STABILITY STUDY ON RENAL TYPE I MINERALOCORTICOID RECEPTOR

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Summary

The purpose of this work is to review stability and activation properties of type I receptor, in order to explain the reasons for its extreme in vitro instability. We demonstrate that the treatment of rat kidney cytosol with H$_2$O$_2$ prevents aldosterone binding, DNA/steroid-receptor complex interactions, and prevents the receptor thermal inactivation. In contrast, exogenous sulfhydryl reducing reagents are necessary to insure maximum binding of mineralocorticoid receptor and DNA/steroid-receptor interaction. However, the presence of β-mercaptoethanol in thermal induced incubations reverts the H$_2$O$_2$ protection. We also demonstrate that contaminations with free or sequestered iron are harmful for both, receptor binding capacity (in a reversible form) and for hormone-receptor /DNA binding properties (in a partially reversible form). We propose a sulfhydryl oxidative mechanism for type I mineralocorticoid receptor inactivation in which iron contaminants might accelerate this process by oxidative catalysis. We also demonstrate that when thiol groups are blocked by specific reagents such as N-ethyl-maleimide or dithionitrobenzoic acid, type I sites loose binding capacity, but the protein is protected from oxidation as well as inactivation.

Key Words: mineralocorticoid receptor, thiol groups, stability, oxidation

Although the primary amino acid sequence for human (1) and rat (2) mineralocorticoid receptor (MCR) has been elucidated, attempts to purify this receptor from kidney preparations has been hampered by its lack of stability.

The binding capacity of cytosol preparations for many steroids is eliminated by sulfhydryl-blocking reagents. A thiol requirement has been inferred for the binding of these hormones and reducing agents in the incubation buffer are required (3,4). The MCR in brain cytosol do not require sulphydryl reducing reagents or EDTA for maximum binding (5) and the addition of 1 mM EDTA to buffers produce no effect on MCR stability. We have observed that renal MCR binding depends greatly on reducing and chelating agents (6). Unoccupied type I and type II receptors differ from each other in their responsiveness to the stabilizing actions of different buffer components, but the binding of both receptors to DNA-cellulose columns is poorly affected when transformation is induced (7). The properties of native as well as cloned MCRs are greatly influenced by buffer conditions, and the apparent subunit composition of recombinant receptors
depends on the presence of molybdate (Mo) or tungstate (8). It was also shown that brain type I MCR may be transformed into a DNA-binding form by means of chaotropic anions in the buffer media (9).

This variety of apparent conflicting observations demonstrates that, whether recombinant or native, MCRs are very different from other steroid receptors. In this work, we analyze systematically the buffer conditions for renal MCR in order to elucidate its stability properties. Specially, we investigate the importance of thiol groups in receptor activation (hormone binding state) and transformation (DNA binding state) events, and its relationship with other factors such as cytosolic iron contaminations.

Materials and Methods

**Isotopes and Chemicals:** [3H]-Aldosterone (ALDO) (SA = 59 Ci/mmol) and [3H]-Corticosterone (B) (SA = 87 Ci/mmol) were purchased from New England Nuclear (USA). RU-28362 was a kind gift from Roussel-Uclaf (France). Radioligand B and ALDO were from Makor Chemical (Israel). DNA-cellulose double-stranded from calf thymus (7 mg DNA/g), aprotinin from bovine lung, trypsin-chymotrypsin inhibitor from soybean, phenylmethylsulfonyl fluoride (PMSF), N-ethylmaleimide (NEM), dithionitrobenzoic acid (DTNB) and α,α'-dipyridyl (DP) were purchased from Sigma (USA). All other reagents were analytic grade. Hematin was prepared from human blood (10) and quantified by the pyridine-hemochromogen assay (11).

