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Modification of an essential amino group in the mineralocorticoid receptor evidences a differential conformational change of the receptor protein upon binding of antagonists, natural agonists and the synthetic agonist 11,19-oxidoprogesterone

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Abstract

The alkylation of amino groups of the mineralocorticoid receptor (MR) with pyridoxal 5′-phosphate or 2,4,6-trinitrobenzenesulphonate (TNBS) under controlled conditions modifies only one lysyl residue, which accounts for a 70% inhibition of steroid binding capacity. The K_d of aldosterone for MR is not affected by the treatment, but the total number of binding sites is greatly decreased. The modified receptor is capable of dynamically conserving its association with the hsp90-based heterocomplex. Importantly, the binding of natural agonists protects the hormone binding capacity of the MR from the inactivating action of alkylating agents. In contrast, antagonistic steroids are totally incapable of providing such protection. Like the antagonistic ligands, and despite its potent mineralocorticoid biological effect, the sole MR specific synthetic agonist known to date, 11,19-oxidoprogesterone (11-OP), shows no protective effect upon treatment of the MR with pyridoxal 5′-phosphate or TNBS. Limited digestion of the MR with α-chymotrypsin generates a 34 kDa fragment, which becomes totally resistant to digestion upon binding of natural agonists, but not upon binding of antagonists. Interestingly, the synthetic 21-deoxypregnanesteroid 11-OP exhibits an intermediate pattern of proteolytic degradation, suggesting that the conformational change generated in the MR is not equivalent to that induced by antagonists or natural agonists. We conclude that in the first steps of activation, the MR changes its conformation upon binding of the ligand. However, the nature of this conformational change depends on the nature of the ligand. The experimental evidence shown in

Abbreviations: MR, mineralocorticoid receptor; hMR, human MR; GR, glucocorticoid receptor; hsp90, 90 kDa heat shock protein; ALDO, aldosterone; CORT, corticosterone; DOC, 11-deoxycorticosterone; 11-OP, 11,19-oxidoprogesterone; 6-OP, 6,19-oxidoprogesterone; PROG, progesterone; SPO, SC9420-spironolactone; T=O, testosterone; PBS, phosphate-buffered saline; PLP, pyridoxal 5′-phosphate; TNBS, 2,4,6-trinitrobenzenesulphonate

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this work suggests that a single lysyl group can determine the hormone specificity of the MR.

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1. Introduction

The steroid hormone receptors belong to a nuclear receptor superfamily of ligand-induced transcription factors which share a common set of three main domains [1]. A central domain binds to hormone response elements in the DNA of target cells, an N-terminal region mediates transactivation, and the C-terminal domain binds the ligand.

Most of the members of the steroid receptor superfamily exist in a heterocomplex with the 90 kDa heat shock protein (hsp90)-based chaperone system [2]. This association stabilises steroid receptors in the hormone binding form and prevents their transcriptional activity. The binding of hormone to the steroid receptor triggers a series of events before the receptor recruits other transcription factors. It is thought that when the steroid binds to the receptor, it generates a conformational change that would lead to the dissociation of the hsp90 heterocomplex (referred to as transformation), followed by receptor dephosphorylation, dimerisation and, for cytoplasmic receptors, translocation into the nucleus. The transformed receptor interacts with specific DNA sequences, undergoes hyperphosphorylation and recruits co-activators or co-repressors to regulate the final biological response. Indeed, the actual temporal sequence of this cascade of events is almost unknown.

Nevertheless, the experimental evidence suggests that the receptor first undergoes dephosphorylation [3] and then hyperphosphorylation [4] during the hormone-dependent activation process. It has also been shown that the dissociation of hsp90 upon steroid binding is not necessarily the first step in the glucocorticoid receptor (GR) signalling pathway [5]. In effect, recent evidence shows that hsp90 may be required for GR trafficking on cytoskeletal tracts [6,7] in a process where cytoplasmic dynein may act as a motor protein for the retrograde movement of the GR [8,9].

The primary event that triggers all these molecular events is the hormone binding to the receptor. Under normal physiological conditions, aldosterone (ALDO) is the endogenous ligand responsible for MR activation in epithelia and vascular smooth muscle cells. However, in non-epithelial cells such as cardiomyocytes and neurones, the physiologic predominant occupant appears to be cortisol, commonly but not unequivocally as an antagonist. The activation of the mineralocorticoid receptor (MR) in kidney leads to the critical regulation of the internal medium by sodium and water retention, and potassium and proton elimination.

The structures of the MR and the GR are very similar [1]. Since their DNA binding domains are 94% homologous, the cross-talk between transformed receptors with their respective hormone response elements is not a surprise. Moreover, the hormone binding domains are 57% homologous, so cross-reaction between glucocorticoid and mineralocorticoid ligands also occurs. In fact, glucocorticoids would also activate the MR if the protective action of the enzyme 11β-hydroxysteroid dehydrogenase would not inactivate glucocorticoids to their 11-keto derivative [10]. Interestingly, it has also been described that certain physiological metabolites of progesterone (PROG) produced during pregnancy inhibit the activity of the enzyme allowing glucocorticoids to acquire mineralocorticoid properties by activation of the renal MR [11].

Despite the high similarity between the GR and the MR, and the overwhelming number of studies performed with the former receptor, at the present time our knowledge about the properties of the MR is very limited. Like the GR, the MR is mainly localised in the cytoplasm in the absence of hormone and translocates into the nucleus upon steroid binding [12–14]. The untransformed MR is an oligomeric complex associated to hsp90 [15–18] and to hsp90 interacting proteins [19]. Like recombinant human MR (hMR) [20], rat kidney MR exhibits features of a phosphoprotein, and it appears to require the activity of a serine-threonine phosphatase to trans-

locate into the cell nucleus as suggested by the inhibitory effect of tautomycin on the nuclear translocation of ALDO/MR complexes in intact renal tissue [14,17,21]. This property has also been reported for the GR [5,22] under in vitro conditions with whole cells in culture.

Cysteine groups of the MR play an essential role in steroid binding as evidenced by its extreme sensitivity to a low redox potential milieu in cell-free system [18,21,23,24]. According to a recent study, Cys_{948} is directly involved in contacting the 20-keto group of the steroid as evidenced by site-directed mutagenesis of the hMR [25]. We have recently provided direct proof that a low redox potential is required in vivo for the mineralocorticoid effect by inhibition of ALDO binding to MR after depletion of tissue glutathione [26]. Thus, the depletion of the most prevalent intracellular thiol directly correlates with the abrogation of both steroid binding capacity of the MR and the mineralocorticoid response.

In a previous work [27], we described that the total binding of ALDO to MR is inhibited when amino groups are modified with alkylating agents. Interestingly, the preincubation of MR with ALDO protected the receptor from such inhibition. Nevertheless, the synthetic 21-deoxypregnane agonist, 11,19-oxidoprogesterone (11-OP), was incapable of protecting the MR from such inactivation. In view of these new and challenging findings, in this work we aimed to dissect the mechanism of inactivation of steroid binding by using selective reagents for lysyl residues under specific reaction conditions, and we studied the putative role of an essential lysine on the steroid binding capacity of native renal MR to discriminate binding between antagonists, natural agonists, and the synthetic agonist 11-OP.

2. Experimental procedures

2.1. Materials

[1,2-^{3}H]ALDO, [1,2,6,7-^{3}H]corticosterone (CORT), 11-[4-^{14}C]deoxycorticosterone (DOC), [^{32}P]orthophosphoric acid, and the ^{125}I-conjugated counterantibodies were purchased from NEN Life Science Products (Boston, MA, USA). RU486 (17β-hydroxy-11β,4-dimethyl-aminophenyl-17α-propynylestra-4,9-diene-3-one) was a kind gift from Rousell-Uclaf (Romainville, France). 11-OP and 6-OP (6,19-oxidoprogesterone) were synthesised in our laboratory as previously described [28]. Unlabelled steroids, PLP (pyridoxal 5′-phosphate) and its derivatives, TNBS (2,4,6-trinitrobenzenesulphonate), sodium borohydride, dimethyl pimelimidate, inhibitors of proteases, α-chymotrypsin from bovine pancreas, L-lysine, N^{α}-acetyl-L-lysine and N^{ϵ}-acetyl-L-lysine and goat anti-mouse IgG-horseradish peroxidase conjugate were from Sigma (St. Louis, MO, USA). [^{32}P]PLP was prepared from pyridoxamine and anhydrous [^{32}P]orthophosphoric acid as described by Cabantchik et al. [29], and the product purity was checked by thin layer chromatography in propanol:water:ammonia (6:1:3). The 3G3 monoclonal IgM antibody against the hsp90 and the BuGR2 monoclonal IgG antibody against the GR were purchased from Affinity Bioreagents (Golden, CO, USA). AC88 monoclonal IgG against hsp90 was from StressGen (Victoria, BC, Canada). The rabbit polyclonal antibody generated against the hMR was described previously [12]. Donkey anti-rabbit IgG-horseradish peroxidase was from Pierce (Rockford, IL, USA).

