Comparative inhibition by hard and soft metal ions of steroid-binding capacity of renal mineralocorticoid receptor cross-linked to the 90-kDa heat-shock protein heterocomplex

Mario D. GALIGNIANA1,2 and Graciela PIWIEN-PILIPUK3
Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, and PHROM-CONICET, 1428 Buenos Aires, Argentina

INTRODUCTION

The mineralocorticoid receptor (MR) belongs to the superfamily of receptors that act as ligand-induced transcription factors [1]. Like other steroid-hormone receptors, native MR is also associated with the 90-kDa heat-shock protein (hsp90) heterocomplex [2]. This association stabilizes corticosteroid receptors in the steroid-binding form and prevents their transcriptional properties (reviewed recently in [3,4]). Some experimental conditions, such as high ionic strength, excessive dilution or alkaline pH, cause the unliganded receptor to lose its steroid-binding activity due to the dissociation of the hsp90 heterocomplex. Cross-linked heterocomplex showed no difference in ligand specificity and affinity with respect to native receptor, but increased stability upon thermal- or ionic-strength-induced destabilization was observed. Treatments in vitro with metal ions in the range $10^{-8}$–$10^{-4}$ M resulted in a differential inhibitory effect for each particular ion on aldosterone binding. Using the negative logarithm of metal concentration for 50% inhibition, the ions could be correlated with their Klopman hardness constants. The analysis of this relationship led us to postulate three types of reaction: with thiol, imidazole and carboxyl groups. The essential role played by these residues in steroid binding was confirmed by chemical modification of cysteines with dithionitrobenzoic acid, histidines with diethyl pyrocarbonate and acidic amino acids with Woodward’s reagent (N-ethyl-5-phenylisoxazolium-3-sulphonate). Importantly, the toxic effects of some metal ions were also observed by treatments in vitro of adrenalectomized rats on both steroid-binding capacity and aldosterone-dependent sodium-retaining properties. We suggest that those amino acid residues are involved in the activation process of the mineralocorticoid receptor upon steroid binding. Thus toxic effects observed with these metal ions may be a consequence of modifications of those essential groups. Our results support the notion that toxicity of metals on renal mineralocorticoid function may be predicted according to their chemical hardness.

Key words: aldosterone, dimethyl pimelimidate, essential amino acids, hsp90, sodium retention.

We analysed the inhibitory effects in vitro and in vitro of several metal ions on aldosterone binding to the rat kidney mineralocorticoid receptor with the purpose of assessing possible toxic effects of those ions on sodium retention, as well as to obtain information on receptor structural requirements for ligand binding. For the assays in vitro, the inhibitory effects of 20 metal ions were analysed on steroid-binding capacity for renal receptor cross-linked to 90-kDa heat-shock protein (hsp90) by pretreatment with dimethyl pimelimidate. Cross-linking prevented the artifactual dissociation of hsp90 (and, consequently, the loss of steroid binding) from the mineralocorticoid receptor due to the presence of high concentrations of salt in the incubation medium. Cross-linked heterocomplex showed no difference in ligand specificity and affinity with respect to native receptor, but increased stability upon thermal- or ionic-strength-induced destabilization was observed. Treatments in vitro with metal ions in the range $10^{-8}$–$10^{-4}$ M resulted in a differential inhibitory effect for each particular ion on aldosterone binding. Using the negative logarithm of metal concentration for 50% inhibition, the ions could be correlated with their Klopman hardness constants. The analysis of this relationship led us to postulate three types of reaction: with thiol, imidazole and carboxyl groups. The essential role played by these residues in steroid binding was confirmed by chemical modification of cysteines with dithionitrobenzoic acid, histidines with diethyl pyrocarbonate and acidic amino acids with Woodward’s reagent (N-ethyl-5-phenylisoxazolium-3-sulphonate). Importantly, the toxic effects of some metal ions were also observed by treatments in vitro of adrenalectomized rats on both steroid-binding capacity and aldosterone-dependent sodium-retaining properties. We suggest that those amino acid residues are involved in the activation process of the mineralocorticoid receptor upon steroid binding. Thus toxic effects observed with these metal ions may be a consequence of modifications of those essential groups. Our results support the notion that toxicity of metals on renal mineralocorticoid function may be predicted according to their chemical hardness.

Key words: aldosterone, dimethyl pimelimidate, essential amino acids, hsp90, sodium retention.

MR behaves like the glucocorticoid receptor in requiring hsp90 for the steroid-binding conformation [2,7,8].

Under physiological conditions, it is thought that the activation of steroid receptors upon steroid binding initiates a series of events that includes changes in the protein conformation, receptor transformation by dissociation of hsp90 (and associated proteins), dimerization, phosphorylation and DNA binding at the hormone-response elements. The temporal sequence of these events is unknown, and the heuristic concept that hsp90 is dissociated from the receptor immediately after steroid binding is now under revision [9].

