purification of this receptor. A number of recent studies have shown that the MCR is present all along the renal tubule, contrary to earlier investigations that had suggested the presence of MCR in the cortical portion only [5].

Data in Fig. 2 show that MCR-positive (ELISA) material retained by the immunoaffinity resin could be eluted as a sharp peak soon after the pH shock of glycine buffer and finally resolved as a single band of approximately 98 kDa in the western blot. The ELISA elution pattern was negative if the anti-MCR antibody was omitted, thereby confirming the technical specificity of the procedure. The purification of almost 200 μg MCR from 50 g tissue was higher than expected from calculations based on rat kidney

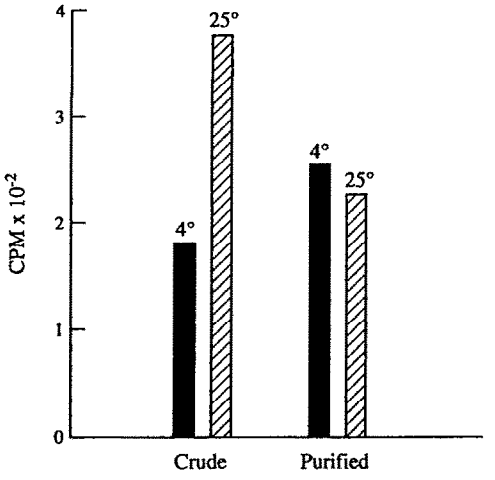

Fig. 4. Immunopurified MCR does not undergo activation. Crude renal cytosol (20 mg) and the immunoaffinity eluate (10 μg) were left for 45 min at either 4° or 25°, in the presence of the MCR-specific ligand RU 26752, and finally processed for binding to DNA-cellulose as described previously (9).

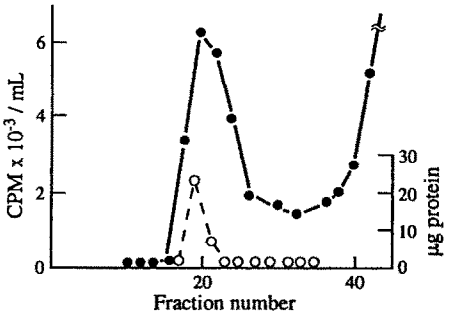

Fig. 5. The purified mineralocorticoid receptor binds ATP. MCR (100 mg) and ATP^{32} (5 mCi) mixtures were passed through a P-10 column. Alternate fractions were processed for the quantitation of protein (○) or radioactivity (●) in 10 ml Picofluor 40.

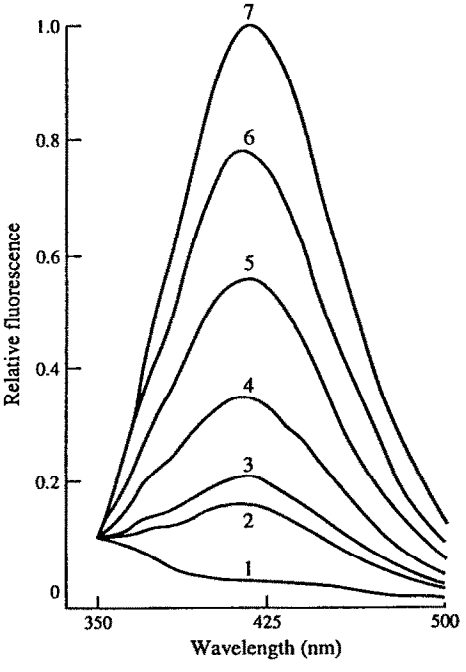

Fig. 6. Quantitation of the binding of etheno-ATP to MCR by fluorescence. The emission spectra of 2.5 mM MCR alone (1) or in the presence of 0.5, 1, 2, 3, 4 and 5 mM etheno-ATP (2–7, respectively) at the excitation wavelength of 310 nm.

[9, 11, 13], confirming the histochemical study in Fig. 1 and other recent reports [5].

Data in Fig. 3 show that the immunoaffinity eluate was excluded as a single peak of approximately 98 kDa during gel permeation chromatography and resolved as a single band after gel electrophoresis, with an absorption maxima at 274 nm. The MCR purified in this manner appeared suitable for further physicochemical studies.

Binding to DNA-cellulose was not altered by heating the immunopurified MCR–[^{3}H]RU 26752 complex for 45 min at 25°, unlike the crude preparation which readily underwent heat activation under these conditions (Fig. 4). The sp. act. of the crude preparation doubled from 11 to 22 dpm per mg protein whereas the immunopurified MCR exhibited comparable sp. act. (33,400 dpm/mg at 4° vs 28,300 dpm/mg at 25°). Thus, immunopurification led to several 1000-fold enrichment although this could not be estimated precisely due to the presence of endogenous steroids and the lack of an appropriate exchange assay for this receptor protein. Conceivably, endogenous steroids in combination with high salt during purification may already lead to maximal activation.

