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Metal Oxyanion Stabilization of the Rat Glucocorticoid Receptor Is Independent of Thiols*

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The ability of sodium molybdate, both to stabilize the steroid binding activity of glucocorticoid receptors and to prevent the activation of receptor-steroid complexes to a DNA binding species, has long been thought to involve thiols. Two receptor thiols in particular, Cys-656 and Cys-661 of rat receptors, have been suspected. The requirements for the action of molybdate, as well as two other metal oxyanions (tungstate and vanadate) known to exert the same effects as molybdate, have now been examined with receptors in which these thiols, or a third cysteine in the steroid binding cavity (Cys-640), have been mutated to serine. No mutation prevented any metal oxyanion from either stabilizing steroid-free receptors or blocking the activation of complexes for binding to nonspecific or specific DNA sequences. Thus, Cys-640, Cys-656, and Cys-661 are not required for any of the effects of molybdate, tungstate, or vanadate with rat glucocorticoid receptors. Studies with hybrid receptors, and with a 16-kDa steroid binding core fragment containing only 3 cysteines at positions 640, 656, and 661, indicated that no cysteine of the rat receptor was needed to maintain responsiveness to molybdate. Even when all of the thiol groups in crude cytosol were blocked by reaction with excess methyl methanethiolsulfonate, each metal oxyanion was still able to stabilize the steroid binding of receptors. These results argue that molybdate, tungstate, and vanadate each interact with the receptor or an associated nonreceptor protein(s) in a manner that does not require thiols.

An indirect mechanism of molybdate action was evaluated in light of the recent report that the whole cell actions are mediated by increased levels of intracellular cGMP. Under cell-free conditions, however, the effects of molybdate could not be reproduced by cGMP derivatives. Evidence consistent with a direct effect was that molybdate, tungstate, or vanadate each modified the kinetics of proteolysis of wild type receptors at 0 °C by trypsin, presumably due to induced conformational changes of the receptor. This alteration of trypsin digestion constitutes yet another effect of metal oxyanions on the glucocorticoid receptor.

Studies on the mechanism of steroid hormone action have been greatly facilitated by the unique effects of sodium molybdate. The normal sequelae for glucocorticoid receptors are steroid binding to the steroid-free receptor, activation to a species with increased affinity for DNA and nuclei, translocation into the nuclei, binding to the biologically active glucocorticoid-responsive elements (GREs),[1] and finally the regulation of transcription of the target genes. Originally, it was reported that millimolar concentrations of molybdate affected the first step by stabilizing the steroid binding activity of unoccupied glucocorticoid receptors (1). Soon, thereafter, it was found that similar concentrations of molybdate also prevented the second step of activation, or transformation, of progesterone receptor-steroid complexes (2). These discoveries subsequently permitted innumerable cell-free studies on the detailed properties of steroid-free and steroid bound receptors, such as the identification and role of heat shock proteins in both steroid binding and activation (3–9), the importance of thiols in steroid binding (10–12), the characterization of a steroid binding core (13), the observation of conformational changes in the receptor during steroid binding (13–15), and the detection of multiple steps in activation (16–19).

The ability of molybdate to stabilize steroid-free receptors and to block activation is general for all of the steroid receptors (20) and seems to be accomplished by preserving the association of hsp90 (and possible other proteins, such as hsp56) to the receptor (21, 22). The oxyanions tungstate (WO_4^{2-}) and vanadate (VO_3^-) are functionally equivalent to molybdate in that they inhibit both the loss of steroid binding activity (23) and activation (24, 25) for glucocorticoid, progesterone (26), mineralocorticoid (27, 28), and androgen (29) receptors. In fact, tungstate is more potent than molybdate (27–30) and vanadate is about five times more potent than molybdate (23).

The discovery of small molecular weight compounds, which mimic the actions of molybdate in intact cells, has meant that the documented properties of molybdate, tungstate, and vanadate are likely to be relevant for the functioning of receptors in whole cells. One endogenous factor was a heat-stable low molecular weight metal ion that contained all of the endogenous molybdenum but was not molybdate (31, 32). A second endogenous species was a novel ether-linked aminophosphoglyceride (33–35). More recent studies in intact cells revealed that cGMP was induced by molybdate and that cGMP derivatives could mimic molybdate in blocking the nuclear binding of vitamin D receptor complexes (36).

Despite the widespread utility and relevance of molybdate in cell-free and whole cell actions of steroid receptors, very little is known at a molecular level about how molybdate works. It has long been suspected that all of the effects of molybdate and related metal oxyanions might involve interactions with the

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[1] The abbreviations used are: GRE, glucocorticoid-responsive element; TAPS, 3-{[tris(hydroxymethyl)-methyl]amino}-propanesulfonic acid; Dex, dexamethasone; Dex-Mes, Dex-21-mesylate; TBS, Tris-buffered saline; Sp-8-BR-cGMPS, *S* configuration of cyclic 8-bromoguanosine-3′,5′-monophosphorothioate; MMTS, methyl methanethiolsulfonate.

thiol groups of glucocorticoid receptors (20), since stable complexes of molybdate with thiols are well known (37–39). Molybdate was also reported to interact directly with cysteines of the steroid binding domain of glucocorticoid receptors to cause a peroxide-mediated loss of steroid binding activity (40). Furthermore, it has been proposed that two thiols (Cys-656 and Cys-661 of rat receptors) in the steroid binding domain are required for molybdate action, although there is no evidence that molybdate directly complexes these thiols (41). Likewise, vanadate interacts with thiols and often undergoes redox reactions with the thiols of cysteines (42). However, it should be noted that, in the absence of exogenous oxidants, thiol-disulfide equilibria do not appear to influence either steroid binding or the activation of glucocorticoid receptors bound by agonists or antagonists (43).

Two interesting studies have lately cast doubt on the working hypothesis that molybdate acts by complexing with receptor thiols. First, the similar ability of molybdate, tungstate, and vanadate to preserve the association of hsp90 with either glucocorticoid receptors or $pp60^{v\text{-}src}$ (44), and of molybdate to stabilize a complex of hsp90 with v-Raf (45), suggested that the oxyanions were interacting with the only common component, *i.e.* hsp90. Second, the fact that cGMP was induced by, and could replace, molybdate to block the nuclear translocation of vitamin D receptors in intact cells (36) argued that the actions of molybdate may be indirect and mediated by cGMP. The purpose of this study was, therefore, to determine whether molybdate actions require a chemical interaction with receptor thiols in general, and with Cys-656 and Cys-661 in particular. At the same time, we examined whether the effects of molybdate on receptors might result from either an indirect effect, such as elevated cGMP, or some direct interaction that might be detected by a physicochemical change, such as the protease digestion pattern of receptors.