In spite of the progress reached during the last decade in the steroid-receptor field, our knowledge about most of the members of this superfamily has advanced unquestionably faster than our understanding about MR. This receptor is sparse in target tissues, highly susceptible to proteolysis and oxidation in cell-free systems, and its hsp90 heterocomplex is easily dissociated. Therefore, it is not surprising that the MR is the only member of

Abbreviations used: MR, mineralocorticoid receptor; hMR, human MR; ALDO, aldosterone; hsp90, 90-kDa heat-shock protein; pME$_{50}$, $-\log_{10}$[metal$^{n+}$] necessary to produce 50% inhibition of steroid binding; ID$_{50}$, dose of metal ion necessary to inhibit 50% of aldosterone-dependent sodium retention; DEPC, diethyl pyrocarbonate; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); RU28362, 17-α-alkanyl-11β,17-dihydroxy-androsterone.

1 Present address: 1301 Medical Science Research Building III, Department of Pharmacology, University of Michigan Medical School, Ann Arbor, MI 48109–0632, U.S.A.
2 To whom correspondence should be addressed (e-mail mgali@umich.edu).
3 Present address: Department of Physiology, University of Michigan Medical School, Ann Arbor, MI 48109, U.S.A.
the superfamily which could not be purified from natural sources. A considerable advance in the field was achieved when the primary amino acid sequences of human MR (hMR) [10] and rat MR [11] were elucidated.

Only a few studies have focused on the molecular mechanism of MR transformation and targeting. One of the most important advances in this field was the demonstration that recombinant hMR overexpressed in insect Sf9 cells undergoes phosphorylation [12]. This property was also demonstrated for native MR [2], and a possible role for serine/threonine phosphatases in MR targeting upon steroid stimulation has also been suggested [2,13].

Similarly, only a few studies have been performed on the structural features of MR. It was demonstrated that rat kidney MR [14] and hMR [15] possess high dependency on thiol groups for steroid binding. The crucial role of essential cysteine groups suggested in those studies was confirmed recently by site-directed mutagenesis of the hMR [16], demonstrating that Cys\(^{410}\) and Cys\(^{412}\) are critical for the ligand-binding process. Another structural study [17] suggested the involvement of the N-terminal region of the hormone-binding domain of MR in ligand binding by using an antibody raised against this region.

It is known that the addition of chelating agents to brain cytosol does not affect MR stability [18]. In contrast, renal-MR stability depends greatly on the presence of chelating agents in buffer preparations. In a previous work, we demonstrated [14] that this contrasting feature for the same protein expressed in different tissues is in part the consequence of harmful effects of contaminant iron. Iron contamination is difficult to avoid in cell-free systems, especially when tissue preparations are obtained from highly irrigated organs such as kidneys. Thus when the cellular compartment is disrupted during the homogenization process, concentrations of iron as high as 0.1 mM can be obtained in kidney cytosol and substantial inactivation of the MR takes place if chelating agents are not present in the buffer. In contrast, brain cytosol possesses 50-fold-lower iron levels and appears to be more stable in the absence of chelators.

Based on the reactive properties of iron, we have ascribed [14] the inactivation of renal MR to putative modification of essential amino acid residues required for the MR function, such as histidines, cysteine and/or carboxyl residues. Contaminant iron may thus inhibit the aldosterone (ALDO) binding due to modification of residues from the steroid-binding domain and/or because other residues not localized in the hormone-binding domain are modified and simply alter the protein structure. Analogously, it is entirely possible that other metal ions normally present in tissues or incorporated to tissues after intoxications could also exert harmful effects on MR activity in a similar fashion.

The transition metals, essential for humans and also found as polluting compounds in the environment, are present in the extracellular and intracellular fluids at considerably lower concentrations than the main group of elements, which includes sodium, potassium, calcium and magnesium. Metal ions may pair off with a ligand and become an essential part of several biochemical processes. A ligand may be loosely defined as a biochemical donor groups such as R-\(^{-}\)COO\(^{-}\) or carboxyl residues. Contaminant iron may thus inhibit the aldosterone (ALDO) binding due to modification of residues from the steroid-binding domain and/or because other residues not localized in the hormone-binding domain are modified and simply alter the protein structure. Analogously, it is entirely possible that other metal ions normally present in tissues or incorporated to tissues after intoxications could also exert harmful effects on MR activity in a similar fashion.

### MATERIALS AND METHODS

#### Reagents

[\(1,2\)-\(^{3}\)H]ALDO (specific activity = 57 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). Unlabelled ALDO, dimethyl pimelimidate, inhibitors of proteases and amino acid-modifying reagents were from Sigma (St. Louis, MO, U.S.A.). RU28362 (17-\(\alpha\)-alkanyl-11\(\beta\)-17-dihydroxy-androsterone) was a kind gift from Roussel-Uclaf (Romainville, France). The 3G3 monoclonal IgM antibody against the hsp90 was purchased from Affinity Bioreagents (Golden, CO, U.S.A.). All inorganic salts were analytical grade and in the chloride form, except vanadyl sulphate.