ATP binding

ATP has often been used to activate various members of the steroid receptor superfamily [20] and alters receptor function in various ways [21]. The coelution of the ATP^{32}–MCR complex from the P-10 column suggests that the receptor binds ATP (Fig. 5). This was quantitated by measuring the fluorescence signal of increasing amounts of etheno-ATP in the presence of a constant concentration of MCR, as shown in Fig. 6. Between 0.5–2 mM etheno-ATP, the receptor attenuated fluorescence intensity, but not the maximum at 412 nm. The fluorescence signal increased proportionately with the dose in the 3–5 mM range of etheno-ATP. Maximum attenuation at 2 mM etheno-ATP, 2.5 mM MCR suggests a 1/1 stoichiometry, considering the difference in molecular weights (ATP = 507 kDa; etheno-ATP = 531 kDa) and residual endogenous ATP.

Calcium binding

The binding of calcium to the chromophores of tryptophan and tyrosine in the MCR [22] should

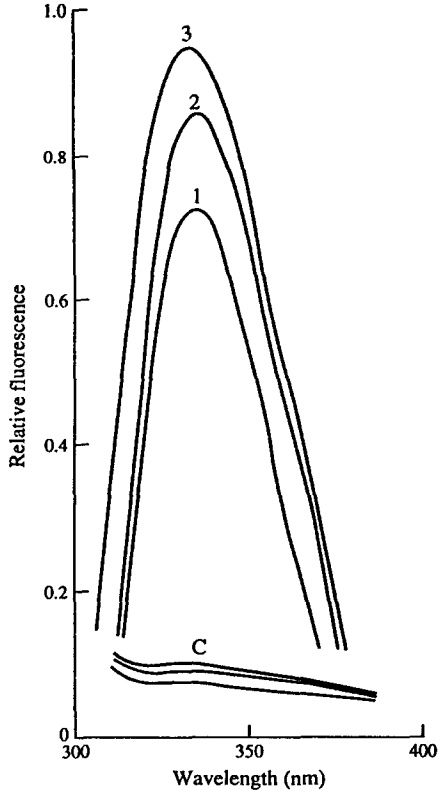

Fig. 7. The mineralocorticoid receptor binds calcium. Tryptophan fluorescence spectra were recorded with 4 mM MCR alone (1), in the presence of 1 mM $CaCl_2$ (3), and after addition of 1 mM EDTA (2). The controls (C), without the MCR, were identical in all cases.

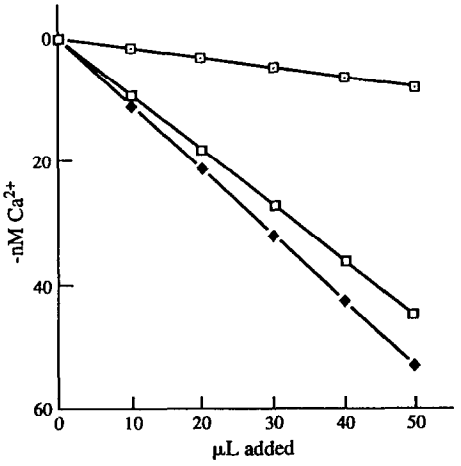

Fig. 8. Quantitation of calcium binding to the MCR. Increasing quantities of PBS (⊡) or MCR (◆) were added to quartz cells containing ionized calcium and Fura-2, and the amount bound specifically (□) was finally calculated.

alter its emission spectrum. The emission maximum of the protein at 327 nm, following excitation at 270 nm, increased by nearly 25% in the presence of calcium (Fig. 7). EDTA lowered the emission intensity of the calcium–MCR complex but did not reverse the effect of calcium. Thus, at least part of the calcium appears to be bound tightly to MCR in a protected, hydrophilic environment. It was estimated by a calcium-specific assay procedure [17] that 1.13 nM calcium could be bound per 0.01 nM of MCR (Fig. 8).

Optical spectroscopy

The far-UV circular dichroism spectrum of the MCR (Fig. 9) is compatible with that of a protein containing substantial α helices: $\theta = -5300$ at 220 nm, −5900 at 208 nm, and +9200 at 190 nm. Analysis by the method of Chang *et al.* [23], using the program of Yang *et al.* [24] gave the following estimates: 33% α helices, 30% β sheets, 12.5% β turns and 24.5% others. These data are in complete agreement with the secondary structure prediction studies for other members of this superfamily which suggested a relatively flat conformation for the receptor protein [25, 26]. In preliminary studies, neither calcium nor ATP altered receptor conformation under these conditions.