**Buffers:** The following buffer formulations (pH 7.4) were used: (I) 0.1 M Tris-HCl, 10 mM EDTA, 23% v/v glycerol, 0.1 mM PMSF, 2 TIU/ml aprotinin and 30 μg/ml trypsin-chymotrypsin inhibitor. (II) buffer II without EDTA. (IV) buffer II plus 20 mM β-mercaptoethanol and 20 mM Na₂MoO₄. All buffers contained 1.0 μM RU-28362 to block type II receptors (12).

**Receptor Preparations and Binding Assays:** Rat kidney MCRs were obtained from 200 g male Sprague-Dawley rats as previously described (13). 500 μl MCR cytosolic fractions were incubated as indicated in each figure with [3H]-ALDO, in the presence or absence of 1000 fold excess of radioligand ligand. Bound steroid was separated from free by adding one volume of 2.0% w/v activated charcoal AR-0.2% w/v dextran 15-20 in buffer I. The radioactivity in 0.4 ml supernatant was counted with 60% efficiency for tritium. Non specific binding was 20 to 30% of the total.

Hippocampal MCRs were obtained from the same rats in the buffer formulation indicated in legends. Incubations were performed as indicated by kidney MCRs, but bound [3H]-ALDO was separated from free [3H]-ALDO on Sephadex G25 columns pre-equilibrated at 4°C and eluted with the same incubation buffer.

**Sulphydryl-Blocking Treatment:** Kidneys were homogenized in buffer II plus 20 mM sodium molybdate (Mo) but without β-mercaptoethanol (2-ME). Receptors were preincubated for 25 min at 25°C with sulphydryl-modifying reagents NEM or DTNB and then, incubated with 5.3 nM [3H]-ALDO in the absence (inhibition assays) or the presence (reversion assays) of 10 mM 2-ME.

**Oxidation of the Receptors:** Kidney cytosols were adjusted to an appropriate buffer formulation (see legends of the figures) plus 20 mM NaN₃, with or without 20 mM molybdate, and were preincubated for 25 min with 10 mM H₂O₂. Then, the cytosol samples were incubated with [3H]-ALDO as described above. An excess of 40 mM 2-ME was used in order to inactivate H₂O₂ or revert MCR induced oxidation.

In other experiments, samples were incubated for 3 h at 0°C and bound steroid was separated from free by Sephadex G25 columns equilibrated in buffer III containing 20 mM NaN₃ (plus 10
mM EDTA or 40 mM 2-ME or 20 mM molybdate). Steroid-receptor complexes were transformed by heating at 25°C for 25 min and then layered on top of minicolumns containing 0.1 g DNA-cellulose equilibrated in the same transformation buffer. The samples were washed once with three volumes of cold (4°C) buffer. This was done at high pressure in order to prevent hormone-receptor dissociation. Adsorbed radioactivity (35% bound to DNA-cellulose with respect to total cytosolic binding) was eluated in batch with 0.5 M phosphate, 0.25% Triton X-100 buffer at pH 6.5.

**Chromatography:** In order to assure the identity of radioactive ALDO after incubations, the samples were extracted with two volumes of CHCl₃, evaporated under nitrogen stream and chromatographed (14). The radioactivity always comigrated with ALDO standard indicating that no oxidation products were generated during the incubations, including H₂O₂-treatment.

**Miscellaneous:** Iron was measured by formation of a chromogen with pyridyl-bis-phenyl-triazine sulphonate making use of a commercial kit (Wiener-lab, Argentina). Proteins were measured according to Bradford (15). Statistics were performed by one-way analysis of non-parametric ANOVA system (Kruskal-Wallis).

**Results**

**Receptor Identity:** Binding activity reached a plateau after 2 h and remained stable for at least 8 h at 0°C. After 12 h of incubation, 90% of the maximum binding capacity could still be measured.

Charcoal was most efficient at final concentration of 1.0% and variations of dextran from 0.1% to 5.0% did not modify specific binding. A final preparation of 1.0% charcoal-0.1% dextran was chosen. We also compared bound from free steroid separation by adsorption on hydroxylapatite (HAP)(16). This method proved to be as efficient as the charcoal-dextran method (r = 0.973).