#### Buffers

The buffers used were as follows. Buffer A: 25 mM Tris/10% glycerol/10 mM β-mercaptoethanol/20 mM sodium molybdate/1 mM PMSF/2 IU/ml aprotinin/30 µg/ml trypsin-chymotrypsin inhibitor/10 mM EDTA, pH 7.4, at 20 °C. Buffer B: buffer A without molybdate. Buffer C: buffer A without β-mercaptoethanol. Buffer D: 25 mM Tris/10%, glycerol/1 mM PMSF/2 IU/ml aprotinin/30 µg/ml trypsin-chymotrypsin inhibitor, pH 7.4, at 20 °C.

#### Receptor preparations

Male Sprague–Dawley rats weighing 200 g underwent adrenal-ectomy 48 h before the experiments. Rats were killed by decapitation and perfused through the aortic artery with more than 100 ml of cold saline solution before the kidneys were blanched. Additional perfusion of kidneys was performed through the renal artery until the renal medulla was completely blanched. Renal cortical and cortex-medullar interphases were homogenized with 2 vols. of buffer A. The homogenate was centrifuged at 67000 g for 30 min at 0 °C and the pellet was discarded. To minimize contaminations with transcontarin and iron of plasmatic and/or renal origin, the supernatant was then adsorbed on hydroxyapatite gel, washed and eluted as described previously [21]. Appropriate dilutions were then performed with the indicated buffers. This final preparation was referred to as ‘cytosol’ and used as a source of MR.
Binding assays

The standard reaction volume used for these studies was 0.5 ml. Binding assays were performed with 20 nM [³H]ALDO for 10–12 h at 0 °C in the presence of 1.0 μM RU28362 to prevent cross-reaction with the glucocorticoid receptor. Bound steroid was separated from free by adding 1 vol. of 25% (w/v) charcoal coated with 0.2% (w/v) dextran 15–20; the mixture was shaken twice for 5 h, maintained on ice for 5 min and centrifuged at 8100 g for 5 min at 4 °C. The radioactivity in 0.5 ml of supernatant was counted with 60% NaI.

Samples were centrifuged for 90 min at 0 °C to remove any steroid that was not removed by adsorption on charcoal.

Metal ions on the capacity of MR to bind ALDO were expressed as -log₁₀[metal⁺] necessary to produce 50% inhibition of steroid binding (pMₑₓₚ).

Scatchard plots

Kidney cytosol (8 mg/ml of protein) prepared in buffer A containing 1.0 μM RU28362 was incubated for 4 h at 0 °C with increasing concentrations of [³H]ALDO (from 1.1 × 10⁻¹¹ M to 7.5 × 10⁻⁹ M) and 500-fold excess of unlabelled ALDO. Bound steroid was separated from free steroid, as described above, and the equilibrium-binding parameters were calculated with the Enzfitter program (Elsevier Biosoft).

Sucrose-gradient ultracentrifugation

We used a modification of a method described previously [2,13]. Briefly, MR was labelled with [³H]ALDO for 4 h at 0 °C, free steroid was removed by adsorption on charcoal/dextran, and samples were centrifuged for 90 min at 0 °C at 463000 g on a 25–20% sucrose gradient in buffer B. When the dissociation of the MR–hsp90 heterocomplex was induced with ionic strength, 0.4 M KCl was added to the buffer containing labelled MR and incubated for 20 min at 20 °C. Fractions (250 μl) were collected by gravity flow. Myoglobin (2 S), BSA (4.6 S), β-amylase (8.9 S) and catalase (11.3 S) were used as external markers.

MR–hsp90 covalent cross-linking

We used a modification of the method described by Aranyi et al. [22]. Triethanolamine (500 μl of 2.0 M, pH 8.0) was added to 5 ml of kidney cytosol in buffer A. The cross-linker reagent dimethyl pimelimidate (20 mM) was added to the medium, and after 30 min at 5 °C the reaction was stopped with 40 mM hydroxylamine.

Modification of amino acids

Due to the possibility of inactivation of diethyl pyrocarbonate (DEPC) in Tris buffer [23], the renal cytosol used with amino acid-modifying reagents was obtained by a modification of the normal method described above. Rat kidneys were homogenized in buffer A and adsorbed on hydroxyapatite gel for 30 min at 0 °C. The adsorbed receptor was washed three times with 3 vols. of ice-cold 10 mM phosphate buffer at pH 6.5 and then eluted from the gel with a minimum volume of ice-cold 250 mM phosphate at pH 6.5. The resultant preparation was diluted with 2 vols. of a solution containing 30 mM molybdate, 15 mM EDTA and inhibitors of proteases (described for buffers A and D). This preparation was used as a source of native MR for experiments where amino acid residues were modified with specific reagents. When cross-linking between MR and the hsp90 complex was required, we followed the same procedure with dimethyl pimelimidate as described in the previous section.