2.2. Source of MR

Male Sprague–Dawley rats weighing 200 g underwent adrenalectomy 48 h before the experiments. Animals were maintained with fresh water and 0.9% NaCl solution ad libitum. Rats were sacrificed by decapitation and perfused through the aortic artery with more than 100 ml of ice-cold saline solution until kidneys were blanched. Additional perfusion of kidneys was performed through the renal artery until the medulla was completely blanched. Kidney papillae were dissected and minced to fine pieces with a scalpel blade. The pieces were either homogenised as described below to obtain 'kidney cytosol', or transferred to culture tubes containing 0.2% collagenase and 0.2% hyaluronidase in Krebs buffer. Collecting duct cells were isolated as described by Husted et al. [30] and cultured in a medium based on a 1:1 mixture of DMEM and Ham's F-12 containing 10% fetal calf serum, 50 μg/ml gentamicin, 100 μg/ml ampicillin, and 5 μg/ml amphotericin B. The medium was replaced daily, and the day before the experi-

ment the regular fetal calf serum was replaced with charcoal-stripped fetal calf serum.

2.3. Preparation of cytosol and immunoprecipitation of MR

Kidney cytosol or collecting duct cell cytosol was obtained from minced papillae or primary cultures of duct cells as follows: either renal papillae or duct cells were homogenised with 1 vol. of PEGM buffer (25 mM phosphate, 10 mM EDTA, 10% glycerol, 20 mM sodium molybdate, 1 mM PMSF, 20 μg/ml aprotinin, 30 μg/ml trypsin–chymotrypsin inhibitor, 20 μg/ml leupeptin, and 20 μg/ml pepstatin, at pH 7.4). The homogenates were centrifuged at $67\,000 \times g$ for 30 min at 0°C and precleared of GR by immunoadsorption with BuGR2 antibody precoupled to protein A-Sepharose as previously described [26]. Then, the MR was partially purified by adsorption to hydroxylapatite gel, eluted and concentrated by ultrafiltration as described in detail [26,31,32]. The resulting preparation is referred to as 'cytosol' and was immediately used for the assays. MR was immunopurified from this cytosol with the anti-MR antibody precoupled to protein A-Sepharose (or non-immune rabbit serum), washed three times with ice-cold PEGM buffer containing 0.02% Nonidet P-40 and twice with the incubation buffer required for each particular experiment.

2.4. Bioassays

The urinary excretion rates for Na^+ and K^+ were measured in adrenalectomised male rats injected with the indicated doses of steroid as described [27,31]. Data were analysed by one-way non-parametric ANOVA followed by Kruskal–Wallis test.

2.5. Binding assays

The standard final volume used for binding studies was 200 μl. Binding assays were performed with 20 nM [^{3}H]ALDO at 0°C for 4 h in PEGM buffer supplemented with 2 mM dithiothreitol and 100 nM RU486. Bound steroid was separated from free by adding 0.25 vol. of a suspension of 4.0% charcoal/0.4% dextran when cytosol was used, or by washing the pellets with PEGM buffer when the assay was performed using immunoadsorbed MR. The non-specific binding was determined with 500-fold excess of radioinert ALDO and subtracted from the total.

2.6. MR–hsp90 covalent cross-linking.

The standard cross-linking reaction was carried out by adding 0.5 ml of 2.0 M triethanolamine at pH 8.0 to 5 ml of kidney cytosol [16,18]. The MR was cross-linked to hsp90 with 20 mM dimethyl pimelimidate for 30 min at 5°C. The reaction was stopped with 0.3 ml of 2 M Tris–HCl buffer at pH 8.0. After 15 min on ice, the samples were loaded onto a Sephadex G-25 minicolumn equilibrated with homogenisation buffer. The MR–hsp90 heterocomplex was eluted from the column by a short centrifugation for 60 s at 1200 rpm, pooled, and concentrated by ultrafiltration.

2.7. Sucrose gradient ultracentrifugation

We followed a standard procedure for sucrose gradient centrifugation as described previously [18]. Briefly, renal cytosol obtained in PEG buffer (PEGM buffer without molybdate) was first labelled with [^{3}H]ALDO for 4 h at 0°C (with or without an excess of unlabelled ALDO), and the free steroid was adsorbed with charcoal/dextran. The resultant [^{3}H]ALDO/MR complexes were centrifuged for 90 min at 0°C at $463\,000 \times g$ on a 5–20% sucrose gradient. The shift of the hsp90-associated receptor was achieved by preincubation of the samples with 30 μg of 3G3 antibody at 0°C for 1 h and then ultracentrifuged. Fractions of 250 μl were collected from the gradient by gravity flow and the specific binding of [^{3}H]ALDO was measured. Myoglobin (2.0 *S*), BSA (4.6 *S*), β-amylase (8.9 *S*), and catalase (11.3 *S*) were used as external markers.

2.8. Lysyl residue modification

Cell duct cytosol or kidney cytosol ($\sim$10 mg protein/ml) in PEGM buffer was incubated at 0°C with the indicated concentration of PLP or TNBS. Aliquots were taken from the reaction mixture at various intervals of time and immediately reduced with a freshly prepared solution of 10 mM sodium borohy-

dride. Once the reaction was complete, an additional 10 mM $NaBH_4$ was added to the samples and then placed on ice for 15 min. Reduced proteins were quickly separated from the excess of reagents in a minicolumn of Sephadex G-25, concentrated by ultrafiltration at 4°C in the dark, and finally incubated with [^{3}H]ALDO to determine the specific binding capacity. When alkylated MR was immunopurified, the receptor was released from the immune pellet by treatment with 1% SDS. Under these conditions, proteins were quantitatively solubilised. Sepharose pellets were discarded by centrifugation and the supernatant was used to perform a spectrophotometric analysis according to Secundo et al. [33] for PLP-treated samples, or according to Habeeb [34] for TNBS-treated pellets. Non-immune pellets were used as blanks of the reaction. We determined the empirical molar extinction coefficient for each product by using a standard curve of L-Lys pretreated with either PLP or TNBS under the same reaction conditions as samples.

2.9. Reconstitution of PLP-modified immunopurified MR

The MR from collecting cortical duct cytosol was immunopurified as described above, and stripped of associated proteins by incubation with 0.5 M KCl for an additional 2 h at 4°C. The hsp90-free immune pellet was modified with 3 mM PLP for 5 min at 0°C, reduced with $NaBH_4$, and washed five times with 1 ml of buffer. This hsp90-free modified MR was then reconstituted with rabbit reticulocyte lysate as the source of chaperones in the presence of an ATP regenerating system [27]. After washing the pellets three times with PEGM buffer, a steroid binding assay was performed using 20 nM [^{3}H]ALDO as tracer as described above. The MR and hsp90 were visualised by Western blotting.

2.10. Limited chymotrypsinisation of MR

Cytosol from cortical collecting duct cells was incubated for 4 h at 0°C with 1 μM steroid. Samples were then treated with [^{32}P]PLP as described above and, after the stabilisation of the Schiff base, the MR was immunopurified in the presence of hormone. Subsequent steps were always performed in the presence of 1 μM steroid. After washing out the supernatant, the immune pellet was resuspended in PEG buffer containing 0.5 M KCl and rotated for an additional 2 h at 4°C. The immune pellet was washed twice with PEG/0.5 M KCl buffer, and three times with phosphate-buffered saline (PBS). Chymotrypsin from bovine pancreas in PBS was then added to the pellet and incubated at 10°C for 1 h. The limited digestion of [^{32}P]PLP-labelled MR was stopped by boiling the samples in SDS-loading buffer for 3 min. The labelled proteolytic fragments were resolved by SDS–PAGE on a 14% gel and autoradiographed.