Binding specificity to the MCR could be accomplish by using the specific glucocorticoid RU-28362 and 5.0 nM [³H]-ALDO or [³H-B] as ligands. Cytosols were also preincubated with HAP in order to exclude transcortin. No difference in specific binding was found for both ligands suggesting identical interactions with MCR. Under these conditions, an excess of radioinert ALDO abolished [³H]-B binding, and vice versa. Scatchard plots indicated only one site with Kᵦᵢᵢᵢ = 0.4 nM in the presence of RU-28362, but a second binding site (Kᵦᵢᵢᵢ = 60 nM) in the absence of this specific type II ligand (data not shown).

**Optimization of MCR Binding Conditions:** Table I lists the influence of each component of the final incubation mixture on ALDO binding. Most remarkable were the effects of 2-ME, EDTA, PMSF, DP and Mo. The stabilizing properties of chelating agents prompted the investigation of presumably inactivating compounds. Thus, 2.5 mM Ca²⁺ decreased the binding 50% while Mg²⁺ was slightly inhibitory. The presence of EDTA or EGTA in homogenizing and incubation buffers prevented these effects. The highly specific chelator for calcium, EGTA, was significantly less effective (P = 0.02) than EDTA. These results suggest that factors others than Ca²⁺ could be involved in MCR inactivation since other metal-ions might be chelated by EDTA, but not by EGTA.

The same components were also assayed under destabilizing conditions. For that purpose, preparations were preincubated at 25°C and then, incubated at 0°C in the presence of [³H]-ALDO. Fig. 1 shows that all components, except CaCl₂ and KCl, could retard the inactivation of unoccupied MCR. Again, EDTA was a better protector than EGTA (p < 0.05). Buffer IV led to an approximately 35% loss binding under this conditions. 0.1 mM PMSF and 20 mM Mo were also effective protecting agents in prevention the loss of binding (Table I and Fig. 1).

The receptor was also preserved from inactivation by adding ALDO to the preincubation medium, while 0.3 M KCl significantly diminished steroid binding capacity and receptor stability in the best buffer formulation. These results are in accordance with previous observations in which
### Table I

**Influence of Buffer Compounds on Aldosterone Binding**

<table>
<thead>
<tr>
<th>Condition</th>
<th>ALDO Bound (fmol/incubation)</th>
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<tbody>
<tr>
<td>Buffer I</td>
<td>24.0 ± 3.6</td>
</tr>
<tr>
<td>Buffer IV</td>
<td>112.7 ± 2.1</td>
</tr>
<tr>
<td>Buffer I plus 1 mM 2-ME</td>
<td>53.9 ± 2.6</td>
</tr>
<tr>
<td>5 mM 2-ME</td>
<td>85.0 ± 3.2</td>
</tr>
<tr>
<td>10 mM 2-ME</td>
<td>89.4 ± 2.3</td>
</tr>
<tr>
<td>100 mM 2-ME</td>
<td>78.9 ± 4.2</td>
</tr>
<tr>
<td>Buffer I plus 20 mM molybdate</td>
<td>57.2 ± 1.4</td>
</tr>
<tr>
<td>Buffer I plus 0.3 M KCl</td>
<td>10.6 ± 1.1</td>
</tr>
<tr>
<td>Buffer IV plus 0.3 M KCl</td>
<td>43.4 ± 1.5</td>
</tr>
<tr>
<td>Buffer I plus 0.1 mM PMSF</td>
<td>63.4 ± 2.2</td>
</tr>
<tr>
<td>Buffer I plus EDTA 1 mM</td>
<td>40.8 ± 2.2</td>
</tr>
<tr>
<td>5 mM</td>
<td>64.2 ± 3.7</td>
</tr>
<tr>
<td>10 mM</td>
<td>72.8 ± 5.6</td>
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<tr>
<td>20 mM</td>
<td>74.5 ± 6.2</td>
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<tr>
<td>Buffer I plus EGTA 1 mM</td>
<td>30.3 ± 2.8</td>
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<tr>
<td>5 mM</td>
<td>44.6 ± 1.5</td>
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<tr>
<td>10 mM</td>
<td>42.9 ± 1.8</td>
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<tr>
<td>20 mM</td>
<td>44.0 ± 2.8</td>
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<tr>
<td>Buffer I plus glycerol 10%</td>
<td>28.3 ± 4.2</td>
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<tr>
<td>30%</td>
<td>32.2 ± 3.6</td>
</tr>
<tr>
<td>Buffer I plus 2.5 mM Ca(^{2+})</td>
<td>13.7 ± 2.2</td>
</tr>
<tr>
<td>Buffer I plus 2.5 mM Ca(^{2+}) 5 mM EDTA</td>
<td>39.6 ± 2.8</td>
</tr>
<tr>
<td>Buffer I plus 2.5 mM Ca(^{2+}) 5 mM EGTA</td>
<td>27.3 ± 2.8</td>
</tr>
<tr>
<td>Buffer I plus 1.5 mM Mg(^{2+})</td>
<td>20.8 ± 0.6</td>
</tr>
<tr>
<td>Buffer I plus 1.5 mM Mg(^{2+}) 5 mM EDTA</td>
<td>49.2 ± 3.8</td>
</tr>
<tr>
<td>Buffer I plus 1.5 mM Mg(^{2+}) 5 mM EGTA</td>
<td>19.9 ± 0.8</td>
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</table>