MATERIALS AND METHODS

Unless otherwise indicated, all operations were performed at 0 °C.

Chemicals—[1,2,4,-^{3}H]Dexamethasone (Dex) was purchased from Amersham Corp. [6,7-^{3}H]Dexamethasone 21-mesylate (Dex-Mes) and ENHANCE were obtained from DuPont NEN. TAPS and dextran were purchased from Calbiochem. Sodium tungstate was from Fluka Chemical Co. (Ronkonkoma, NY), and V8 protease was obtained from U. S. Biochemical Corp. Sodium molybdate was from Baker Chemical Co. (Phillipsburg, NJ). Tween, acrylamide, bisacrylamide, and SDS were purchased from Bio-Rad. All other chemicals were obtained from Sigma. The monoclonal antibody BuGR2 was purchased from Affinity Bioreagents (Neshanic Station, NJ) and the polyclonal antibody aP1, which was raised against the carboxyl-terminal region of the rat glucocorticoid receptor (46), was a gift from Dr. Bernd Groner (Friedrich Miescher-Institut, Basel, Switzerland). Sp-8-Br-cGMPS (*S* configuration of cyclic 8-bromoguanosine-3′,5′-monophosphorothioate) was a gift from Dr. Julia Barsony (National Institutes of Health).

Buffers and Solutions—TAPS buffer (25 mM TAPS, 1 mM EDTA, and 10% glycerol) was adjusted to pH 8.8 or 9.5 at 0 °C with NaOH. Tris-buffered saline (TBS) contained 20 mM Tris and 0.28 M sodium chloride, pH 7.5. The 2 × SDS sample buffer contained 0.6 M Tris, pH 8.85, 0.2 M dithiothreitol, 2% SDS, 20% glycerol, and bromphenol blue. Western blot transfer buffer was comprised of 25 mM Tris, pH 8.3, 192 mM glycine, and 20% methanol.

Cells—HTC spinner cultures were grown in Swim's S77 medium supplemented with 5% each of heat-inactivated (50 °C/60 min) newborn and fetal calf serum and 0.03% glutamine. The construction of wild type C640S and C656,661S mutant glucocorticoid receptors and their calcium phosphate-mediated transient transfection into monolayer cultures of COS-7 cells were as described previously (12). Transfection of the Z540C fusion plasmid (a gift from Dr. Keith R. Yamamoto, University of California, San Francisco via Dr. William Pratt, University of Michigan) was the same as the mutant glucocorticoid receptors.

Preparation of Cytosol—HTC cell cytosolic receptors were prepared by freeze-thaw lysis and centrifugation at 100,000 × *g* as described previously (47). Transfected receptors in COS-7 cell cytosol were prepared by resuspending the frozen cell pellet in TAPS, pH 9.5, buffer, slowly thawing the pellet on ice, followed by centrifugation at 17,000 × *g*. The supernatants were used as cytosol.

Proteolytic Digestion and Steroid Binding Assays of Receptors—The 16-kDa receptor fragment was generated by proteolysis of COS-7 cell cytosol with 20–50 μg/ml of trypsin for 1 h at 0 °C. A 10-fold (w/w) excess of soybean trypsin inhibitor was then added to prevent further proteolysis. Full-length or 16-kDa receptors ± 20 mM molybdate, 20 mM sodium tungstate, or 5 mM metavanadate were incubated under destabilizing conditions (30 min at 20 °C for the 98-kDa receptor or 1.5–2.5 h at 0–10 °C for 16-kDa receptors) before determining the residual steroid binding activity. Specific binding was quantitated by treating the receptor solutions with 50 nM [^{3}H]Dex for 2.5 h at 0–4 °C, followed by 10% dextran-coated charcoal suspension (20% with 16-kDa complexes) to remove free steroid, and subtracting the nonspecific binding seen in the presence of a 500-fold excess of unlabeled Dex. Receptor solutions were covalently labeled for 2.5 h at 0–4 °C with 150 nM [^{3}H]Dex-Mes ± a 100-fold excess of unlabeled Dex.

HTC receptors ± 20 mM molybdate, 20 mM tungstate, or 5 mM metavanadate were incubated various concentrations of either trypsin, chymotrypsin, or V8 protease for 1 h at 0–4 °C. A 10-fold (w/w) excess of either soybean trypsin inhibitor (for trypsin) or aprotinin (for chymotrypsin and V8 protease) was then added to prevent further proteolysis. After digestion, the samples were labeled with [^{3}H]Dex-Mes for fluorography or bound with [^{3}H]Dex to determine specific steroid binding as described above.

SDS Gel Chromatography and Fluorography—Samples were diluted 1:2 in 2 × SDS sample buffer, heated for 5 min in a boiling water bath, and analyzed on either 10.8 or 12% polyacrylamide gels run in a water cooled Bio-Rad Protean II slab gel apparatus. Gels were fixed and stained in a 50% methanol, 7.5% acetic acid, 0.01% Coomassie Blue R-250 solution for 30 min at room temperature. The gels were destained overnight in a 10% methanol, 7.5% acetic acid solution, and then incubated with constant shaking in ENHANCE for 1 h followed by a 10% polyethylene glycol 8000 solution for 30 min at room temperature. The gels were dried on a Bio-Rad model 443 slab gel drier at 60 °C for 2 h and exposed to Kodak X-Omat XAR-5 film at −80 °C for at least 2 weeks.