Modification of cysteines was achieved with 1 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) as described previously [14]. Histidines were modified with 1 mM DEPC at pH 6.5 for 15 min at room temperature [23]. Carboxyl groups were modified with 40 mM Woodward’s reagent (N-ethyl-5-phenylisoxazolium-3'-sulphonate) at pH 6.5 for 15 min at room temperature [24]. When the reversal of the reaction was attempted, 10 mM dithiothreitol (for DTNB-treated samples) or 100 mM hydroxylamine (for DEPC- and Woodward’s reagent-treated samples) was added to the medium and incubated at room temperature for 15 min. A steroid-binding assay for native receptor was performed with 20 nM [³H]ALDO for 10 h at 0 °C as described above, whereas cross-linked receptor was incubated with the radioactive ligand for 1 h at 20 °C.

Inhibition of ALDO-dependent biological effect by metal ions

Intraperitoneal injections of inorganic salts ranging between 0.5 and 50 mg of ion/kg (30 mg/kg for copper) were given to adrenalectomized male Sprague-Dawley rats every 12 h for 4 days (eight doses per ion, 10 rats per dose). Rats were fed with Purina Diet 1 and saline solution ad libitum during that period and divided into two groups. Sodium retention was measured in the first group (six rats) after the injection of 1 μg of ALDO/100 g of body weight, exactly as we have described in detail previously [21]. Kidneys were isolated from the second group (four rats) and cytosol was prepared in buffer A. [³H]ALDO-binding capacity was measured as described in the Binding assays section. All the bioassays performed with rats followed the ethical requirements ruled by national authority.

Statistical tests

Data were analysed by one-way non-parametric analysis of variance, followed by Kruskal–Wallis test.

RESULTS

Biochemical properties of the cross-linked receptor

The oligomeric structure of the MR tightly bound to the hsp90 heterocomplex is an indispensable requirement for hormone binding. Inasmuch as treatments with high concentrations of salt dissociate the hsp90 complex from the MR, covalent cross-linking between these proteins is required to analyse the toxic effects of metal ions on ALDO binding when different concentrations of salts are used. Since the interaction of the MR with the hsp90 heterocomplex is the weakest of the entire superfamily of steroid receptors, such cross-linking is particularly necessary for this receptor under those experimental conditions.

Figure 1 depicts some properties of the cross-linked MR heterocomplex in kidney cytosol. In order to verify the specificity of the [³H]ALDO binding to the cross-linked receptor, competition curves were performed with unlabelled steroids (Figure 1A). ALDO, 11-deoxycorticosterone and corticosterone exhibited equivalent affinity for cross-linked MR, dexamethasone showed 40-fold less affinity than ALDO, and dihydrotestosterone and 17β-oestradiol did not compete with the tracer. Therefore, cross-linked MR exhibited identical specificity as native MR.

Scatchard plots (Figure 1B) show indistinguishable single slopes for both native MR (Kᵦ = 0.18 ± 0.02 nM) and cross-linked MR (Kᵦ = 0.12 ± 0.04 nM). The maximum binding capacity measured with cross-linked MR (95 ± 3 fmol/mg) was

© 1999 Biochemical Society
approx. 25% higher than the binding capacity measured with native MR (75 ± 2 fmol/mg), reflecting the stabilizing effect of hsp90 cross-linked with MR in a cell-free system. This is clearly evident in Figure 1(C), where heat inactivation at 20 °C of native MR (black symbols) and cross-linked MR (open symbols) incubated in buffer A is shown. Samples were taken at the indicated times and steroid-binding assays was then performed at 0 °C. Unoccupied native MR lost approx. 80% of the steroid-binding capacity after preincubation at 20 °C for 40 min. This inactivation was faster if 4 M urea or 0.4 M KCl was added to the incubation medium. In contrast, the cross-linked MR was stable after 60 min at 20 °C and neither 4 M urea nor 0.4 M KCl affected the stability.