Kidney MCR were prepared in buffer I supplemented with the components indicated, or in buffer IV. Incubations with 5.0 nM \[^{3}H\]-ALDO in the presence of 1.0 μM RU-28362 were performed at 0°C for 12 h. The results are the means ± SD (n=6).

**FIG. 1**

**Kidney MCR Stability at High Temperature:** Kidneys were homogenized in buffer I supplemented with: (1) 10 mM EDTA. (2) 10 mM EGTA. (3) 5 mM CaCl\(_2\) plus 10 mM EGTA. (4) 2.5 mM CaCl\(_2\). (5) 20 mM molybdate. (6) 10 mM 2-ME. (7) 23% v/v glycerol. (8) nothing. (9) 5.0 nM \[^{3}H\]-ALDO. (10) buffer IV. (11) buffer IV plus 0.3 M KCl. (13) PMSF: a) 1.0 mM, b) 0.1 mM, c) 0.03 mM, d) 0.005 mM. The incubations were performed at 25°C at indicated times in order to destabilize MCR-preparations (10.6 mg protein/ml) and then, 0.5 ml aliquots were incubated at 0°C during 12 h with 5.0 nM \[^{3}H\]-ALDO (except incubation 9) in the presence of 1.0 μM RU-28362. Zero times points represent binding detectable at 0°C without preincubation. The data are the means ± SD (n=6).
Effects of Sulfhydryl Reagents on ALDO Binding: Kidneys were homogenized in buffer II with 20 mM Mo, but without 2-ME. Preincubations were performed 25 min at 25°C in the presence of NEM or DTNB and then, incubated at 0°C with 5.3 nM [\(^{3}H\)]-ALDO. Black bars = controls with or without 10 mM 2-ME. Hatched bars = preincubations at 25°C with 10\(^{-6}\) to 10\(^{-5}\) M thiol blocking reagent, and incubation at 0°C. Heavy hatched bars = Preincubations at 25°C with 10\(^{-3}\) M DTNB or NEM and incubations at 0°C in the presence of 10 mM 2-ME (reversion assay). Protein concentration: 12.2 mg/ml. Bars are the means ± SD (n=5).

Temperature and ionic strength were found to affect steroid receptor stability (17-19). Optimum concentrations of PMSF (0.1 to 1 mM) led to a 50% residual binding capacity after 50 min at 25°C. The effect of this inhibitor of serine-proteases was shown to be dose-dependent since concentrations lower than 0.1 mM were less effective. Such protective action was of interest since other protease-inhibitors failed to prevent the loss of MCR in rat kidney cytosols (17). However, PMSF did not preserve binding entirely. In effect, steroid binding decreased during a 24 min incubation in the presence of optimum PMSF concentrations, but then, no significant decay was observed suggesting that the limit of PMSF-protective action has been reached.