Western Blotting—Samples were diluted with 2 × SDS sample buffer and analyzed on polyacrylamide gels as described above. The gels were equilibrated in transfer buffer for 30 min at room temperature prior to electrophoretic transfer of receptor to nitrocellulose membranes in a Bio-Rad Transblot Apparatus (100 mA overnight followed by 250 mA for 2 h). The nitrocellulose was stained in Ponceau S (0.02% Ponceau S and 0.04% glacial acetic acid in water) to localize molecular weight markers, incubated with 10% Carnation non-fat dry milk in 0.1TTBS (TBS containing 0.1% Tween) for 45 min and then washed with 0.1TTBS for 15 min. Primary antibody was diluted in 0.1TTBS (1:10,000 for aP1 or 1:5000 for BuGR2) and added to the nitrocellulose for a 2 h incubation at room temperature. Biotinylated secondary antibody and ABC reagents (each diluted 1:1000; Vector Laboratories, Burlingame, CA) were each added for sequential 30-min incubations at room temperature. After each incubation, the nitrocellulose was washed for 15 min with 0.1TTBS, except for an additional wash with TBS containing 0.3% Tween immediately after incubation with the ABC reagents. Detection of signal was performed by enhanced chemiluminescence (Amersham) using the recommended protocol of the supplier.

Mini-column and ABCD Assays—Mini-column assays were performed as described (48). Briefly, glucocorticoid receptors from HTC cytosol were incubated with ethanol ± 50 nM [^{3}H]Dex ± a 500-fold excess of unlabeled Dex, in the absence or presence of metal oxyanions, for 2.5 h at 0 °C. Activation was affected by incubation at 20 °C for 30 min. The activated complexes were loaded onto a tandem column containing DNA-cellulose (Pharmacia Biotech Inc.) in the upper column and DEAE-cellulose (Whatman, Hillsboro, OR) in the lower column. After the cytosol (50 μl) had flowed into the DNA-cellulose bed, 7 ml of TAPS buffer, pH 8.8, was forced through the tandem columns using a syringe. The resins were dried and assayed for radioactivity. The receptor retained by the DNA-cellulose was calculated as a percentage of total receptor recovered from both columns.

For the ABCD assays, 25 μl of activated receptor-steroid complexes prepared as above for the mini-column cytosols was incubated for 1 h at 0 °C with 6 pmol of the below oligonucleotide in 15 μl of TAPS 8.8 buffer (total volume = 100 μl). This oligonucleotide,

5′-GATCCTGTACAGGATGTTCTAGCTACA-3′
3′-CTAGGACATGTCCTACAAGATCGATGT-5′

OLIGONUCLEOTIDE 1

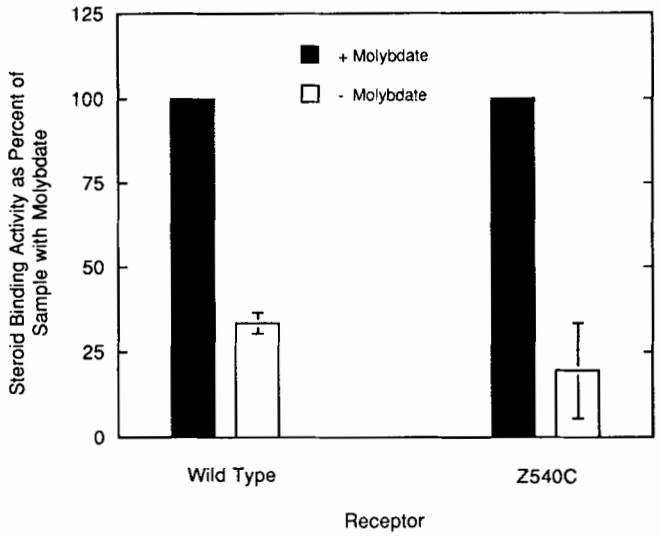

FIG. 1. **Molybdate stabilization of steroid binding activity with wild type and Z540C receptors.** Duplicate cytosols containing wild type or Z540C receptors from transiently transfected COS-7 cells were incubated ± 20 mM molybdate at 20 °C for 30 min before determining the specific binding of [^{3}H]Dex as detailed under "Materials and Methods." In each case, binding was expressed as percent of total binding in the presence of molybdate (±S.D., n = 8 for wild type; ± range, n = 2 for Z540C).

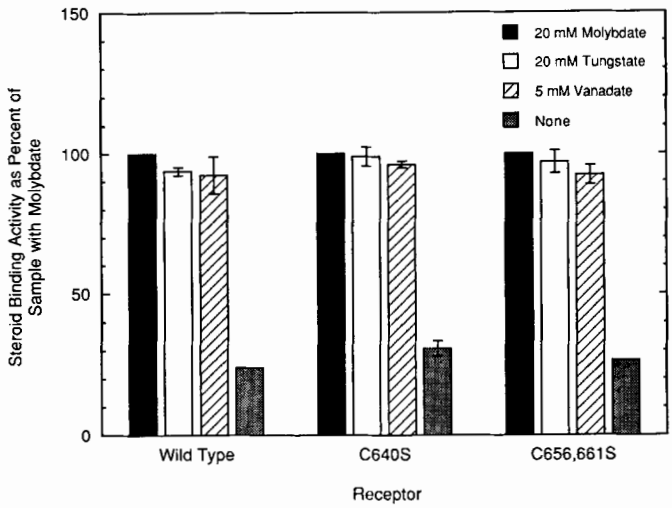

FIG. 2. **Stabilization of steroid binding activity for wild type and mutant receptors by molybdate, tungstate, and vanadate.** Duplicate cytosols containing wild type or mutant (C640S or C656,661S) receptors from transiently transfected COS-7 cells were incubated ± oxyanion (20 mM molybdate, 20 mM tungstate, or 5 mM vanadate) at 20 °C for 30 min before determining the specific binding of [^{3}H]Dex as detailed under "Materials and Methods." In each case, binding was expressed as percent of total binding in the presence of molybdate (±range, n = 2). The binding seen after heating in the presence of molybdate was 100 ± 3% of that seen at 0 °C in the absence of molybdate.

contains a GRE (underlined) of the rat tyrosine aminotransferase gene. The 5'-end of each strand of DNA was labeled with one molecule of biotin. The glucocorticoid receptor·steroid GRE complex was then bound to streptavidin-agarose (Life Technologies, Inc.), added as 30 μl of a 50% slurry (in 10 mM Tris, pH 7.5, 25 mM NaCl, 1 mM dithiothreitol, and 10% glycerol), during a 1-h incubation at 0 °C. This glucocorticoid receptor·steroid GRE-streptavidin-agarose pellet was washed twice with 1 ml of 20 mM Tris, pH 7.5, and assayed for radioactivity.