The interpretation that thermal stabilization is due to an efficient cross-linking of MR with hsp90 is strengthened by sucrose-gradient-ultracentrifugation studies depicted in Figure 1(D). The sedimentation profile of the [3H]ALDO–MR complex incubated at 0 °C in the presence of molybdate showed a major peak at 9.2 S (marker of untransformed MR) and a minor peak at 5.1 S. The addition of 0.4 M KCl to the medium (in the absence of molybdate) resulted in a large increase of the 5.1-S peak, with a concomitant loss of the 9.2-S peak. After cross-linking with dimethyl pimelimidate, the 9.2-S peak remained stable (slightly but consistently shifted to 9.7 S) in the absence (peak not shown) or presence of 0.4 M KCl (*). The 5.1-S species were not evident in any preparation of cross-linked MR due to the fact that the hsp90–MR complex was bound covalently. Incubations with the specific antibody 3G3 against hsp90 shifted the 9.7-S peak to 11.3 S (+), demonstrating that hsp90 was present in the untransformed MR heterocomplex. Control gradients (results not shown) showed no shift of native and cross-linked MR when the 3G3 antibody was
replaced by a non-immune IgM antibody. Pooled 5.1-S peaks from non-cross-linked MR treated with 3G3 antibody underwent no shift (results not shown), confirming a previous report [2], where the association of hsp90 with the MR was exclusively demonstrated in untransformed species.

**Inhibitory effect of metal ions on steroid binding**

We further characterized the requirement of chelating agents for native and cross-linked MR to preserve steroid-binding capacity upon thermal inactivation at 20 °C. The results are summarized in Table 1. Conditions I and II represent controls for the maximum steroid-binding capacity achieved for receptors preincubated at 0 °C or 20 °C in buffer A. When native and cross-linked MR were incubated in buffer D (lacking EDTA, molybdate and reducing agents), a significant loss of steroid-binding capacity was observed for both preparations (condition III). As expected, native MR was affected more than the stabilized cross-linked MR. This inactivation was more efficiently reduced if EDTA (condition IV) rather than EGTA (condition V) was present in the buffer, suggesting that metal ions different from Ca$^{2+}$ may also affect the ligand-binding capacity of cross-linked MR. Consistent with a conserved association between MR and hsp90, cross-linked MR remained unaffected by treatments with EDTA containing 1 mM KCl (conditions VI and VII). In contrast, the steroid-binding capacity of native MR was totally abolished in a high-ionic-strength medium regardless of the addition of EDTA (condition VI) or EGTA (condition VII).

The experiments described above prompted us to study the putative harmful action of metal ions other than Ca$^{2+}$ on hormone-binding capacity of cross-linked MR. Figure 2 shows the inhibitory effects of five transition-metal ions as compared with Ca$^{2+}$ over the concentration range 10$^{-8}$–10$^{-1}$M. The pME$_{50}$ values (shown in parentheses) were calculated following a log-log four-parameters function, resulting in the following inhibitory potencies for [H]$^{3}$ALDO binding: Hg$^{2+}$ (6.05) > Zn$^{2+}$ (5.97) > Cu$^{2+}$ (5.05) > Fe$^{3+}$ (4.1) > Ni$^{2+}$ (2.25) > Ca$^{2+}$ (0.98).

In order to determine a pattern of reactivity for metal ions with MR heterocomplexes, the study described above was extended to a total of 20 ions. All curves showed a sigmoidal shape similar to those depicted in Figure 2. Calculated pME$_{50}$ values were then plotted against positions of metals in the periodic table (Figure 3). Ions appeared to fall into four categories, characterized by pME$_{50}$ values, which differed by 1.5 units from each other.

The first group included alkaline and alkaline earth ions, which exhibited low reactivity: Na$^{+}$ (0.11), Be$^{2+}$ (1.01), Mg$^{2+}$ (0.65), Ca$^{2+}$ (0.73) and Ba$^{2+}$ (0.55), as well as Co$^{2+}$ (1.51). Except sodium, all are divalent metal ions. The second group involved tetravalent titanium and divalent cations (including vanadyl and uranyl ions), with higher reactivities than the former group. The pME$_{50}$s were similar and may be clustered near a value of 2.25 in only one well-defined sigmoid curve: La$^{3+}$ (2.51), Ti$^{4+}$ (2.11),

---

**Table 1  Protective action of chelating agents on MR steroid binding**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Native MR</th>
<th>Cross-linked MR</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Control</td>
<td>2312 ± 134</td>
<td>2798 ± 98</td>
</tr>
<tr>
<td>II. Buffer A</td>
<td>1924 ± 101</td>
<td>2701 ± 127</td>
</tr>
<tr>
<td>III. Buffer D</td>
<td>751 ± 111</td>
<td>1898 ± 107</td>
</tr>
<tr>
<td>IV. Buffer D + 10 mM EDTA</td>
<td>1526 ± 129</td>
<td>2612 ± 163</td>
</tr>
<tr>
<td>V. Buffer D + 10 mM EDTA</td>
<td>1050 ± 89</td>
<td>2246 ± 89</td>
</tr>
<tr>
<td>VI. Buffer D + 10 mM EDTA + 0.4 M KCl</td>
<td>101 ± 44</td>
<td>2650 ± 148</td>
</tr>
<tr>
<td>VII. Buffer D + 10 mM EDTA + 0.4 M KCl</td>
<td>54 ± 32</td>
<td>2288 ± 100</td>
</tr>
</tbody>
</table>