Requirement for free thiol functions: 2-ME was unable to protect MCR in spite of being one of the most important supplements for binding in buffer formulations. Since 2-ME increased specific binding more than three times, a series of experiments were undertaken in which thiol groups were blocked with specific reagents such as DTNB or NEM (Fig.2). Both sulfhydryl-modifying reagents inhibited binding in a dose-dependent mode. When an excess of 10 mM 2-ME was added to the preparation previously incubated with 1 mM NEM or DTNB, the blocking effects were reversed.

Inactivation by Iron: It is known that iron can react with Cys, His, Asp and Glu residues in proteins, and that it is also an efficient catalyst from thiol oxidation process (20). In addition, the former experiments showed that the nonspecific chelating agent, EDTA, increased specific binding

### TABLE II

<table>
<thead>
<tr>
<th>Condition</th>
<th>ALDO Bound (fmol/incubation)</th>
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<tbody>
<tr>
<td>Buffer I</td>
<td>22.1 ± 4.5</td>
</tr>
<tr>
<td>Buffer I + DP 1 mM</td>
<td>33.1 ± 3.8</td>
</tr>
<tr>
<td>5 mM</td>
<td>60.8 ± 4.7</td>
</tr>
<tr>
<td>10 mM</td>
<td>59.5 ± 3.7</td>
</tr>
<tr>
<td>Buffer IV</td>
<td>114 6 ± 6.0</td>
</tr>
<tr>
<td>Buffer IV + 5 mM DP</td>
<td>115.6 ± 4.8</td>
</tr>
<tr>
<td>Buffer IV (without EDTA) + 5 mM DP and preincubated at 25°C for 50 min</td>
<td>44.8 ± 2.7</td>
</tr>
<tr>
<td>Buffer IV preincubated at 25°C for 50 min</td>
<td>48.1 ± 4.0</td>
</tr>
</tbody>
</table>

Cytosols were obtained in buffer I or IV, with or without EDTA or DP. Incubations with 5.0 nM [\(^{3}H\)]-ALDO were performed as in Table 1. The results are the means ± SD (n=6).
FIG. 3

MCR Inactivation by Iron:
A. Kidney MCR was incubated 12 h at 0°C with \(10^{-4}\) to \(10^{-2}\) M FeCl₃ in buffer IV lacking EDTA, with (■-■) or without (▲-▲) 10 mM DP (n=6). B. Incubations with hematin in buffer IV lacking EDTA, with (■-■) or without (▲-▲) 10 mM DP (n=4). Proteins: 5.9 mg/incubation.

and protected the receptors from the inactivation more efficiently than EGTA, a specific chelating agent for Ca²⁺, suggesting that cations other than Ca²⁺ are involved in MCR inactivation. Consequently, iron was postulated as a putative inhibitory contaminant. In order to confirm the above hypothesis, a specific chelating agent for iron was used. Table II lists the influence of dipyridyl (DP) on ALDO binding capacity and thermal stability of MCR. The results are similar to EDTA in both instances and no additive effect was observed. This indicates that DP and EDTA might act through the same mechanism and that iron is more destabilizing than Ca²⁺.

Figure 3-A depicts inactivation of MCR as a function of increasing concentrations of exogenous ferric ions in a buffer lacking EDTA, also demonstrated that DP stabilizers the unbound MCR. Not only free iron, but also hemic iron inhibited steroid binding (Fig.3-B). Absolute values (fmol/mg protein) for maximum binding measured in the presence or absence of DP were, respectively, 19.1 and 11.2 in panel A, and 17.8 and 9.8 in panel B.