3. Results

3.1. Reaction of PLP with cytosolic renal MR

To address the question whether amino residues of the MR are essential for steroid binding, we treated partially purified renal cytosol with PLP and measured the specific hormone binding capacity in the chemically modified preparation. The phosphate group of PLP is important for the initial interaction of the inhibitor with the target protein, whereas the formyl moiety on C_4 is required for the formation of a covalent imine or Schiff base with a primary amino group. Since the initial imine bond formed by PLP with the ε-amino group of lysine is hydrolysed in aqueous solution, the primary reaction with PLP was followed by reduction to the secondary amine with $NaBH_4$. This reaction also serves to inactivate any unreacted PLP by reduction of its aldehyde group. Inasmuch as the reagent is in excess with respect to the number of reactive lysyl groups, the reaction should follow pseudo-first order kinetics according to the following equilibrium:

$$\mathrm{MR} + \mathrm{PLP} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} [\mathrm{MR_S} - \mathrm{PLP}] \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \mathrm{MR_C} - \mathrm{PLP} \qquad (1)$$

where MR_S-PLP represents the initial hydrolysable imine formed by reaction of amino groups in the receptor and the carbonyl group of PLP, MR_C is the secondary amine obtained upon reduction of the unstable intermediate Schiff base, and k are the rate constants for each hemireaction. As demonstrated later, it can be assumed that the first hemi-

reaction is extremely rapid in both directions, whereas the first order interconversion of MR_S-PLP and the covalent complex MR_C-PLP is slow. Due to the stability of this covalent complex, k_{-2} is considered to be negligible with respect to the other rate constants. Therefore, an overall apparent rate constant of inhibition, K_i, should be proportional to the $(k_1 \times k_2)/k_{-1}$ ratio.

Fig. 1A depicts the time courses of inhibition of [^{3}H]ALDO binding after pretreatment of cytosol at 0°C with PLP in the concentration range of 0.5–20 mM for various periods of time. In order to determine if any inactivation of the MR can be ascribed to the conditions of the reaction rather than the reactivity with PLP, controls were treated without the addition of PLP (0 mM). As can be seen, the inhibition of steroid binding was time- and PLP concentration-dependent. The reaction followed a pseudo-first order kinetics during the first 6 min of incubation, and the maximal inhibitory effect was reached at 10 mM PLP. This is more clearly seen in the graph depicted in Fig. 1B where the initial rate of inactivation of MR (a condition where the back reaction is not significant) was plotted against the concentration of inhibitor. The function shows a plateau at concentrations of inhibitor higher than 10 mM. This saturating effect is consistent with the properties of an affinity label and suggests that PLP inhibition of the MR binding capacity probably occurs through binding to specific sites.

The inset in Fig. 1B depicts a double-reciprocal plot, from which a k_2 value equal to 0.044 ± 0.001 min^{-1} mM^{-1} was calculated from the slope. Because the double-reciprocal plot is linearly related to the concentration of reagent, no reversible complex should be formed prior to the inactivation process, therefore the k_{-2} value should be indeed negligible as compared to k_2, k_{-1}, and k_1.

To test the specificity of reaction with PLP, chemically related derivatives of PLP such as pyridoxine, pyridoxal, pyridoxamine, and pyridoxamine 5′-phosphate were assayed at a concentration of 10 mM, and no significant inhibitory effect on the MR binding capacity was observed (data not shown). In order to confirm that the inhibitory effect of PLP occurs via reversible imine bond formation, we attempted to reverse the reaction. Therefore, renal cytosol was first incubated with 3 mM PLP. The reaction mixture was

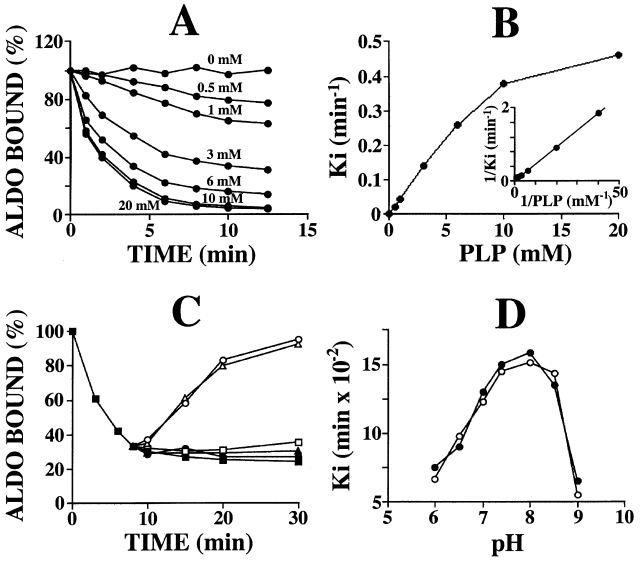

Fig. 1. Kinetics of PLP inhibition of renal MR. (A) Inhibition of ALDO binding to MR by PLP is concentration- and time-dependent. Renal cytosol in PEGM buffer was preincubated at 0°C with PLP at a final concentration ranging from 0.5 mM to 20 mM. At the indicated times, aliquots of 0.5 ml were removed and reduced by adding 8.3 μl of 0.6 M $NaBH_4$. When the reaction was exhausted, the treatment with $NaBH_4$ was repeated. The excess of reagent was cleared by a quick centrifugation over a Sephadex G-25 minicolumn. Controls were treated as the samples but without the addition of PLP (0 mM). The medium was supplemented with 2 mM dithiothreitol, 100 nM RU28362, and 20 nM [^{3}H]ALDO (±500-fold radioinert ALDO) then incubated at 0°C for 4 h. Bound steroid was separated from free with charcoal/dextran, and the remaining specific binding was plotted against the preincubation time with PLP. (B) The inactivation of the MR by PLP follows saturable pseudo-first order kinetics. The apparent rate constants of inhibition, K_i, were calculated from the graphics shown in A and plotted as a function of the PLP concentration. The insert depicts a double-reciprocal plot. (C) Inactivation of MR by treatment with PLP is reversible. Kidney cytosol was incubated with 3 mM PLP for 8 min at 0°C. The samples were cleared of excess reagent by a quick centrifugation over Sephadex G-25 minicolumns. The eluates were divided into two equal fractions. One half was first reduced with $NaBH_4$ and then treated with 60 mM of primary amines (white symbols) and the other half was first treated with the amines and then reduced (black symbols). Aliquots were removed at different times, cleared by gel filtration, and the [^{3}H]ALDO binding capacity was measured. Symbols represent the following treatments: (■) no treatment; (○) reversion by L-Lys, (△) N^{α}-acetyl-Lys or (□) N^{ε}-acetyl-Lys followed by reduction with $NaBH_4$; reduction with $NaBH_4$ followed by incubation with (●) L-lysine or (▲) N^{α}-acetyl-Lys. (D) PLP inhibition of native MR is pH-dependent. Kidney cytosol was treated with 3 mM PLP (as shown in A) in buffer adjusted to the indicated pH. Binding of [^{3}H]ALDO (●) or [^{3}H]CORT (○) was measured and the K_i was then plotted against the pH of the reaction.

immediately loaded onto a Sephadex G-25 minicolumn and centrifuged quickly to remove the excess of reagent. Samples were then split in half, one half was first reduced with $NaBH_4$ and then treated with a 60 mM excess of primary amines, and the other half was treated first with the amines and then reduced. Aliquots were removed from the reaction at different times, cleared by a quick gel filtration, and steroid binding was assayed. Results are shown in Fig. 1C.

The addition of the primary amines L-lysine and N^{α}-acetyl-lysine prior to reduction with $NaBH_4$ resulted in reversal of the inhibition, whereas N^{ε}-acetyl-lysine was incapable of reversing the reaction. On the other hand, the addition of amines after reduction with $NaBH_4$ failed to reverse the inhibitory effect of PLP. Therefore, it is likely that the inhibition of the steroid binding capacity is a consequence of reversible imine bond formation on the MR. Inasmuch as the reactivities of L-Lys and N^{α}-acetyl-Lys are similar, it is also likely that under these experimental conditions the ε-amino group of lysine is the reactive species rather than α-amino groups. This is more clearly inferred from the inability of N^{ε}-acetyl-lysine to reverse the reaction. A k_{-1} value equal to 0.21 ± 0.03 min^{-1} mM^{-1} was measured for the reversion reaction shown in Fig. 1C, therefore a similar k_1 value (0.20 min^{-1} mM^{-1}) could be calculated.