**Figure 2  Inhibitory effect of metal ions on MR steroid binding**

Cross-linked MR in buffer D containing 1 mM β-mercaptoethanol and 1.0 μM RU28362 was incubated for 10 h at 0 °C with 20 nM [3H]$^{3}$ALDO in the presence of the following metal ions: □ Hg$^{2+}$, ○ Zn$^{2+}$, ● Cu$^{2+}$, ◇ Fe$^{3+}$, △ Ni$^{2+}$ and (△) Ca$^{2+}$. Results are expressed as percentages of specific binding measured in the absence of metals.
Soft ions are usually found bound to soft ligands such as sulphur than bigger ions such as Ba$^{2+}$, which is as reactive as the bigger Fe$^{2+}$ (2.25). The third group included trivalent metal ions with pME$_{50}$ values of approx. 4.2, fitting all three cations into only one sigmoid: Cr$^{3+}$ (4.22), Fe$^{3+}$ (4.1) and Al$^{3+}$ (4.26). In the last group, heavy divalent metal ions and trivalent thallium appeared with pME$_{50}$ values above 4.5, most of them close to 5.9 except copper, which was slightly less reactive: Cu$^{2+}$ (5.05), Zn$^{2+}$ (5.97), Cd$^{2+}$ (5.81), Hg$^{II}$ (6.15), Tl$^{III}$ (5.80) and Pb$^{2+}$ (5.96). Although there was a tendency to increase the inhibitory effects on steroid binding with the electronic configuration of each metal ion and, consequently, with the position in the periodic table, there were ions that escaped from that tendency. The most notorious were manganese, aluminium and, especially, the triad of group VIII (iron, cobalt and nickel). Attempts to correlate ion reactivity with MR and ionic radius (results not shown) also failed. For example, the small ion AI$^{3+}$ ($r = 0.45\ \text{Å}$) is as reactive as the bigger Fe$^{3+}$ ($r = 0.64\ \text{Å}$), and more reactive than bigger ions such as Ba$^{2+}$ ($r = 1.29\ \text{Å}$) or La$^{3+}$ ($r = 1.15\ \text{Å}$), but also smaller ions such as Be$^{+}$ ($r = 0.30\ \text{Å}$).

**Relationship between Klopman constants and pME$_{50}$**

Klopman constants ($\sigma_r$) for metal ions were derived mainly from two types of information [19,25]: frontier orbital electronegativity, which describes the ability of the metal ion to polarize charge from a ligand, and desolvation energy, which represents the endothermic term required for removal of solvent before a bond can form to a new ligand molecule or anion. Accordingly, ions that bind preferentially to electronnegative or hard donor atoms have been called ‘a type’ or ‘hard’, whereas those binding to polarizable or soft atoms are called ‘b type’ or ‘soft’ cations.

When the pME$_{50}$ values obtained above were replotted against the Klopman constant ($\sigma_r$) for each ion, new and valuable information about the MR heterocomplex was obtained. Figure 4 depicts three families of ions (connected by a line in the plot), which share common properties. The softest metal ions, with $\sigma_r \leq -1$ (Hg$^{II}$, Tl$^{III}$, Cd$^{II}$ and Cu$^{2+}$), were strongly inhibitory. Soft ions are usually found bound to soft ligands such as sulphur electron donors, in this case most probably cysteines of the MR–hsp90 heterocomplex.

The intermediate metal ions between $-1$ and +4 on the Klopman scale fell into two distinct groups. Those with d-valence electrons were stronger inhibitory ions (Cr$^{3+}$ and Fe$^{3+}$) than those with s-valence orbitals (Na$^+$, Ba$^{2+}$, Mg$^{2+}$, Ca$^{2+}$, Be$^{2+}$, Ni$^+$, Co$^{2+}$, Mn$^{2+}$ and vanadyl). In the last group, there was a clear distinction in pME$_{50}$ values between those metal ions rich in d electrons (Ni$^{2+}$, Co$^{2+}$, Mn$^{2+}$ and vanadyl), which were moderately inhibitory, and those without d electrons (Na$^+$, Ba$^{2+}$, Mg$^{2+}$, Ca$^{2+}$ and Be$^{2+}$). The main difference between Cr$^{3+}$ and Fe$^{3+}$ and the rest was that ions with d-valence electrons were capable of accepting $\pi$ electrons from ligands, whereas the others were not. This type of $\pi$ charge is available on tertiary aromatic nitrogen. Therefore, the inactivation of the steroid-binding capacity may be ascribed to reactivity with histidine amino acids.