RESULTS

Molybdate Stabilization of Steroid Binding to Receptors Does Not Require Amino-terminal Sequences—The most straightforward approach for defining the possible role of each of the 20 cysteines of the rat glucocorticoid receptor (49) in mediating the effects of molybdate is to express truncated or mutant receptors from the appropriate recombinant cDNAs. Therefore, we first examined the hybrid protein Z540C, which had been constructed by fusing the cDNA elements of the entire β-galactosidase to the amino-terminal end of amino acids 540–795 of the rat receptor (50). This hybrid receptor binds steroid with high affinity (50)[2] and is associated with hsp90 (51) but is totally free of the amino-terminal 539 amino acids of the receptor. The data of Fig. 1 show that molybdate was equally capable of stabilizing the steroid binding of intact 98-kDa and hybrid Z540C receptors. Therefore, none of the 15 cysteines, or any other amino acid, between positions 1 and 539 of the rat receptor are necessary for molybdate stabilization. Any required receptor thiols would be among the remaining five cysteines located in the steroid binding domain.

Metal Oxyanion Stabilization of Steroid Binding to C640S and C656,661S Mutant Receptors—In order to determine the role of the three thiols in the core of the steroid binding domain (13), we examined the properties of two receptors in which these cysteines had been mutated to serine: C640S and C656,661S. We have previously reported that the C640S has a 3-fold reduced affinity for Dex but wild type properties with regard to the specificity of binding (52) and reaction with thiol reagents (12). The C656,661S double mutant displayed wild type behavior with regard to Dex binding affinity and reaction with thiol reagents (12). Therefore, there is nothing highly abnormal about these two mutant receptors.

The steroid binding activity of all three receptors was similarly stabilized by 20 mM molybdate when heated at 20 °C for 30 min (Fig. 2). Furthermore, 20 mM tungstate and 5 mM vanadate were equally effective in preserving the steroid binding capacity of receptors upon heating. Thus Cys-640, Cys-656, and Cys-661 are not required for the stabilization of steroid binding activity in 98-kDa receptors by any of the metal oxyanions.

With 17 other cysteines in the rat receptor, it seemed possible that other cysteines in the 98-kDa receptor might be able to substitute for one or more of the mutated cysteines. To rule out this possibility, we looked at the ability of molybdate to preserve the binding activity of the 16-kDa steroid binding core fragment formed by trypsin digestion of steroid-free receptors (13). This 16-kDa fragment contains only three thiols (Cys-640, Cys-656, and Cys-661) and has been shown (12) not to be associated with the ~15-kDa DNA binding domain that is also generated by trypsin digestion (43, 53, 54). There was a dramatic effect of molybdate with each 16-kDa steroid binding core fragment (Fig. 3). Not only was the 16-kDa fragment sequence of 537–673 sufficient for molybdate stabilization but also none of the three thiols present (*i.e.* Cys-640, Cys-656, or Cys-661) were required for stabilization of steroid binding activity by molybdate. From these results, we conclude that molybdate stabilization of the steroid binding activity of rat glucocorticoid receptors does not involve a direct interaction with any specific thiol of the receptor.

No Cytosolic Thiols Are Required for Metal Oxyanion Stabilization of Receptors—To confirm the above conclusion that no thiol of the glucocorticoid receptor is required for molybdate stabilization of steroid binding, we inquired whether a thiol group(s) of any protein was required by looking at the action of molybdate under conditions where all cytosolic thiols have been blocked. We had previously documented that the addition of 3 mM of the thiol-specific reagent methyl methanethiolsulfonate (MMTS) (55) to crude receptor solutions containing 20 mM molybdate both reduced the total thiol content to 0–5% of the initial levels of 0.8 mM and eliminated all covalent labeling of

[2] M. Xu and S. S. Simons, unpublished results.

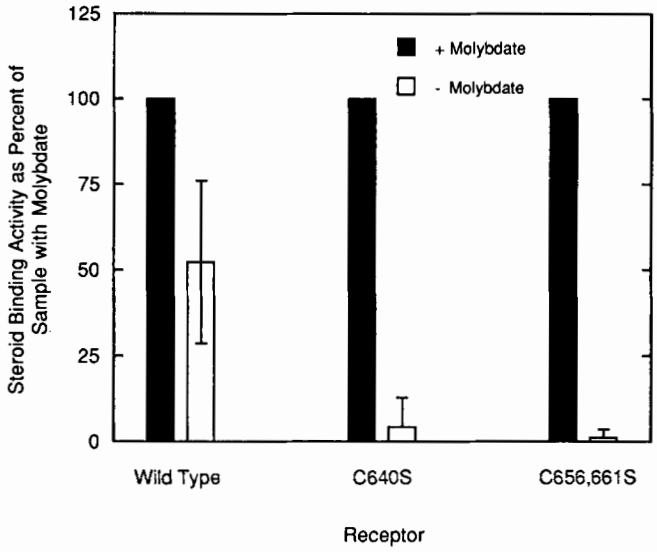

FIG. 3. **Molybdate stabilization of steroid binding activity with 16-kDa tryptic fragments of wild type and mutant receptors.** Duplicate cytosols containing wild type or mutant receptors from transiently transfected COS-7 cells were digested with trypsin and then incubated ± 20 mM molybdate at 10 °C for 2.5 h (wild type and C656,661S) or 90 min (C640S) before determining the specific binding of [^{3}H]Dex as detailed under "Materials and Methods." In each case, binding was expressed as percent of control receptors in the presence of molybdate (±S.D., n = 5 for wild type, = 4 for C640S and C656,661S). In each experiment, 16-kDa fragment preparations were found, by affinity labeling with [^{3}H]Dex-Mes and/or by Western blotting with aP1 anti-receptor antibody, to contain no full-length 98-kDa receptors and ≤15% of the 30-kDa mero-receptor (not shown).

proteins by Dex-Mes (10). Under these conditions, where all free thiols of the cytosol preparations have been converted to mixed disulfides, steroid-free rat (10) and mouse (56) receptors retained ~50% of the initial binding activity.