Since the reaction of PLP proceeds by nucleophilic attack of the carbonyl group of its aldehyde moiety on amino groups to yield a Schiff base [35], an increase of the K_i at higher pH values due to the deprotonation of the ε-amino group is expected. Fig. 1D, however, shows an unexpected function for this relationship. In effect, the K_i value increased as a function of the pH, but it reached a plateau between pH 7.4 and 8.5 and is dramatically decreased at pH 9.0. This pH is close to the pK_a value of ε-amino groups and should be expected to exhibit maximum deprotonation and reactivity with PLP. It should be noted that not only the inhibition rate constant, but also the maximum ALDO binding capacity of the MR dramatically decreased at pH 9.0. When [^{3}H]CORT was used as ligand, similar results to those observed with ALDO were also obtained, demonstrating the independence of this reaction with the agonist.

3.2. Stabilisation of the MR–hsp90 association by cross-linking

It is known that the multimeric structure of a steroid receptor in tight association with hsp90 is a sine qua non requirement for steroid binding. This is more evident for MR, GR and progesterone receptor than for oestrogen receptor, although it has been clearly demonstrated that hsp90 is also essential for optimal oestradiol binding to the oestrogen receptor [36]. The physiological signal for the dissociation of hsp90 from the MR is the binding of steroid, but transformation can be artificially generated in vitro in the absence of hormone, e.g. high ionic strength or at extreme pH [2]. Because we utilised buffers at high alkaline pH, the latter can be the case to explain why the K_i decreases at a pH >8.0. Therefore, we cross-linked the MR to the endogenous hsp90-based chaperone system to stabilise its oligomeric structure. The hydrodynamic profile of cross-linked products was analysed by ultracentrifugation over a sucrose gradient.

Fig. 2A shows the sedimentation profiles of [^{3}H]-ALDO–MR native complexes at various pH values. The native ALDO/MR complex shows a major peak at 9.3 *S* (untransformed receptor) and a minor peak at 5.0 *S* (transformed receptor) at pH 7.4. Alkalinisation of the medium increased the transformation of the MR, so a lower 9.3 *S*/5.0 *S* ratio was consistently obtained at pH 8.5 and pH 9.5. This observation justifies both the decrease of the total steroid binding capacity of the MR and the decreased inhibition rate constant obtained when the maximum deprotonation of the ε-amino group occurs. We confirmed that hsp90 is present in the 9.3 *S*, untransformed form, by shifting the 9.3 *S* peak to 11.4 *S* by preincubating samples with the anti-hsp90 3G3 antibody. On the other hand, the 5.0 *S* peak underwent no shift under the same experimental conditions, indicating that the hsp90 complex is dissociated from MR in the 5.3 *S* form.

Fig. 2B depicts the sedimentation profiles for cross-linked MR. When the pH was 9.5, the MR was recovered in the untransformed form. This fully conserved form of MR was also obtained in a high ionic strength buffer containing 0.5 M KCl, a condition which transforms the native receptor. When cross-linked MR was incubated with the anti-hsp90

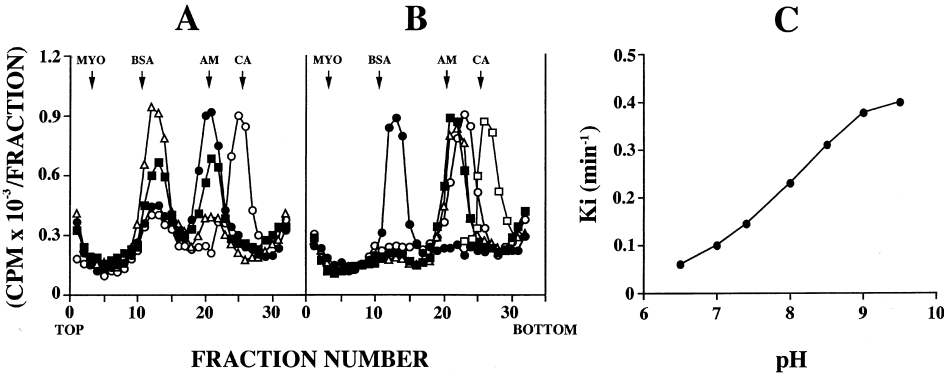

Fig. 2. Stabilisation of the MR oligomeric complex. (A) Hydrodynamic profiles of native MR. Kidney cytosol in PEG buffer was labelled with [^{3}H]ALDO for 4 h at 0°C. After adsorbing free steroid, the [^{3}H]steroid/MR complexes were resolved on a 5–20% sucrose gradient. Fractions were collected by gravity flow and the radioactivity counted. Conditions are: ●, native MR at pH 7.4; ○, native MR incubated with 3G3 anti-hsp90 antibody before centrifugation; ■, native MR at pH 8.5; △, native MR at pH 9.5. (B) Hydrodynamic profiles of cross-linked MR. Dimethyl pimelimidate cross-linked MR was labelled with [^{3}H]ALDO as described for A and centrifuged on a sucrose gradient. For comparative purposes, the elution profile of native MR disassembled by treatment with high ionic strength buffer is also shown. Conditions are: ■, cross-linked MR at pH 7.4; □, cross-linked MR preincubated with 3G3 anti-hsp90 antibody before centrifugation; ○, cross-linked MR at pH 9.5; △, cross-linked MR in buffer containing 0.5 M KCl; ●, native MR in buffer containing 0.5 M KCl. (C) pH dependence for PLP inhibition of cross-linked MR. Cross-linked MR from kidney cytosol was treated with 3 mM PLP at several pH values. The K_i was plotted against the pH of the reaction.

antibody, a shift to the 11.4 *S* form was also obtained. These results demonstrate that hsp90 was covalently and stably associated to the MR regardless of the pH or ionic strength of the medium.

We next confirmed the inhibitory effect of PLP on steroid binding using cross-linked MR. The inactivation curves by PLP were indistinguishable from those obtained in Fig. 1 with native MR. Fig. 2C depicts the variation of the K_i with the pH for cross-linked MR. The pH dependence for the inactivation of MR correlates well with a theoretical curve calculated from the equation:

$$K_i = \frac{k_{max}}{1 + ([H^+]/K_a)} \quad (2)$$

As can be seen, the inactivation rate increases with the pH and reaches a plateau at pH ≥9.0. This is consistent with optimal reactivity of deprotonated ε-amino groups at a pH that approaches the pK_a of a lysine ε-amino group. In effect, from the intercept and slope of a plot of $1/K_i$ vs. [H^+], we calculated a k_{max} of 0.51 min^{-1} (the pH-independent value) and a pK_a of 9.4, a value that is expected for the ε-amino of a lysine group. We therefore conclude that lysine ε-amino groups are modified by PLP. In addition, since we worked with cross-linked MR, the results argue against the possibility that the receptor inactivation by PLP observed in Fig. 1 could have been generated by the dissociation of hsp90.

3.3. Aminoalkylation of the MR by TNBS

In order to confirm that the previously described inactivation of MR from kidney cytosol is due to modification of ε-amino groups, we used TNBS, another specific aminoalkylating agent [34], to modify cross-linked MR in collecting duct cell cytosol. As

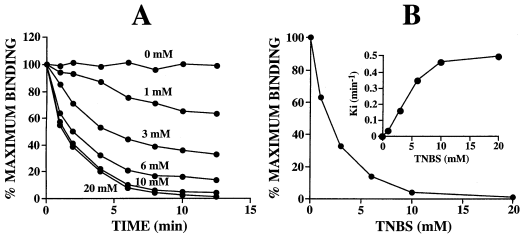

Fig. 3. Kinetics of TNBS inhibition on cross-linked MR from collecting duct cell cytosol. (A) TNBS-dependent inhibition of steroid binding. Dimethyl pimelimidate cross-linked cytosol from duct cells was preincubated for the indicated times at 0°C with TNBS. The [^{3}H]ALDO binding capacity was measured after reduction of the alkylated complex with $NaBH_4$. (B) Pseudo-first order kinetics of TNBS-dependent inhibition. The residual binding was plotted against the TNBS concentration. The inset shows the variation of K_i as a function of the concentration of the reagent.

shown in Fig. 3A, the treatment with TNBS also abolished ALDO binding in a time- and concentration-dependent fashion. Moreover, the extent of inhibition with TNBS is equivalent to that obtained in Fig. 1A for equivalent concentrations of PLP. Fig. 3B depicts the TNBS concentration-dependent curve. Consistent with a pseudo-first order reaction (Fig. 3B, inset), a value equal to 0.051 min^{-1} mM^{-1} was calculated for k_2. Therefore, this rate constant of inactivation is similar to the k_2 measured for treatments with PLP (Fig. 1B).