A third trend that involved $\sigma$ charge and exhibited stronger inhibitory properties with increased hardness was also evident from the plot. Starting at Na$^+$, a line that includes Ba$^{2+}$, Mg$^{2+}$, Ca$^{2+}$ and Be$^{2+}$, showed a growing trend of reactivity with hardness. These s orbitals are almost always involved in reactions with oxygen-donor ligands. Therefore, this type of inhibition may be attributed mainly to carboxyl groups.

### Table 2 Inhibition of ALDO binding in chemically modified MR

<table>
<thead>
<tr>
<th>Condition</th>
<th>Native MR</th>
<th>Cross-linked MR</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Control</td>
<td>2452 ± 154</td>
<td>3158 ± 98</td>
</tr>
<tr>
<td>II. DTNB</td>
<td>104 ± 96</td>
<td>192 ± 107</td>
</tr>
<tr>
<td>III. DTNB +-dithiothreitol</td>
<td>2111 ± 149</td>
<td>2718 ± 138</td>
</tr>
<tr>
<td>IV. DEPC</td>
<td>450 ± 88</td>
<td>312 ± 103</td>
</tr>
<tr>
<td>V. DEPC + hydroxylamine</td>
<td>1500 ± 176</td>
<td>2556 ± 386</td>
</tr>
<tr>
<td>VI. WR</td>
<td>301 ± 24</td>
<td>504 ± 110</td>
</tr>
<tr>
<td>VII. WR + hydroxylamine</td>
<td>1714 ± 269</td>
<td>2708 ± 112</td>
</tr>
</tbody>
</table>

Specific binding (c.p.m./mg of protein)

© 1999 Biochemical Society
DTNB) or hydroxylamine (to reverse the effects of DEPC and Woodward’s reagent). The steroid-binding capacity was recovered to similar values as those observed in untreated controls.

We achieved the above-described modifying reactions following standard conditions [23,24], which minimized putative non-specific modification of other amino acid residues. To control such specificity, we attempted transformations of the cross-linked MR with each specific reagent in the presence of a twofold excess of cysteine, histidine, lysine, tyrosine or glutamic acid (results not shown). Under these conditions, the inhibition of the steroid binding by treatment with DTNB was selectively abolished by cysteine, histidine suppressed the inhibitory effect of DEPC and glutamic acid abolished the inhibition observed with Woodward’s reagent. In all the cases, more than 90% of the steroid-binding capacity measured in untreated controls was recovered when the specific aforementioned amino acid was used. On the other hand, negligible inhibition (from 4 to 13%o) was obtained when any of the other four amino acids were used in each reaction. These results confirmed that the modifying reactions performed under these experimental conditions were specific.

As predicted from the analysis of reactivity with metal ions, modifications on thiol, histidyl and/or carboxyl groups do affect the steroid-binding capacity of the MR-hsp90 heterocomplex.

Inhibitory effects in vivo of metal ions on steroid binding and sodium retention

To assess the biological relevance in vivo of these studies in vitro, we injected adrenalectomized rats every 12 h with eight different intraperitoneal doses of inorganic salts, ranging from 0.5 to 50 mg of ion/kg. Due to the high toxicity observed with copper chloride, the maximum dose used for this salt was only 30 mg of ion/kg. After 4 days of treatment, the capacity of ALDO (1 μg/100 g of body weight) to promote maximum Na+ retention [21] was evaluated. Controls were injected with vehicle alone.

After 2.5 h rats were killed, urine was aspirated from bladders, and sodium elimination was measured by flame photometry. No differences were observed between metal-ion-treated animals (5.3±0.6 μEq of Na+ per mg of creatine) and untreated animals injected with vehicle alone (5.8±0.4 μEq of Na+/min per mg of creatine). When untreated rats were injected with ALDO (1 μg/100 g), only 17% of the Na+ excretion measured in controls was quantified (1.0 μEq of Na+/min per mg of creatine). However, this effect was inhibited in a dose-dependent fashion in rats treated with metal ions. The ID50 values (the dose of metal ion necessary to inhibit 50% of ALDO-dependent Na+ retention, in mg/kg) obtained for each treatment were as follows: Hg²⁺, 2.5±1.1; Cu²⁺, 3.0±1.3; Cd²⁺, 7.1±2.0; Al³⁺, 10.7±2.8; Fe³⁺, 18.3±5.3; VO⁴⁺, 22.5±4.6; Co²⁺, 23.3±3.8; La³⁺, 24.5±3.3; and Ni²⁺, 26.7±4.0.

Kidneys excised from a second group of rats treated as described for the bioassay, but not injected with ALDO, were homogenized in buffer A and the specific binding of [3H]ALDO was measured in cytosol. Consistent with the ID50 values obtained in vitro, the pME50 values obtained for treated rats were as follows: Hg²⁺, 4.5±0.5; Cu²⁺, 4.9±0.8; Cd²⁺, 4.7±0.4; Al³⁺, 2.6±0.2; Fe³⁺, 3.8±0.7; VO⁴⁺, 1.0±0.8; Co²⁺, 1.0±0.6; La³⁺, 1.2±0.3; and Ni²⁺, 1.5±0.4.