Receptors that were preincubated with 3 mM MMTS in the absence of molybdate were unstable and lost steroid binding activity with a $t_{1/2}$ of 0.99 ± 0.13 h (S.D., n = 4). However, the addition of 20 mM sodium molybdate to MMTS pretreated receptors caused a pronounced stabilization of the steroid binding activity (Fig. 4; $t_{1/2}$ = 4.3 ± 1.1 h (S.D., n = 4)). Similarly, tungstate and vanadate each stabilized the steroid binding of MMTS pretreated receptors (Fig. 4; $t_{1/2}$ = 4.9 ± 0.6 h and 3.4 ± 0.4 h (range, n = 2), respectively). Under these conditions, all covalent labeling of protein thiols by [^{3}H]Dex-Mes was prevented (see *inset* of Fig. 4). These results argue that metal oxyanion stabilization of the steroid binding activity occurs even when all accessible thiols have been blocked by conversion to a mixed disulfide. Thus, molybdate, tungstate, and vanadate stabilization does not require free thiols in either the receptor or in any other cytosolic protein.

Specific Thiols of Receptor Are Not Required for Prevention of Activation by Metal Oxyanions—A second property of molybdate, tungstate, and vanadate is their ability to block the activation of receptor-steroid complexes to a form with increased affinity for DNA (24). The association of hsp90 is required to preserve the unactivated state of receptor-steroid complexes, and the steroid binding state of free receptors (6, 8). However, it is possible that the contact points between hsp90 and receptor could be altered by the conformational changes attending steroid binding to receptors, thus exposing additional amino acids that might interact with molybdate (13). For this reason, Cys-640, Cys-656, and Cys-661 still might be involved in molybdate inhibition of activation, even though they are not required for molybdate stabilization of steroid binding. To examine this possibility, we used the previously well characterized mini-column assay (19, 48) to determine the binding of complexes to calf thymus DNA. As shown in Fig. 5*A*, the ability of molybdate to block the activation of receptors is unaffected by the mutation of those cysteines in the steroid binding domain that have been proposed to interact with molybdate (41).

The effects of molybdate on the binding of receptor-steroid complexes to biologically active DNA sequences containing the second GRE of the tyrosine aminotransferase gene (57) were examined next. Gel mobility shift assays could not be used because the comigration of receptor and nonreceptor proteins from crude HTC cell cytosol solutions prevented the quantitation of bound receptors (data not shown). Such an identical mobility of receptor and nonreceptor bands has also been reported for chick intestinal nuclear extract binding to vitamin D response elements (59). In contrast, easily quantifiable binding of receptors was seen with an avidin-biotin-coupled DNA (ABCD) binding assay. DNA binding was increased by activation and was prevented by increasing salt (data not shown), just as has been reported for DNA-cellulose column assays (60, 61) and other solution DNA binding assays (58). Despite the low amount of DNA used, >50% of the added activated complexes usually bound to DNA. The ABCD assay had the added advantages that receptor binding was followed directly, since the radioactive label was on the glucocorticoid receptor as opposed to the DNA, and easily quantitated by counting the final pellets. Using this assay, we found that mutation of cysteines 640, 656, and 661 did not prevent metal oxyanions from blocking activation and the subsequent binding to a GRE (Fig. 5*B*). Thus, the ability of molybdate, tungstate, and vanadate to block receptor activation to specific DNA binding species did not require Cys-640, Cys-656, or Cys-661.

The binding to specific DNA sequences by C656,661S mutant receptors, relative to wild type receptors (Fig. 5*B*), was less than that to nonspecific DNA (Fig. 5*A*). This may reflect an altered affinity of C656,661S receptors for specific, but not nonspecific, DNA sequences. However, we cannot yet rule out differences in activation efficiency, because the experiments of Fig. 5, *A* and *B*, were not performed concomitantly.

Molybdate Stabilization of Steroid Binding Activity of Cell-free Receptors Is Not an Indirect Effect of cGMP—In the absence of any evidence for a direct effect of molybdate on the glucocorticoid receptor protein, it seemed possible that the effects of molybdate might be indirect and mediated by nonreceptor molecules. In fact, it has recently been proposed that the intracellular action of molybdate to block nuclear binding of vitamin D receptors is mediated by increases in intracellular 3′,5′-cGMP. Furthermore, 1 mM dibutyryl cGMP could mimic all of the whole cell effects of 10 mM sodium molybdate (36). We therefore examined whether cGMP was as effective as molybdate in stabilizing the steroid binding activity of cell-free receptors heated at 20 °C for 30 min. In these experiments, both dibutyryl cGMP and freshly prepared solutions of a more esterase-resistant derivative Sp-8-Br-cGMPS[3] were used either without and with 0.5 mM isobutylmethylxanthine to inhibit phosphodiesterase activity. Surprisingly, 0.01–10 mM dibutyryl cGMP, or 1–5 mM Sp-8-Br-cGMPS, were not only unable to cause any stabilization of steroid binding activity but also, with added isobutylmethylxanthine, decreased the specific binding below that seen with just buffer (data not shown). Thus, cGMP could not substitute for molybdate in the cell-free stabilization of steroid binding activity and was not an obligatory intermediate for molybdate stabilization of cell-free receptors.

Effect of Metal Oxyanions on the Proteolysis of Receptors—Proteases have proved very useful in detecting steroid-induced conformational changes in receptors (13–15). As a means of

[3] J. Barsony, personal communication.