3.4. Modification of amino groups of the MR does not affect the K_d for ALDO

To determine whether or not the modification of MR lysyl groups affects the total number of binding sites and/or the affinity of MR for the steroid, we treated native MR from duct cell cytosol with either 3 mM PLP or 3 mM TNBS, and after stabilising the aminoalkylated complex, we performed Scatchard plots using [^{3}H]ALDO, [^{3}H]CORT or [^{14}C]DOC as tracers. All the steroids showed identical results, so only the plots obtained with [^{3}H]ALDO are depicted in Fig. 4A. As can be seen, the K_d for untreated MR (0.64 ± 0.12 nM) was not different from the K_d measured for PLP-treated MR (0.53 ± 0.04 nM) or TNBS-treated MR (0.50 ± 0.03 nM). However, the concentration of binding sites was about one-third lower for modified receptor than for untreated MR.

It can be entirely possible that the alkylation of any amino group in the chaperone protein can account for the loss of steroid binding capacity rather than a modification on the receptor itself. To study this possibility, we first treated duct cell cytosol with PLP followed by reduction with $NaBH_4$. Controls were treated in a similar form but without the addition of PLP. Then, the modified MR was immunoadsorbed and the hsp90-based chaperone heterocomplex was stripped off the receptor with 0.5 M KCl. The oligomeric structure of the MR with hsp90 was finally reassembled by reconstitution with rabbit reticulocyte lysate and an ATP regenerating system. The binding of [^{3}H]ALDO was measured as a quantitative parameter for the refolding reaction (Fig. 4B, bar graphs), and the association of hsp90 to the MR was visualised by Western blot analysis (shown below the bar graph).

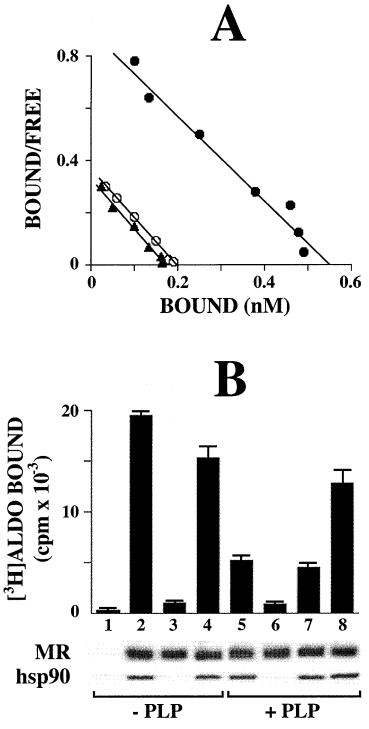

Fig. 4. Aminoalkylation of MR does not affect the K_d for ALDO nor the dynamic association with hsp90. (A) Scatchard analysis for PLP- and TNBS-treated MR. Cross-linked MR from duct cell cytosol was treated with 3 mM PLP (○) or 3 mM TNBS (▲), or underwent the same treatment as the reaction with the alkylating compound, but without adding reagent to the medium (●). A Scatchard plot was performed using [^{3}H]ALDO as tracer. (B) Association of hsp90 to the MR. Native duct cell cytosol treated (+PLP) or not (−PLP) with PLP was immunoadsorbed and stripped by high ionic strength where indicated. The [^{3}H]ALDO binding capacity (bar graph) and the association with hsp90 (Western blots shown below each bar) were evaluated before and after the treatment with PLP. Conditions are: (1) non-immune pellet; (2) immune pellet; (3) immune pellet stripped with 0.5 M KCl; (4) the stripped immune pellet reconstituted with rabbit reticulocyte lysate; (5) cytosol was treated with 3 mM PLP for 6 min at 0°C and, following reductive stabilisation of the product, the modified MR was immunoprecipitated; (6) the immune pellet of PLP-modified MR was stripped with 0.5 M KCl; (7) hsp90-free unmodified MR immune pellet was incubated with 3 mM PLP for 6 min at 0°C and, after reductive stabilisation of the product, the heterocomplex was reconstituted with rabbit reticulocyte lysate; (8) hsp90-free unmodified MR immune pellet was first incubated with 3 mM PLP for 6 min at 0°C, and then incubated with 60 mM L-Lys for 30 min before reduction with $NaBH_4$ followed by reconstitution with rabbit reticulocyte lysate.

The association of hsp90 with unmodified MR was lost, and also the steroid binding, by incubating the co-immunoprecipitated MR–hsp90 complex with 0.5 M KCl (condition 3 vs. condition 2). Reconstituting the oligomeric complex restored 80% of the ALDO binding capacity of MR (condition 4). On the other hand, when the MR was treated with PLP and immunoprecipitated, the ALDO binding capacity was only 30% (condition 5) of that measured in the unmodified MR (condition 2). It is important to emphasise that hsp90 was recovered in this immune pellet of PLP-treated MR. The association of hsp90 with MR was lost by treatment with high ionic strength and, consequently, the steroid binding capacity was fully abolished (condition 6). Condition 7 represents immunoprecipitated native receptor that was stripped of associated proteins followed by treatment with PLP. After alkylation of the hsp90-free MR, its oligomeric complex was reconstituted by incubation with reticulocyte lysate. The binding capacity for ALDO was restored to 80% of the level of PLP-modified MR shown in condition 5. Importantly, this result and the Western blot analysis clearly demonstrate that the alkylated MR fully preserved its capability to reassociate with hsp90 after alkylation, which is a direct evidence that the PLP-modified MR is not effected in its dynamic equilibrium with the proteins of the hsp90 heterocomplex.

Finally, when the unstable Schiff base generated by reaction of PLP with the stripped MR was reversed by incubation with an excess of L-lysine prior to the reaction with $NaBH_4$ (condition 8), full reconstitution of the MR–hsp90 heterocomplex was achieved (compare with condition 4), indicating that the chemical treatment did not affect per se the capability of the MR to interact with hsp90.

3.5. *Determination of the number of amino groups involved in the inactivation of the MR by alkylating agents*

The stoichiometry for MR alkylation was determined for PLP-treated or TNBS-treated MR immunoprecipitated from duct cell cytosol. The immune pellet was divided into two fractions. After stripping off the hsp90 complex from one of the fractions, the MR associated to the Sepharose pellet was extracted and the number of reactive lysines was quantified by spectrophotometry. The other fraction was used to measure the [^{3}H]ALDO binding capacity. Inasmuch as one molecule of ALDO binds to one molecule of MR, we therefore calculated the number of reactive lysines per molecule of receptor. The results are shown in Fig. 5A.

The plots obtained with both reagents are similar and do not follow a linear function. This indicates that not all of the amino residues are modified at the same rate which, in turn, can be determined by the accessibility of such residues to the reagent. The extrapolation of the linear phase of the plot to zero binding capacity shows an average stoichiometry equal to 1.15 ± 0.18 lysine per molecule of MR. In other words, it appears that modification of only one residue accounts for 70% of the total inhibition of ALDO binding capacity to MR.