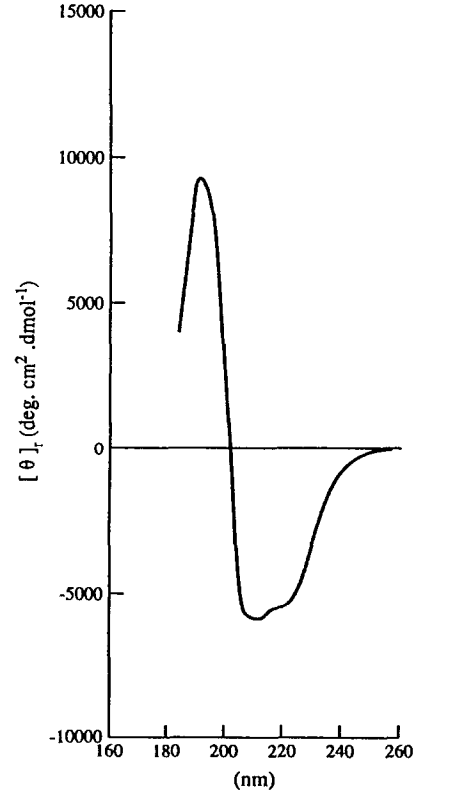

Fig. 9. The far-UV circular dichroism spectra of the mineralocorticoid receptor. A protein concentration of 10 µM (*ca.* 1 mg/mL) was used along a light path of 0.01 cm and the data were processed for the calculation of protein conformations [23, 24, 35].

Glycosylation

In gas chromatography, the bovine renal MCR exhibited asparagine-linked N-glycosylation consisting of galactose (29.3 nM), mannose (51.5 nM) and N-acetyl-glucosamine (239 nM). This suggests a complex molecular branching on asparagine side chains in which all of the glucosamines do not bear galactose. The assessment of the number of glycosylated asparagine residues per MCR molecule will require painstaking studies in a specialized environment. Nevertheless, glycosylation may alter the assessment of the molecular mass of the receptor protein.

DISCUSSION

The technique of immunopurification [14, 19] has rarely been used for steroid hormone receptors, perhaps because most members of this superfamily have been cloned and overexpressed [1, 2, 22]. Little attention is currently paid to possible post-translational modifications that permit tissue, organ, and species dependent response from a highly conserved gene [3, 5]. Thus, glycosylation would go entirely undetected in proteins overexpressed in bacterial systems.

The antibody used in these studies was directed against the MCR purified from rat kidneys in the presence of RU 26752—a ligand specific to this receptor [10, 11]. The antibody has been rigorously characterized by several techniques including immuneprecipitation, macroaggregation, western blots and photoaffinity [12, 13]. It precipitates the RU 26752–MCR complex from rat kidney cytosol but does not recognize RU 26572 bound nonspecifically to brain proteins since the MCR in the central nervous system does not bind this ligand, further confirming specificity [19]. The rich MCR-specific immunostaining in bovine kidney, in contrast to limited quantities in rodent tissues, is compatible with other observations where this receptor was shown to be distributed all along the renal tubule [5]. Although it was not possible to assess receptor concentration in bovine renal cytosol due to endogenous steroids, the yield of approximately 200 μg/50 g tissue permitted the purification of sufficient MCR for further structural studies.

The lack of activation of the purified MCR contrasts with the activation of receptor in crude cytosol and questions the relevance of this process *in vivo*. Possibly, the cytosolic components may merely limit the access of MCR to promoter sites in DNA. Since the purified MCR accepted the ligand just as readily as the crude preparation, the activation process does not provoke irreversible alteration in the steroid binding domain. The relatively flat conformation, predicted by circular dichroism, is likely required for ligand binding and attests to the purity of our product relative to other preparations [25, 26].

Consensus sequences that bind ATP (Rossmann folds) have been identified in several proteins [27, 28], and are homologous to residues 745–768 in the MCR [26]. The binding of ATP to MCR in 1/1 stoichiometry is compatible with the theoretical prediction [26] and may be responsible for receptor activation [20] and receptor–ligand interaction [21]. Given the conserved domain structure in this superfamily of genes [22], other receptor proteins are also likely to bind ATP.

Calcium influences a wide range of cellular processes [29–31]. Glucocorticoids [32] and estroprogestatives [33] increase intracellular calcium level. Of the 170 or so calcium-binding proteins, glucocorticoids have been reported to induce calmodulin [34]. The physiological action of some of these calciphilics is achieved via a calcium-dependent interaction with other proteins [31]. Thus, calcium may be an intracellular messenger for steroid hormones, at least under certain conditions. Computer simulation has shown the presence of several putative calcium binding domains in the MCR *viz*: 90–100, 110–130 and 490–450 (Dr J. P. Mornon, personal communication). We provide here experimental proof for this prediction although the functional significance of calcium–receptor interactions remains to be elucidated.

Optical spectroscopy permits analysis of secondary structure difficult to obtain by other experimental techniques [35] and suggests a relatively flat conformation of the native MCR, as for other members of this superfamily [25, 26]. In future, circular dichroism may be useful in detecting possible conformational changes following the association of cofactors (ATP, calcium) with the MCR protein. Similarly, this technique may help distinguish between native vs denatured forms of the receptor, between active (agonist bound) vs inactive (antagonist bound) configurations, and between cloned vs innate protein conformations.

Finally, the binding of Ca and ATP to the MCR, in accordance with the predicted computer simulation, should permit delineation of precise three-dimensional configuration of this superfamily of proteins. Functional analysis of other receptor classes, where structure prediction has already been attempted [26], may benefit from techniques established here. Such post-translational modifications may assure tissue-specific pharmacological action from a highly conserved gene [36, 37]. Immunoaffinity purification of still larger quantities of the MCR should permit further chemical, physical and crystallographic characterization of the native receptor.

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