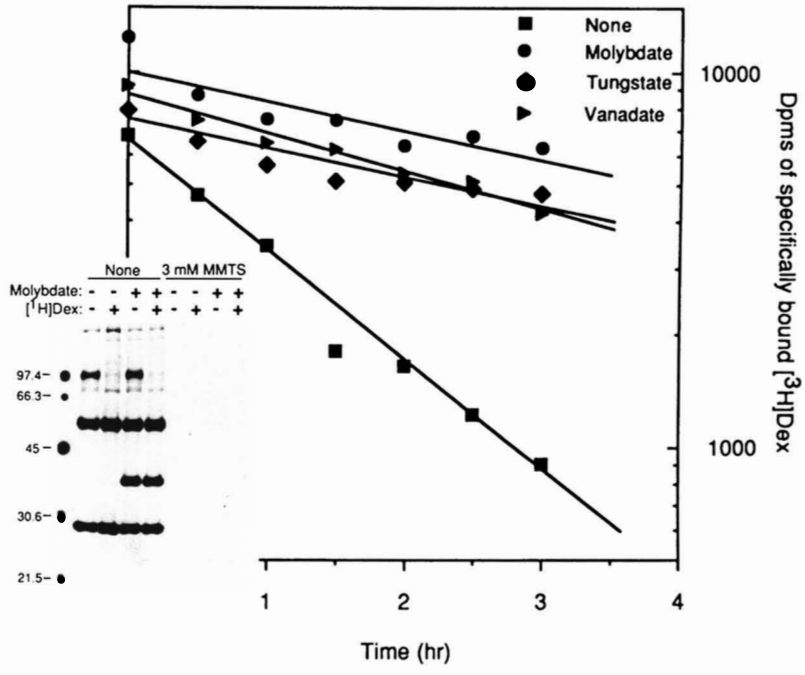

FIG. 4. **Metal oxyanion stabilization of steroid binding in cytosols reacted with excess MMTS.** Duplicate HTC cell cytosols containing endogenous receptors were treated with buffer ± 3 mM MMTS for 30 min at 0 °C to block all free -SH groups and then adjusted to contain 0 or 20 mM molybdate, 20 mM tungstate, or 5 mM vanadate. At various times after the addition of metal oxyanion, the receptors were incubated with [^{3}H]Dex ± 500-fold excess of nonradioactive Dex for 2 h at 0 °C. The specific binding of [^{3}H]Dex to receptors was then determined as described under "Materials and Methods" and plotted *versus* the time of incubation with metal oxyanion before adding steroid. *Inset*, fluorography of [^{3}H]Dex-Mes-labeled MMTS-pretreated cytosol. MMTS preincubated cytosols ± 20 mM molybdate were incubated with [^{3}H]Dex-Mes ± 100-fold excess of nonradioactive Dex to label the receptors and other thiol-containing proteins. The proteins were separated by SDS-PAGE and visualized as described under "Materials and Methods." Similar results were obtained in a second experiment.

determining whether molybdate, tungstate, or vanadate directly interact with the glucocorticoid receptor, we therefore determined the protease digestion patterns of receptors ± metal oxyanion. Digestion was performed with steroid-free receptors because of the above demonstrated ability of metal oxyanions to stabilize steroid-free receptors (see Figs. 1–4). Also, sequences near the proposed molybdate interaction site(s) from 568 to 671 (14, 62) in the steroid binding domain are known to be much more susceptible to trypsin digestion in ligand-free than in ligand-bound receptors (7, 13, 63). Molybdate was found to retard the digestion of steroid-free receptors by trypsin, as determined by the kinetics both of the formation of affinity-labeled (Fig. 6*A*) and Western blotted (data not shown) fragments and of the reduction of steroid binding activity (Fig. 6*B*). A similar inhibition of trypsin digestion was afforded by tungstate and vanadate (data not shown and Fig. 6*B*). Only minor differences ± molybdate were seen in the steroid binding or rate of formation of affinity labeled digestion products with chymotrypsin and no differences accompanied V8 protease digestions (data not shown). This decreased rate of proteolysis with trypsin does not appear to be due to an inhibition of trypsin activity by metal oxyanion, since digestion of other [^{3}H]Dex-Mes-labeled proteins (Fig. 6*A*) and of the general Coomassie Blue-stained proteins (data not shown) was relatively unaffected. Thus, trypsin appears able to detect subtle conformational changes in the receptor protein that are caused by metal oxyanions.

DISCUSSION

Two effects of the metal oxyanions molybdate, tungstate, and vanadate on glucocorticoid receptor function have been reported in the literature: stabilization of steroid binding and blockage of activation. It has long been suspected that both effects involve interactions with the thiol group of one or more cysteines. Cysteines 656 and 661 of the rat receptor have been proposed as being intimately involved in molybdate action (41). We now find that receptors in which Cys-656 and Cys-661, or Cys-640, have been mutated to serine remained fully sensitive to molybdate, tungstate, and vanadate with regard to both stabilization of steroid binding (Fig. 2) and inhibition of activation to a species that will bind to nonspecific and/or specific DNA sequences (Fig. 5). Thus, these 3 cysteines are not required for the actions of metal oxyanions. Further studies with wild type and mutant receptors in the context of the full-length and truncated proteins revealed that no thiol of the receptor is required for molybdate stabilization of steroid binding (Figs. 1–3). These conclusions were confirmed by the observation that metal oxyanions could stabilize the steroid binding of receptors, even when all of the exposed cysteines were blocked by conversion to mixed disulfides (Fig. 4). A similar stabilization of MMTS-treated receptors by molybdate had been reported (64). However, the concentration of MMTS used in this earlier study greatly reduced the steroid binding. Since much more steroid binding is retained when all, as opposed to some, of the thiols are blocked (10), not all of the thiols may have been modified by MMTS in this earlier study. The current data, with MMTS concentrations that afford apparently complete blockage of receptor SH groups (Fig. 4), argue that metal oxyanion action does not require the thiol group of any molecule, even hsp90.

What functional group, then, might molybdate, tungstate, and vanadate be interacting with to stabilize the steroid binding activity of receptors? Complexes in which molybdenum (VI) is bound to a two sulfur atom bridging unit have been described (65), but we are not aware of any examples in which molybdate (MoO_4^{2-}) associates with a disulfide bond. Thus, it is unlikely that the capacity of molybdate to stabilize the steroid binding of MMTS-treated receptors is due to complexation with the disulfide form of a cysteine that was initially present as a thiol. Molybdate, tungstate, and vanadate have each been observed to form chelates with a variety of functional groups (20, 66). In particular, molybdate also associates with carboxyl, imino, amino, and phosphate groups (18, 38, 39). Vanadate forms complexes with amines and alcohols and possibly with amides (42). Thus, these oxyanions could stabilize the steroid binding of receptors by interacting with a variety of functional groups other than thiols.

It has yet to be unequivocally established that the mechanisms for metal oxyanion stabilization of steroid binding, and prevention of activation, of receptors are the same. In fact, fluoride and glucose 1-phosphate preserve steroid binding activity but are unable to inhibit activation (24). Studies with full-length mutant receptors have now eliminated any requirement of Cys-640, Cys-656, or Cys-661 for metal oxyanion inhibition of activation (Fig. 5). However, we could not use the