In order to determine whether the presence of ligand can prevent the modification of this Lys group,

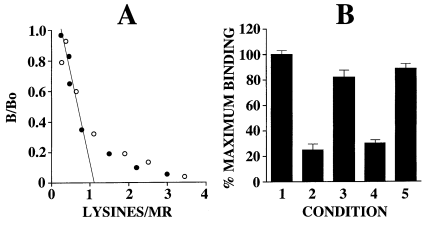

Fig. 5. Aminoalkylation by PLP or TNBS of a sole lysine residue is impeded by occupancy of the ligand binding pocket of the MR. (A) Number of Lys modified per molecule of receptor. Native MR immunopellet from duct cell cytosol was divided into two fractions. One fraction was used to measure the [^{3}H]ALDO binding capacity to determine the number of MR molecules in the immune pellet. The other fraction was treated with several concentrations of either PLP (○) or TNBS (●), and the number of reactive lysines was determined by spectrophotometry of the products as described in Section 2. The plot depicts the fraction of remaining bound steroid (Bo) with respect to untreated MR (B) versus the number of modified Lys per molecule of MR. (B) Protective effect of ALDO on inactivation by aminoalkylation. Immunopurified cross-linked MR from duct cell cytosol was incubated for 4 h at 0°C with 20 nM ALDO followed by alkylation with 3 mM PLP or 3 mM TNBS for 6 min at 0°C. After stabilising the product by reduction with $NaBH_4$, the bound steroid was dissociated by an incubation at 37°C for 1 h. The released ALDO was washed out and the immune pellet was reincubated with 20 nM [^{3}H]ALDO to measure the steroid binding capacity. Conditions are: (1) immune pellet of untreated MR; (2) immune pellet of PLP-treated MR; (3) immune pellet of PLP-treated MR preincubated with ALDO; (4) immune pellet of TNBS-treated MR; (5) immune pellet of TNBS-treated MR preincubated with ALDO.

we preincubated cross-linked MR with an excess of ALDO and then carried out the reaction with either PLP or TNBS. Fig. 5B clearly shows that the presence of ALDO in the steroid binding pocket prevents the inactivation by both alkylating reagents (bars 3 and 5) as compared to the respective control incubations performed with unliganded MR (bars 2 and 4). Although this ligand protection is not conclusive, it suggests that the amino group involved in the reaction with PLP or TNBS might lie in or near the steroid binding pocket of the MR. On the other hand, it may well reflect a differential protrusion of a specific reactive amino group upon steroid binding.

3.6. Mineralocorticoid features of synthetic and natural ligands

It is classically accepted that C_{21}-hydroxylation enhances the mineralocorticoid properties of steroid ligands. A typical example of this requirement is the weak activity exhibited by PROG as compared to its 21-hydroxy derivative, DOC. However, we have postulated [27,31,37] that the mineralocorticoid effect of a given steroid is mainly due to its planar conformation rather than the functional groups present in the molecule. Thus, the biological properties of the flat synthetic 21-deoxysteroid 11-OP were compared to those exhibited by natural agonists.

Fig. 6A depicts the competition curves between unlabelled steroid and [^{3}H]ALDO. The relative affinity obtained for each steroid is (EC_{50} values in nM are given in parentheses): ALDO (2.3 ± 0.3) = DOC (2.8 ± 0.6) = CORT (3.1 ± 0.6) > SC9420-spironolactone (SPO) (9.1 ± 3.5) = PROG (15.1 ± 7) > 11-OP (43.2 ± 3.0) ≫ 6-OP and testosterone (T = O) (no significant binding). Fig. 6B depicts the dose–response curves for the steroids as urinary Na^+/K^+ ratio. Due to the combined protective action of several factors such as the activity of 11β-hydroxysteroid dehydrogenase [10], binding to transcortin and other plasma proteins [38], and/or the putative selectivity of the MR itself [39], the in vivo biological effect of CORT is impaired in spite of its high affinity for MR in the absence of those regulatory factors, as it is well known. Interestingly, the affinity of 11-OP is about 20-fold lower than the affinity of DOC for the MR, but the in vivo effect of 11-OP is as potent as that exhibited by DOC at low doses and even approaches that of ALDO at doses ≥10 μg. These observations confirm our previous reports [27,31]. On the other hand, the antagonist steroids PROG and SPO, and the inactive steroids 6-OP and testosterone

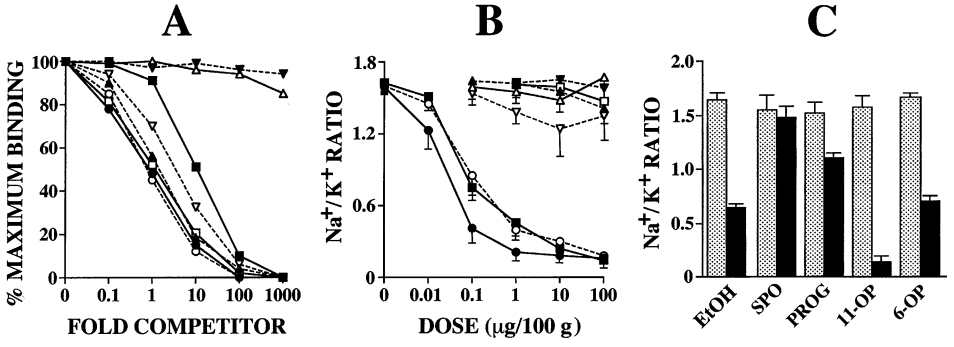

Fig. 6. Mineralocorticoid properties of the ligands. (A) Steroid competition curves for binding to MR. Kidney cytosol in PEGM buffer containing 2 mM dithiothreitol and 1 μM RU486 was incubated with 5 nM [^{3}H]ALDO (± 10 μM non-radioactive ALDO) and increasing concentrations of the indicated steroids. After 12 h at 0°C, bound steroid was separated from free with charcoal/dextran, and the specifically bound radioactivity was plotted against the relative concentration of competitor. Competitor ligands are: ●, ALDO: ○, DOC; □, SPO; ▲, CORT; ▽, PROG; ■, 11-OP; △, 6-OP; ▼, T = O. (B) Mineralocorticoid effect. Steroids were injected into adrenalectomised male rats and the natriuresis decrease and kaliuretic increase were measured in urine. Values of Na^+/K^+ ratio represent the mean ± S.E.M. of six animals per dose. (C) 11-OP potentiates ALDO mineralocorticoid effect. The mineralocorticoid biological effect of a suboptimal dose of 0.06 μg ALDO (black bars) was measured by co-injecting either vehicle (EtOH), 100 μg SPO, 100 μg PROG, 0.6 ng 11-OP, or 100 ng 6-OP. Dotted bars represent the Na^+/K^+ ratio for control values obtained by injecting the steroid (or EtOH) without co-injecting ALDO. Data are the mean ± S.E.M. of eight animals per group. The following co-treatments with ALDO are statistically different from the control co-injected with EtOH: SPO at $P < 0.003$, PROG at $P < 0.010$, and 11-OP at $P < 0.001$.

are very weak mineralocorticoids, if they are not totally inactive. It is interesting to emphasise that the maximum activity of 11-OP in transcription assays is as efficient as that measured for ALDO, CORT, cortisol and DOC (data not shown).

The bar graph in Fig. 6C shows that ~40% of the maximum mineralocorticoid effect can be measured using a suboptimal dose of 0.06 μg of ALDO. As expected, this effect was totally abolished by co-treatment with SPO and partially inhibited by co-treatment with PROG. Importantly, as low a dose of 11-OP as 0.6 ng, which is per se ineffective to promote any biological effect, potentiated the effect of 0.06 μg of ALDO, so that the maximal mineralocorticoid effect was reached. On the other hand, the inactive stereoisomer 6-OP does not affect the ALDO-dependent response.

These results can be due to several reasons, but more likely because 11-OP binds to a putative second binding site on the MR different from the ALDO binding site, or because the binding of 11-OP induces a different conformational change on the MR with respect to the conformation adopted by the MR upon ALDO binding.

3.7. *The ligand in the steroid binding pocket protects the MR from the inhibitory effect of PLP*

In the experiment shown in Fig. 5B we demonstrated that preincubation of MR with ALDO prevents the inactivating effect of PLP. Therefore, we addressed the question whether this protective action of the steroid is a common feature for all MR ligands. Thus, we first treated duct cell cytosol with the cross-linking agent dimethyl pimelimidate, and then isolated the MR oligomeric complex by immunoprecipitation. The immune pellet was incubated with steroid for 4 h at 0°C in PEGM buffer at pH 7.4, and the free ligand was washed out. This ligand-occupied MR pellet was treated with 3 mM PLP for 6 min. After the reductive stabilisation of the Schiff base, the samples were incubated at 37°C for 1 h to dissociate bound steroid. Consistent with a previous work [27], control experiments demonstrated that there is no change in the steroid binding capacity of cross-linked MR after this incubation. Therefore, after washing the immune pellets, a reincubation with [^{3}H]ALDO for 4 h at 0°C was performed and the specific [^{3}H]ALDO binding capacity was measured.