Kidneys are highly vascular organs with high iron levels (21). A thorough perfusion aimed at eliminating free and hemic iron is therefore thought to be an essential requirement. In order to test this requirement, rat kidneys were perfused to different blanching degrees with cold 0.9% NaCl solution. Iron contents in cytosol and binding capacity were then correlated (Figure 4). An inverse relation was observed between endogenous iron levels and MCR binding. Endogenous iron was also measured in plasma (30 μM), hippocampus (2.6 μM) and whole brain (3.1 μM).

At variance with kidney MCR, it has been demonstrated that EDTA is not necessary for steroid binding in brain MCR buffer formulations (9). Since iron levels are, at least, ten times lower in brain cytosol than in kidney cytosol, this might be the reason kidney MCR showed such a dependence on EDTA in a cell free system. If this hypothesis is valid, one would expect that stability properties of brain MCR might be different from kidney MCR under destabilizing conditions. This is exactly what Fig 5 demonstrates. Hippocampal MCR exhibited 50% of maximum binding.
capacity after 12 min at 25°C preincubation in buffer I in the absence of chelators and exogenous hematin. On the other hand, kidney MCR was unable to bind steroid under the same condition. EDTA and DP protected hippocampal MCR as well as kidney MCR against inactivation induced by the presence of exogenous hematin. However, incubations performed with chelators only, demonstrated the lack of effect of EDTA and DP on hippocampus -confirming S.Emadian's previous report (5)-, but not on kidney where endogenous iron levels are higher.

![Graph](image)

**FIG. 5**

**Hippocampus MCR Stability at High Temperature:** MCRs from rat hippocampus (panel A) or kidney (panel B) were destabilized by preincubation at 25°C in buffer I in the absence or presence of 0.1 mg hematin/mL and/or 10 mM chelators. Results represent the means of three independent replicate experiments.

**FIG. 6**

**Hydrogen Peroxide Inactivation:** MCR was prepared in buffer II without (panel A) or with (panel B) 20 mM molybdate (Mo). Preincubations for 25 min at 25°C (pre) and incubations with 10.1 nM [3H]-ALDO at 0°C for 12 h (post) were carried out in the presence or in absence of H₂O₂ or 2-ME (1x = 10 mM). Results (mean ± SD, n=4) are expressed as % maximum binding obtained in the best condition (B'). Significant differences (p < 0.001) are: panel A vs panel B; [D] vs [A,B,C,E,F]; [B'] vs [A',C',D',E',F']; [E'] vs [B',F']; [A'] vs [C',F']; [C'] vs [D',E',F']; [D'] vs [E',F'].

**Inhibition of Receptor Activation and Transformation by H₂O₂:** It is known that H₂O₂ inhibits reversibly glucocorticoid binding to liver cytosol in the absence of thiol reagents. However H₂O₂ and molybdate protect glucocorticoid receptors against thermal inactivation (22).
Stability of MCR in Cell-free Systems

Vol. 59, No. 7, 1996

Molybdate 20 mM - + - - - - -
2-ME 20 mM + + - - + + +
2-ME 40 mM - - - + - - -
H₂O₂ 10 mM - - + + - - -
EDTA 10 mM + + + + - - -
DP 10 mM - - - - - - +
Iron 100 μM - - - - + + +

**FIG. 7**

**DNA-Cellulose Binding of Steroid-Receptor Complexes:** [¹H]ALDO-MCR complexes were obtained by incubation for 3 h at 0°C in buffer IV. After a gel filtration through Sephadex G-25 equilibrated in buffer III, complexes were transformed by heating (25 min at 25°C) in buffer III supplemented as indicated the bottom of the figure. Then, complexes were sown on DNA-cellulose columns equilibrated in the same transformation buffer, washed and eluted with 0.5 M phosphate-Triton X-100 buffer at pH 6.5. Bars represent the mean ± SD (n=6). Significant differences are (p < 0.001): [A] vs [B,C,E,F,G] [D] vs [B,C,E,F,G]; [F] vs [A,B,C,D,E]; [G] vs [A,B,C,D,E]; (p < 0.010): [F] vs [G].