Figure 5 shows a plot of these pME50 and ID50 values against FRC. There was a correlation between the ALDO-dependent biological response (□) and inhibition of ALDO binding (●). Importantly, the patterns of both the biochemical effect of metal ions on ALDO binding and the inhibition of ALDO binding are closely related to the pattern observed for assays in vitro shown in Figure 4. Thus the assumption that these ions may be potentially toxic and able to affect the structure and, consequently, the function of the MR heterocomplex can be correlated with the treatment in vivo.

DISCUSSION

The MR-hsp90 cross-linked heterocomplex has properties that resembles those observed for native MR in conditions where cross-reactions with the glucocorticoid receptor are prevented. Treatment with dimethyl pimelimidate did not affect ligand selectivity or affinity compared with native or recombinant MR [10,12,21]. In contrast, the stability of the heterocomplex was greatly improved with respect to the non-cross-linked heterocomplex [14]. Therefore, the inhibitory effect on steroid binding observed for treatments with salts cannot be attributed to a transformation process. A controversial question as to whether or not hsp90 was present in untransformed receptor [26] was answered clearly (Figure 1D) when samples were preincubated with 3G3 antibody and then resolved in a sucrose ultracentrifugation gradient. A clear shift from 9.7 to 11.3 S could be observed due to the antibody binding to the heterocomplex. The presence of hsp90 in untransformed steroid-receptor complexes confirmed previous reports for other steroid receptors [22,27] and, particularly, the native rat kidney MR [2].

The differential efficiency observed with chelating agents on native MR stability [14] could also be seen for cross-linked MR, suggesting that metal ions may be involved in the inactivation process. It is entirely possible that, under circumstances where the cellular compartmentalization is altered (i.e. tissue homogenization), free or complexed metals present as physiological compounds in tissues (i.e. iron) or as contaminants in buffers (i.e. aluminium) can exert effects on protein stability and reactivity. It should be pointed out that most of the metal ions studied here are able to form stable chelates with EDTA.
We used salts of several metal ions to inactivate MR. The metal cations, like protons, attack negative sites. For all ions tested, the inhibition curves displayed a simple sigmoidal shape similar to conventional titration curves. A rational relationship for the inactivation of MR with hard-and-soft-acids theory was obtained. The hypothesis that reactivity patterns may be related to chemical hardness was suggested several years ago [19,23]. Thus the analysis of our results based on that model predicts that hard ions (Al\(^{3+}\), La\(^{3+}\), Be\(^{2+}\), etc.) prefer to bind to oxygen or nitrogen, whereas soft ions (Cu\(^{2+}\), Hg\(^{2+}\), Cd\(^{2+}\), etc.) prefer sulphur. Borderline (intermediate) ions (Fe\(^{2+}\), Cr\(^{3+}\), Co\(^{2+}\), Ca\(^{2+}\), etc.) differ among themselves with some pronounced differences for nitrogen, sulphur and oxygen. On the basis of Klopman theory, cleavage of RNA induced by hard and soft metal ions was also explained by the tendency of these ions to react with oxygen and nitrogen atoms of nucleic acid bases [28,29].

Doubts were raised [30] about whether the same types of reactions between metal ions and cellular components that are known to occur in vitro do in fact also occur in vivo. Results shown in Figure 5 (treatments in vitro) clearly argued in favour of the possibility that the relationship in vitro obtained for pME\(_{50}\) and \(\sigma\) (Figure 4) correlates with effects in vivo.

The assumption that metal ions may inactivate MR by reacting with cysteines, histidines and acidic amino acids is also suggested by treatments with specific modifying reagents, strengthening the notion that these amino acids are essential for steroid binding. The chemical modification of MR was reversed with dithiothreitol and hydroxylamine and totally prevented in the presence of cysteines, histidines and acidic amino acids is also suggested by our data. This conclusion is independent of the mechanistic nature of the inhibition observed for both ALDO binding to the MR and sodium retention.

As the number and variety of chemicals entering the environment from energy technologies increase, it becomes more and more desirable to identify physical and biological concepts and principles on which systems of health protection from chemical pollutants can be based. The classification of metal ions into hard and soft classes is a useful one, but may not necessarily lead to a universal order of reactivity. Nevertheless, this work shows that a correlation between toxicity of metal ions and ALDO-dependent sodium retention may exist and, consequently, could be predicted.

We thank Professor C. P. Lantos (University of Buenos Aires) for his criticism and K. C. Kanelakis (University of Michigan) for the kind revision of this manuscript.

REFERENCES


Received 17 March 1996/21 April 1996; accepted 20 May 1999

© 1999 Biochemical Society