Fig. 7A shows that the steroid binding capacity of cross-linked MR preincubated with vehicle (EtOH) was inhibited by further alkylation with PLP as compared to untreated control. The protective effect of the ligand on the PLP-dependent inactivation of cross-linked MR could be evidenced not only for ALDO, but also for DOC and CORT. On the other hand, when MR was prebound to the antagonists PROG and SPO, as well as the inactive ligands T=O and 6-OP, no protection was evidenced upon treatment with PLP followed by rebinding of [^{3}H]-ALDO. Interestingly, the synthetic agonist 11-OP was also incapable of protecting the MR, demonstrating that the binding of 11-OP to the MR exhibits different properties with respect to natural agonists. It should be noted that identical results (not shown) were also obtained with TNBS-treated MR.

Fig. 7B depicts the MR inactivation rate upon treatment with PLP at different pH values. As shown in Fig. 2C for kidney cytosol, the maximal inactiva-

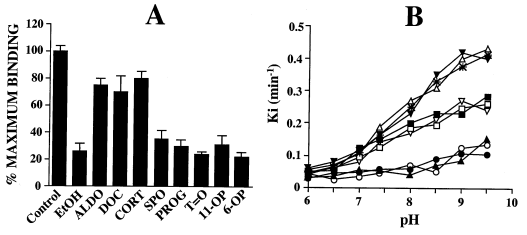

Fig. 7. Differential steroid binding to the MR is evidenced by receptor alkylation. (A) Protective effect of ligands. Immunopurified cross-linked MR was preincubated with steroid and then treated with PLP exactly as described for Fig. 5B. Control represents immunopellets not treated with PLP. EtOH represents preincubation with vehicle instead of steroid. Results are the mean ± S.E.M. of three experiments each performed in duplicate. (B) pH dependence of PLP inhibition on immunopurified cross-linked MR protected by ligands. Cross-linked MR was immunoadsorbed from duct cell cytosol and the indicated steroids were bound to the receptor. Hence, a reaction of the steroid/receptor complexes with PLP was carried out at various pH values for short incubation times (as described for Fig. 1A and 3A) followed by reduction with $NaBH_4$. Steroids were dissociated from the MR by incubating the samples at 37°C for 1 h. After washing the immune pellets with buffer, the [^{3}H]-ALDO binding capacity was measured. From the slopes of binding vs. time plots, K_i was calculated and then plotted versus the pH of the reaction. The MR was bound to: *, unliganded MR; ▼, T=O; △, 6-OP; ■, 11-OP; □, SPO; ▽, PROG; ●, ALDO; ○ DOC; ▲, CORT.

tion of unliganded receptor is reached at pH ≥9.0, suggesting the blockade of an ε-amino group. Similarly, steroids that do not exhibit binding properties to the MR such as testosterone and 6-OP were incapable of affecting the inactivation rate of the MR which is indistinguishable from the control. In contrast, the physiological agonists ALDO, DOC and CORT protected the MR from inactivation in the full range of pH values. Consistent with the notion that the binding of 11-OP to the MR is not equivalent to the binding of natural agonists, the synthetic agonist 11-OP shows a similar function as the inactive steroids at pH ≤8.0, but a partial protective action at higher pH values. Such an effect is clearly different from the observations obtained with both natural agonists and inactive steroids. Interestingly, a similar effect as that of 11-OP can be described for SPO and PROG, antagonistic steroids whose rate constants of inhibition are not distinguishable from the K_i obtained with 11-OP in the complete range of pH values assayed.

Taken together, these results demonstrate that the binding of natural agonists to the MR is comparable to neither the binding observed for 11-OP nor the binding of the antagonists SPO and PROG. Remarkably, 11-OP is not an antagonist, but an agonist that not only possesses mineralocorticoid effects per se but is also able to potentiate the ALDO biological effect.

3.8. Analysis of the MR conformation by limited proteolysis

In view of the peculiar results obtained in Fig. 7B for 11-OP and the antagonistic ligands, we addressed the hypothesis that all these steroids can induce a differential conformational change in the MR as compared to natural agonists. Therefore, we incubated the MR from cortical collecting duct cytosol with steroid and then modified the receptor with [^{32}P]PLP. The labelled modified receptor was concentrated by immunoprecipitation and digested by limited proteolysis with α-chymotrypsin for 1 h at 10°C. Fig. 8 shows that the treatment of unliganded MR with [^{32}P]PLP followed by proteolysis with 10 U/ml of α-chymotrypsin yielded several proteolytic fragments ranging from the size of full-length MR (~110 kDa) and 95 kDa. In addition, two smaller fragments of 67 kDa and 34 kDa were also generated during the limited proteolysis. This pattern was not affected by binding of steroid.

When the concentration of α-chymotrypsin was increased 4-fold, full degradation of unliganded MR was observed. However, the ALDO-bound MR complex still shows the 34 kDa band as a predominant intermediate proteolytic product. Similar results were also obtained with the agonist CORT. On the other hand, the antagonist PROG was unable to protect against the degradation of both the full-length MR and its fragments. These observations clearly indicate that following agonist binding only the 34 kDa fragment becomes resistant to α-chymotrypsin. This was assigned to a particular conformation acquired by MR according to the nature of the bound hormone.

Interestingly, the synthetic agonist 11-OP cannot be grouped with any of those two classes of ligands. In effect, the MR that is bound to 11-OP, showed intermediate pattern degradation upon digestion with α-chymotrypsin. Thus, ~40–50% of the 34 kDa fragment is still intact as compared to the 80% still remaining for the ALDO- or CORT-bound MR. On the other hand, the bent stereoisomer 6-OP is totally

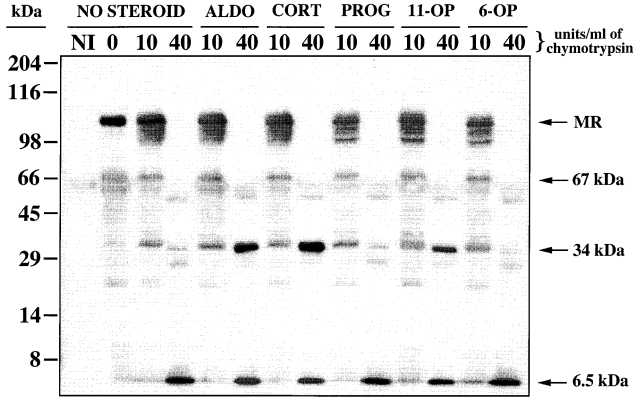

Fig. 8. Limited chymotrypsinisation of steroid/MR complexes. Cytosol from duct cells was incubated with 1 μM steroid for 4 h at 0°C. Samples were then incubated with [^{32}P]PLP (3 mM total PLP) for 6 min at 0°C and reduced with $NaBH_4$. The MR was immunoprecipitated, stripped with PEG/0.5 M KCl buffer, and resuspended in PBS containing α-chymotrypsin from bovine pancreas at the concentrations indicated above the graph. The digestion was performed for 1 h at 10°C. The reaction was stopped by boiling the samples in SDS-loading buffer. [^{32}P]PLP-labelled proteins were resolved by electrophoresis on a 14% SDS–polyacrylamide gel, and visualised by autoradiography.

ineffective in protecting the MR from degradation. We would like to emphasise that both compounds, 11-OP and 6-OP, are 21-deoxysteroids, which share equivalent functionalities, but exhibit opposite overall conformations.

4. Discussion

The rat MR possesses a total of 53 Lys residues, 18 of which are localised in the steroid binding domain [40]. This led us to postulate that some lysyl groups may be essential for the binding of steroid to the MR. In this work, we provide evidence that this may be the case due to the substantial inhibition of hormone binding to MR upon modification of amino groups with PLP and TNBS. According to the data obtained in the experiment shown in Fig. 5, a stoichiometry equal to one Lys residue per molecule of receptor accounts for about a 70% inhibition of steroid binding. This suggests a critical role for only one lysyl group in the MR among those 53 total residues. Regardless of the exact location and number of residues modified by the treatment of the MR, the importance of this observation is that such inhibition is prevented if a natural agonist (i.e. ALDO, DOC or CORT) is bound to the receptor, but it is not protected upon antagonist binding (i.e. PROG or SPO). In this sense, the synthetic agonist 11-OP remarkably resembles the properties of antagonistic compounds rather than natural agonistic steroids (Fig. 7).