In the preceding section we demonstrated the inhibitory properties of tissue iron in homogenates and suggested that this cation could be responsible for the non-proteolytic inactivation of kidney MCR. Iron may exert its actions by reacting with carboxyl, imidazole and thiol groups. Iron may also act by catalyzing the oxidation of Cys. The latter possibility would lead to a receptor form comparable to the H₂O₂-treated glucocorticoid receptor form. According to this hypothesis, kidney MCR would have similar properties in an oxidative environment. In order to test this, kidney cytosol was prepared in the presence or absence of molybdate, and preincubated 25 min at 25°C with or without H₂O₂ and/or 2-ME. The buffers contained 20 mM NaN₃ to fully inhibit endogenous catalase (final preparations were negative on o-phenylenediamine test). Controls demonstrated that 20 mM NaN₃ had no effect on steroid binding.

As shown in Fig. 6, kidney cytosol loses its [¹H]-ALDO binding capacity when it is preincubated for 25 min at 25°C in the absence of molybdate (panel A). But thermal inactivation was prevented if molybdate was present during the preincubations (panel B). Due to the oxidation of thiol groups required for steroid binding, we could not measure binding capacity for 10 mM H₂O₂-treated receptors in the absence of 2-ME (in the presence or absence of molybdate). Similar properties were previously described for glucocorticoid receptor (22).

The addition of 2-ME to cytosol incubation media significantly stabilized steroid-binding capacity (columns D, D’ and F’). But the destabilizing effect of H₂O₂ is prevented if 2-ME is present in the preincubation (columns E and F). Our results suggest that both, GCR and MCR, have equivalent mechanism for activation/inactivation processes.

In order to test the possibility that oxidation inhibits receptor binding to DNA-cellulose, cytosol was first incubated under the most favorable condition with [¹H]-ALDO in the presence of 1.0 μM RU-28362, and then, chromatographed on Sephadex G-25 to remove molybdate, EDTA, 2-ME and free steroid from steroid-receptor complexes (23). These complexes were then transformed
by heating at 25°C for 25 min and the ability to bind DNA-cellulose was measured (Fig.7). Molybdate as well as pretreatment with 10 mM H₂O₂ inhibited over 80% of DNA-cellulose binding. However, the complex was protected by this oxidation from thermal inactivation. In effect, an excess of 40 mM 2-ME added after H₂O₂-pretreatment restored DNA-binding capacity. Iron totally abolished DNA-binding, but 10 mM EDTA restored 43% and 5 mM DP 65% of this capacity.

This showed that iron was also harmful for transformation, which was not entirely reverted by chelators as with the activation event.

Discussion

In this paper we demonstrated that kidney MCR is particularly sensitive to oxidation processes. This high sensitivity might be attributed to cysteins residues due to the high reactivity exhibited with specific sulfhydryl reagents such as NEM and DTNB. If true, this would explain the high sensitivity to metal-ions (such as iron) and the H₂O₂-effects.

Proteolytic inactivation may be partially responsible for the lack of MCR stability in cell-free systems. It has been reported (17) that a large series of serine-protease inhibitors failed in protecting MCR. However, we showed (Fig.1) that the presence of PMSF (not assayed in that series) in buffer formulations protected MCR binding capacity in a dose-dependent fashion. This effect was only partial suggesting that other mechanisms might be involved besides the putative proteolytic effect. S. Ganesan et al (24) and L. Lemesle et al. (25) have reported that PMSF also reacts with thiol groups. Consequently, it is unclear whether PMSF-stabilizing effect on MCR is due to its ability to inhibit proteases or because it reacts with MCR sulfhydryl groups and prevents the inactivation, similar to the reaction observed in DTNB- and NEM-reversion assays (Fig.2).