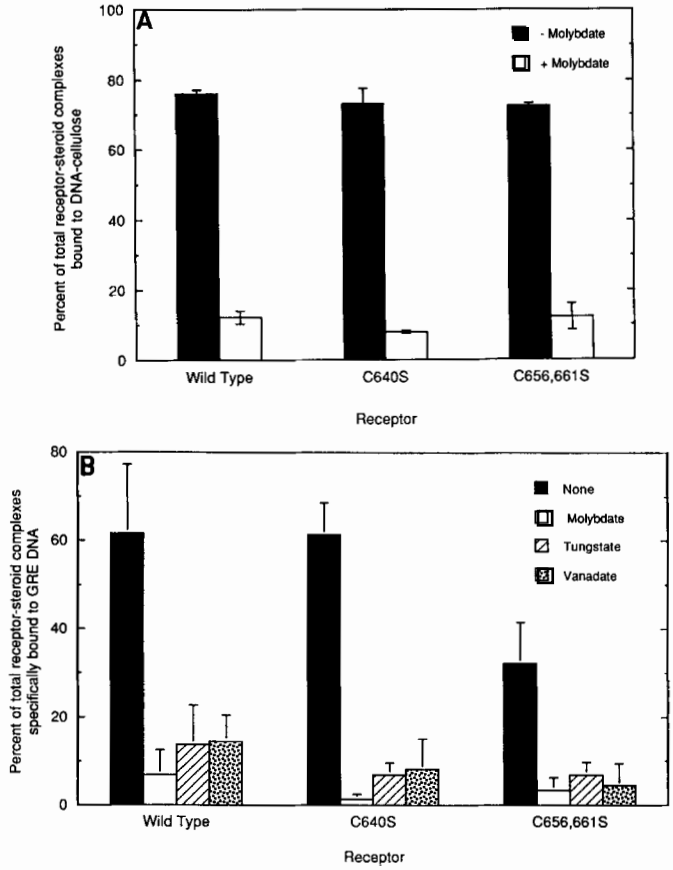

FIG. 5. **Ability of metal oxyanions to block the activation of wild type and mutant receptors for binding to nonspecific (*A*) or specific DNA sequences (*B*).** Cytosols containing wild type or mutant receptors from transiently transfected COS-7 cells were bound with [^{3}H]Dex ± 500-fold excess nonradioactive Dex and then treated ± 20 mM molybdate, 20 mM tungstate, or 5 mM vanadate before being activated at 20 °C for 30 min. The binding of duplicate samples to nonspecific calf thymus DNA (*A*) and specific GRE DNA (*B*) was determined as detailed under "Materials and Methods." The binding was expressed as percent of total added receptors and the average of two experiments (±range) (*A*) or three or five experiments (±S.D.) (*B*) was plotted.

16-kDa core fragment to rule out the other thiols because the 16-kDa fragment does not include the DNA binding domain (13) and has not been observed to bind DNA (53, 54). Mutations of the cysteines in the DNA binding domain could not be used, since they eliminate DNA binding (reviewed in Ref. 49). Finally, we could not use MMTS to examine the requirement of free thiols in molybdate inhibition of activation. Although MMTS is known not to prevent activation (67), the level of DNA binding of MMTS-treated complexes is severely reduced (19, 48, 67, 68).

Several molecules besides the steroid receptors are stabilized by metal oxyanions and thus could be the target(s) of metal oxyanion action. Molybdate preserves both the high molecular weight oligomeric structures of the dioxin receptor (69, 70), which has been shown to contain hsp90 (71), and a v-Raf·hsp90 complex (45). Similarly, the integrity of the complex between pp60$^{v\text{-}src}$ (44) and hsp90 is maintained by molybdate, tungstate, and vanadate, which led to the proposal that each case of metal oxyanion stabilization proceeded via an interaction with the common subunit of hsp90. In fact, both molybdate and vanadate have recently been observed to directly interact with hsp90 (72). On the other hand, the facts that relatively high concentrations of metal oxyanions are necessary for an effect (~1–20 mM) and that molybdate stabilization of hsp56 association with hsp90 requires the presence of glucocorticoid receptors (73), coupled with the probability that metal oxyanions can interact with at least one type of functional group other than a thiol, leaves open the possibility of more than one target.

The mechanism of molybdate action is currently obscure. We have ruled out an indirect response via increased levels of cGMP (36) in broken cell systems. Vanadate has a higher binding affinity for hsp90 than molybdate (72), in line with vanadate being effective at lower concentrations than molybdate in stabilizing the steroid binding of receptors (Ref. 23 and Fig. 2). Both vanadate and molybdate have been reported to cause the same increase in β-sheet content of hsp90 as ATP or heat (72). However, since heat or ATP both cause activation (18, 75), it is difficult to see how the same increased β-sheet formation can be related to the prevention of receptor-steroid complex activation by molybdate or vanadate.

An increased resistance of glucocorticoid receptors to trypsin digestion has been noted upon adding steroid (13) or metal oxyanions (Fig. 6) to steroid-free receptors. The effect of added steroid appears to be due to a conformationally induced change following steroid binding to the receptor (13). Similarly, the effect of added metal oxyanions is compatible with a conformationally induced change resulting from a direct contact between the oxyanion and receptor. Alternatively, these effects could reflect metal oxyanion-induced changes in the tertiary structure of an associated molecule, such as hsp90 (44). In this specific case, however, it is important to realize that the more rapid digestion in the absence of molybdate could not be due to the dissociation of hsp90 since all bands are still specifically labeled by [^{3}H]Dex-Mes (47), and hsp90 is required at least for the competitive steroid binding by excess Dex (8, 76). Although molybdate has been reported to be an inhibitor of general protein (77) and receptor (64) digestion by the endogenous proteases of rat liver cytosols (77), we do not feel that the reduced rate of receptor proteolysis by trypsin in HTC cell cytosols was due to an inhibition of protease activity by the metal oxyanions for two reasons. First, molybdate has no effect on chymotrypsin or papain digestion of receptors (24). Those proteases that are inhibited by molybdate could not be shown to attack receptors (77). Second, we have seen no effect of metal oxyanions on the trypsin digestion of other affinity labeled or Coomassie Blue-stained proteins (Fig. 6 and data not shown). It is interesting that we found a significant metal oxyanion inhibition of receptor proteolysis only with trypsin and not with chymotrypsin or V8 protease. This selectivity is reminiscent of the protease-detected steroid-induced conformational change in glucocorticoid receptors that was also seen only with trypsin (13).