Nevertheless, limited proteolysis of the liganded MR (Fig. 8) shows that the digestion products obtained depend on the nature of the ligand bound to the receptor. Thus, controlled proteolysis generated a key 34 kDa fragment that is totally resistant to degradation only when natural agonists are bound to MR. Although 11-OP is also a potent agonist its protective effect is only partial, which strengthens the notion that 11-OP binding to the MR is not equivalent to that observed for ALDO. On the other hand, digestion of PROG/MR or 6-OP/MR complexes showed a similar degradation pattern as the unliganded MR. This suggests that the induced conformational change of the MR upon PROG or 6-OP binding, if any, is not as dramatic as the structural change generated by ALDO or CORT. It should also be pointed out that, in contrast to these findings with the MR, antagonists rather than agonists induce protection of proteolytic fragments for the progesterone receptor [41], the oestrogen receptor [42] and the retinoic acid receptor [43].

Although the number of lysine groups present in the protein is high, the saturation curves depicted in Fig. 1 indicate that only a limited number of residues can be modified by the reagent. Actually, the Lys/MR stoichiometry measured in Fig. 5A demonstrates that the steroid binding capacity of MR is totally abolished when three or four lysine groups have been modified. Due to the bulky nature of the alkylating reagents used in these experiments and the conditions of the reaction, it is unlikely that these residues are buried in the receptor structure.

Although our results cannot provide conclusive evidence as to assign the localisation of the essential lysine to the ligand binding pocket of the MR, the differential protective effect obtained with natural agonists and antagonists leads us to consider that it is entirely possible that a Lys residue could be located in a topological region which is vital for conferring (directly or indirectly) an appropriate environment around the steroid binding pocket of the MR. Interestingly, only full natural agonists exert efficient protection against inactivation by alkylation. This is supported by the experiments described in Fig. 5B and 7. Importantly, the synthetic agonist 11-OP shows discrepancies with natural agonists both in the protective effect against MR alkylation and the pattern of proteolytic fragments generated by limited digestion with α-chymotrypsin.

We have previously postulated that the synthetic steroid 11-OP may exert its biological effect by binding to an alternative site on the MR [37]. Other possible biopharmacological properties that can account for 11-OP's biological effect were studied and ruled out in a previous work [27] (i.e. a longer in vivo half-life, stronger in vivo binding to renal MR, non-genomic effects, etc.). In spite of the potent biological action of 11-OP, its protective effect against inactivation by PLP at high pH is just partial and closer to that observed with PROG and SPO (Fig. 7B). Again, this supports the notion that only agonists bound to the ALDO binding pocket with an appropriate orientation of the molecule are capable of fully protecting the amino group(s) from alkylating agents. A reasonable explanation for the differential protective

effect observed with different ligands is the putative differential orientation of the ligand in the steroid binding pocket or its binding to a completely different binding site. Accordingly, a recent publication by Geller et al. [44] described cases of hypertension and pregnancy-related hypertension by a substitution of Leu_{810} by Ser in the hMR. This substitution alters receptor specificity, where PROG, SPO and other 21-deoxysteroids become mineralocorticoids. Moreover, Auzou et al. [45] have reported that a mutant of hMR in Ala_{773} also confers 11β-substituted spirolactones agonistic properties. Both mutations, Leu_{810} and Ala_{773}, are localised in the hormone binding domain of the MR.

In this work, we also present evidence that a partial agonist/antagonist like PROG and an antagonist like SPO were incapable of protecting the MR at physiological pH, and only a partially protective effect was detected at alkaline pH for both compounds. The actual mechanism of action for SPO is still controversial. It was initially thought that SPO competitively inhibits ALDO binding to the MR and prevents its translocation to the nucleus [46]. However, it was shown that SPO translocates into the nucleus associated to MR [47] and can bind to the DNA in vitro [48]. Both processes seem to be less efficient than for ALDO. Recently, a cell-dependent partial agonistic activity of SPO was also reported [49]. One possible explanation for these features may also be found in a differential positioning of SPO in the MR. Our results may be explained by a similar argument. In effect, even when SPO was present at saturating concentrations, only a minimal protective effect against the treatment with PLP could be evidenced, suggesting that the steroid can be placed in the ligand binding pocket, but has a different orientation of the steroid frame and/or functional groups. Therefore, modified lysyl groups required for ALDO binding remain exposed and, consequently, unprotected.

An interesting property is that the steroid placement in the ligand binding site of the MR is mainly due to the overall ligand planarity and/or the ligand's ability to adapt its molecule to a very narrow interacting site in the protein, so the $C_3=O$ group of the steroid A-ring can be anchored to the protein [31,37]. In contrast, the C_{11} and C_{19} groups of the steroid should be interacting with a more spacious area within the hormone binding domain as judged by the capacity of bulky ligands to bind to the MR. Therefore, one can envisage that an all or nothing event upon hormone binding should be unlikely in view of the fact that the hormone binding is an adaptive process where the whole structure of the protein changes upon steroid binding. Thus, agonists and antagonists can overlap their respective orientations in the binding site, although the ligand-dependent rearrangement of the MR conformation may be different. For example, it is possible that PROG can bind to the MR at the same site as ALDO. However, the total length of the PROG (11.31 Å for O_3–O_{21}) molecule is not enough as to properly fold the protein as compared to ALDO (12.45 Å). This inconvenience can be solved by hydroxylation of C_{21}, so that DOC (12.30 Å) acquires potent biological activity. The acquisition of mineralocorticoid biological activity by DOC may be the consequence of a more efficient interaction of the steroid with the helix H11 of rat MR by anchoring the steroid's C_{21}-hydroxyl group to the Cys_{939} (or Cys_{948} of hMR). Accordingly, similar arguments can explain the mineralocorticoid effect of CORT (12.24 Å) if the other environmental regulating factors (i.e. sequestering proteins, metabolising enzymes, etc.) would not interfere with binding to MR in epithelia and vascular smooth muscle cells. It is worth underlining that, although cortisol dissociates more rapidly from the MR than ALDO [24], it also antagonises ALDO in non-epithelial tissues.

On the other hand, 11-OP fulfils the requirement for optimal planarity and strongly conserves its conformation due to the presence of an extra ether ring, which confers an extreme rigidity to the molecule. This rigidity is also shared by the natural agonist ALDO, a steroid that also exhibits an extra stabilising ring on the β face of the molecule, although this extra ring in ALDO opens reversibly under physiological conditions, a property that is not exhibited by 11-OP. Although 11-OP is a flat steroid which does behave as a potent mineralocorticoid, the length the molecule is shorter (11.38 Å) than the average length of natural agonists. Inasmuch as the molecular frame of 11-OP is rigid and stable, it is possible that a different orientation of the ligand is required to fit in the ALDO binding site. This 'enforced' positioning of 11-OP may induce a particular conformational

change of the MR, which can be evidenced by partial proteolysis with α-chymotrypsin. On the other hand, due to the fact that 11-OP potentiates the biological effect of suboptimal doses of ALDO, it can also be speculated that the binding of this synthetic steroid to the receptor does not occur exactly in the same site where the natural agonists bind to the MR. Such a putative site can function as a stabiliser of the active conformation of the MR. This hypothesis is inferred from the experiments shown in this work as well as from previously published experiments [27] in which we showed that inactive concentrations of 11-OP increased the affinity of ALDO for the MR, whereas higher concentrations of 11-OP (capable of competing with ALDO by binding to the ligand binding site of the MR) exhibited a competitive inhibitory effect on ALDO binding. Moreover, the off-rate of ALDO is 3 times lower in the presence of concentrations of 11-OP that fail to compete with ALDO [27]. Interestingly, the 21-hydroxylated derivative of 11-OP has a similar length (12.23 Å) as the length of natural agonists, but it exhibits very weak mineralocorticoid effect and low affinity for the MR (unpublished results). This unexpected observation agrees with the notion that 11-OP (and possibly its 21-hydroxy derivative) binds to a different binding site than ALDO, DOC and CORT.

Finally, the differential binding properties of several ligands evidenced by the existence of a crucial amino group in the MR can open new perspectives on our understanding of steroid–receptor interactions and further activation of this transcription factor. The use of the sole MR synthetic agonist to date, 11-OP, can also be extremely useful to unravel the still controversial and poorly understood structural and functional regulation of the MR.

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