The non proteolytic inhibition should not be assigned to Ca²⁺. In effect, the nonspecific chelator EDTA (which can also chelate iron) was more efficient than the Ca²⁺-specific chelator, EGTA. On the other hand, free or hemic iron could greatly diminish MCR binding capacity, while dipyridyl prevented that inhibitory effect.

The ability of iron to facilitate electron transfers predicts the potential for toxicity that accompanies its ubiquity in cells. It is not surprising that oxidative phenomena have also been implicated when its cellular compartment is disrupted in homogenization processes. Nature generally enforces a strict physical separation of iron using several strategies to avoid the initiation or potentiation of peroxidations (26). Thus, under circumstances where compartmentalization is altered, free or complexed iron can exert profound effects on proteins. In effect, iron may react with amino acids groups and disturb the structure (and the activity) of the protein, but it can also catalyzes oxidations even when it is chelated (26). Thus, brain receptor might be much more stable than kidney receptor in a less oxidative environment, and their properties may be different in cell-free systems. Whatever the actual mechanism (oxidation, reactivity with certain amino acids, etc.) the evidence suggests that higher levels of iron lowers MCR binding capacity (Fig.4).

Iron’s redox potential predicts the oxidation of Cys to disulfide form. The latter oxidation would lead to a MCR state comparable to the reversible peroxide-oxidized form. On the other hand, iron pretreatment inhibits DNA-binding, but EDTA and DP revert only partially the inhibition. Our observations on MCR properties are similar to those described for GCR (4,18,28). According to the general properties of steroid receptors, it can be hypothesized that MCR conformation may be altered in certain conditions by the presence of iron or other oxidants. One possible reason may be the high protrusion and, therefore, reactivity of cysteins present in the DNA-binding domain. In effect, J.M. Berg (27) demonstrated that nucleic acid binding proteins with periodic Cys residues (such as steroid receptors) also act as transition metal-binding factors.
This might explain the effect of iron on MCR-steroid binding and MCR-DNA binding capacities, specially in the absence of molybdate where Cys are more exposed in the DNA-binding domain of the transformed steroid receptors as well as in the hormone binding domain of untransformed receptors (4,22,28).

J.Bodwell et al.(4) have demonstrated that in GCR most sulfhydryl-modifying reagents (including DTNB) had little effect on steroid binding and that these compounds greatly interfere with the activation process. GCR also depends on reducing reagents in the 1 to 100 mM DTf range (3), while in MCR reached a plateau with a little on 5 mM 2-ME. This and the receptor's high sensitivity to NEM, DTNB and free or chelated iron, would indicate that thiol groups may be more exposed in MCR. So, we can expect a high reactivity (specially in the presence of oxidants) and this may be one of the most important reasons by which with the best buffer formulations we still observe a time- and temperature-dependent loss of MCR in kidney tissue. In this aspect, MCR differs from GCR.

We observed that 30 mM H$_2$O$_2$ or more prolonged incubation than 30 min at 25°C also inhibits ALDO binding in a partially reversible form and the inhibition of MCR transformation is now totally irreversible (data not shown). Limited peroxide-treatment inhibited steroid binding and DNA binding capacity (Figs. 6 and 7), but in this state MCR was protected from thermal inactivation. In effect, when a low potential environment was restored, hormone and DNA binding capacity were recovered quantitatively. Interestingly, the recovery of binding capacity after treatment with NEM and DTNB also occurs and no loss of binding capacity takes place at 25°C. Thus, the protection of thiol groups of kidney MCR with blocking reagents from oxidative inactivation suggests the possibility that this mechanism might be involved in activation-inactivation process.

The study of such oxidative process observed artefactually in vitro suggests a possible in vivo modulation of activated MCR availability. The potential role of oxidation/reduction in regulating the function of proteins has been described in protooncogenes (29) and GCR (30,31). Preliminary experiments we have just performed using reduced and oxidized physiological thiol compounds such as thioredoxin, glutathion and free cystein, showed results (unpublished) similar to those presented with sulfhydryl reagents. Currently, we are carrying out experiments designed to obtain a more definitive answer to this question.

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