Other possible mechanisms of action of metal oxyanions include forming bridges between the receptor and some other molecule, such as hsp90, and providing a template to help form and reinforce those tertiary structures of the receptor protein that are required for functional activity. The more rapid protease digestion of steroid-free receptors in the absence of metal oxyanions (Fig. 6) is consistent with the first hypothesis. The second hypothesis is supported by the fact that the regeneration of steroid binding activity with dithiothreitol after the reaction of receptors with high concentrations of MMTS (4 mM) was increased when molybdate was added along with the dithiothreitol (10). This last property of molybdate would not reflect a reassociation of hsp90 and thus increased steroid binding, since MMTS has been found not to cause any dissociation of hsp90 from glucocorticoid receptors (56). Furthermore, cell-free reassociation of hsp90 to glucocorticoid receptors occurs to any appreciable extent only at 30 °C in the presence of reticulocyte lysates with receptors that are immobilized on a matrix (76).

It has been reported that reduced thiols are required for the dissociation of hsp90 from glucocorticoid receptors to give acti-

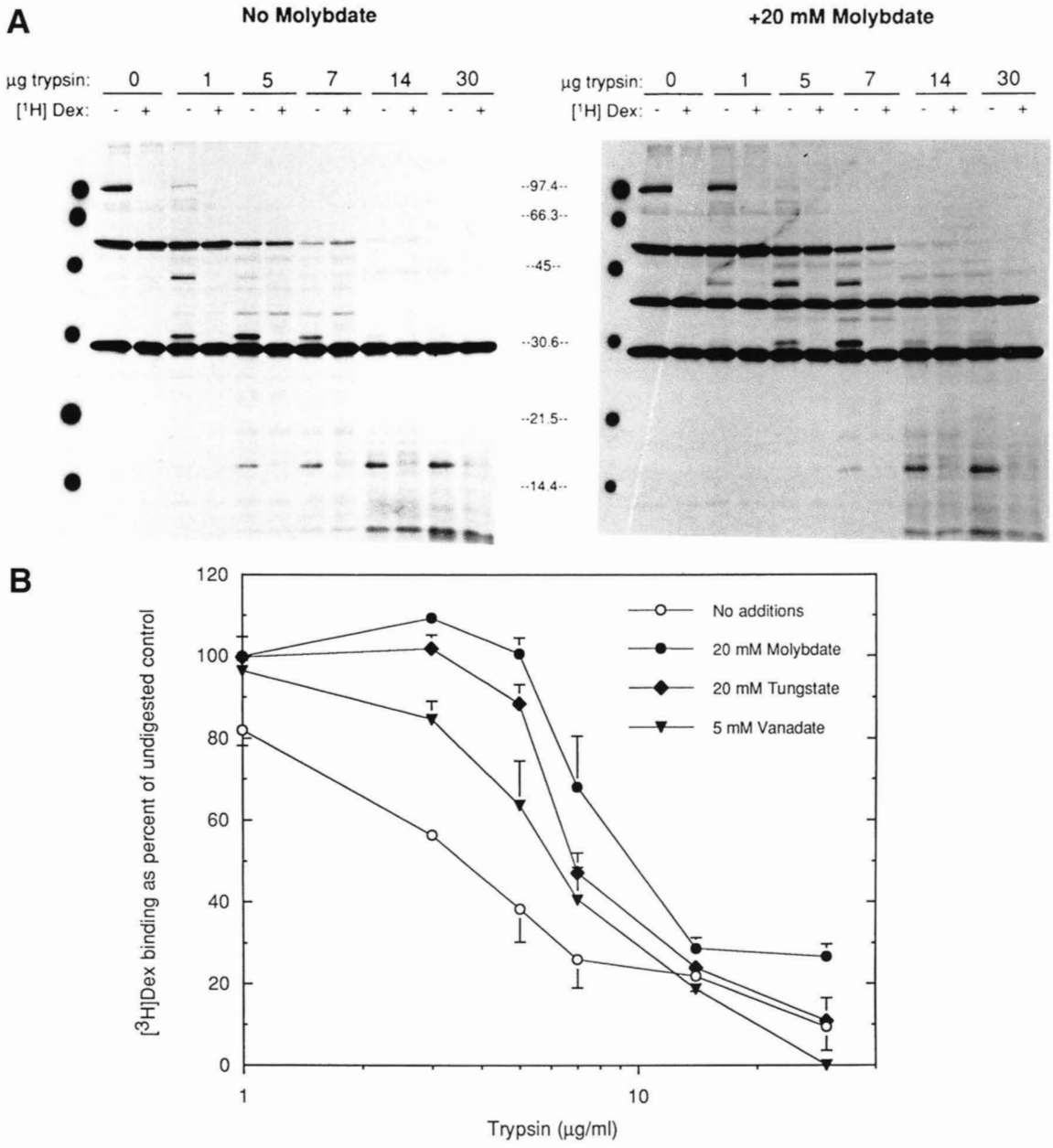

FIG. 6. **Effect of metal oxyanions on trypsin digestion pattern of wild type glucocorticoid receptors.** Cytosols containing HTC cell receptors were digested with increasing concentrations of trypsin ± 20 mM molybdate, 20 mM tungstate, or 5 mM vanadate at 0 °C for 60 min before adding an excess of soybean trypsin inhibitor to block further digestion. The samples were then either affinity labeled with [^{3}H]Dex-Mes ± 100-fold excess nonradioactive Dex or bound with [^{3}H]Dex ± 500-fold excess nonradioactive Dex. The affinity-labeled receptor fragments ± molybdate at 42, 30, and 16 kDa, the labeling of which was blocked by excess nonradioactive Dex, were visualized by fluorography after SDS gel chromatography (*A*), whereas the amount of [^{3}H]Dex binding was determined after the addition of dextran-coated charcoal (*B*) as described under "Materials and Methods." The molecular weights of the marker proteins, located in *A* by the spots at the left of each gel, are indicated by the numbers between the two gels. Similar results were obtained in a second experiment.

vated, or transformed, receptors (64, 67). However, the data of Figs. 2 and 5 establish that the thiols present in the proposed hsp90 binding sites (41, 62) are not required either for hsp90 association, to give the steroid binding form of receptors, or for the dissociation of hsp90 that accompanies activation.

In summary, the ability of the metal oxyanions molybdate, tungstate, and vanadate to stabilize the steroid binding activity of rat glucocorticoid receptors does not require thiol groups of the receptor, or of nonreceptor proteins, as was previously thought. Likewise, cysteines at positions 640, 656, and 661 are not necessary for these metal oxyanions to block activation of the receptor steroid complex. Thus, a variety of new mechanisms need to be explored in explaining the important properties of metal oxyanions with the steroid receptors.

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