Differential Effects of the Reversible Thiol-Reactive Agents Arsenite and Methyl Methanethiosulfonate on Steroid Binding by the Glucocorticoid Receptor†

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ABSTRACT: The hormone binding domain of the glucocorticoid receptor contains a unique vicinally spaced dithiol, and when it is bound by arsenite under conditions that are specific for reaction with vicinally spaced dithiols versus monothiols, steroid binding activity is eliminated [Simons, S. S., Jr., Chakraborti, P. K., & Cavanaugh, A. H. (1990) *J. Biol. Chem. 265*, 1938–1945]. The vicinally spaced dithiol lies in a region of the receptor that appears to be a contact site for hsp90, which is required for the high-affinity steroid binding conformation of the glucocorticoid receptor [Dalman, F. C., Scherrer, L. C., Taylor, L. P., Akil, H., & Pratt, W. B. (1991) *J. Biol. Chem. 266*, 3482–3490]. As part of a long-term project to develop a vicinal dithiol-specific agent that will permit studies of ligand-induced conformational changes in this region of the receptor, we have examined here the differential effects of two reversible thiol-reactive agents, arsenite and MMTS. At low concentration, arsenite inactivates the steroid binding activity of the unliganded receptor in a vicinal dithiol-specific manner, whereas dissociation of steroid from untransformed, transformed, or DNA-bound transformed receptors occurs only at concentrations typical of monothiol interactions. MMTS produces a unique bimodal effect on the steroid binding capacity of the unliganded receptor at pH 9 that is pH-dependent and becomes essentially unimodal at physiological pH. Whereas arsenite disrupts the dexamethasone–receptor complex more readily than the triamcinolone acetonide–receptor complex , MMTS has the opposite effect. During treatment for 1 h at 0 °C, neither reagent causes dissociation of hsp90 from the receptor. Also, pretreatment of the hsp90-free unliganded receptor with the covalent sulfhydryl-modifying agents *N*-ethylmalemide or iodoacetamide does not affect the ability of the receptor to be enzymatically refolded into a heterocomplex with hsp90 by reticulocyte lysate. The observations of this work are consistent with the concept that the vicinally spaced dithiol lies in a portion of the binding pocket that is critical for binding the D-ring region of the steroid, and they suggest that labeling of the receptor with a vicinally spaced dithiol-specific derivatizing agent may allow detection of conformational changes likely to occur in a critical region of the hormone binding domain on dissociation of hsp90.

To understand the molecular mechanism of glucocorticoid hormone action, we must define those features of glucocorticoid receptor (GR)[1] secondary and tertiary structure that determine the high-affinity steroid binding conformation. The most important of these determinants defined to date are the presence or absence of an intramolecular disulfide for a vicinally spaced pair of cysteine SH groups lying in the GR steroid binding domain (Miller & Simons, 1988; Chakraborti et al., 1992) and the association of the GR with hsp90 (Bresnick et al., 1989; Scherrer et al., 1990).

Early studies demonstrated that the steroid binding capacity of unliganded receptors was inactivated by treatment of cytosol with irreversible sulfhydryl-modifying agents, such as *N*-ethylmaleimide (Baxter & Tomkins, 1971; Koblinski et al., 1972; Rees & Bell, 1975), iodoacetamide (Young et al., 1975), and *p*-(chloromercuri)benzenesulfonate (Harrison et al., 1983). Bodwell et al. (1984) first introduced the use of the reversible reagent methyl methanethiosulfonate (MMTS) for the study of receptor SH groups. MMTS treatment reduces the affinity of the GR for the traditional agonists dexamethasone and cortisol by about a factor of 5, while there is very little, if any, decrease in the affinity for the nonconventional steroids deacylcortivazol and RU38486 (Miller & Simons, 1988). MMTS reacts in a highly specific manner with protein thiols to derivatize them by adding a thiomethyl group through formation of a mixded disulfide (Brocklehurst, 1979). The SH group can be regenerated by reagents that promote thiol–disulfide interchange. At low concentrations, MMTS appears to promote the formation of an intramolecular disulfide bond in the receptor (Miller & Simons, 1988; Chakraborti et al., 1990, 1992), implying that two thiol groups must be located very close to each other in the properly folded high-affinity steroid binding conformation of the hormone binding domain.

Studies with sodium arsenite, which at low concentrations is a vicinal thiol-specific reagent (Zahler & Cleland, 1968; Gaber & Fluharty, 1972), confirmed the importance of a vicinally spaced thiol pair in the steroid binding site (Chakraborti et al., 1990; Simons et al., 1990). Because other steroid receptors do not possess a vicinally spaced dithiol in the hormone binding domain, low concentrations of arsenite uniquely inhibit steroid binding by glucocorticoid receptors (Lopez et al., 1990). The vicinally spaced dithiols reacting

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[1] Abbreviations: GR, glucocorticoid receptor; hsp90, the 90-kDa heat shock protein; triamcinolone acetonide, 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetonide; dexamethasone, 9α-fluoro-16α-methyl-11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DTT, dithiothreitol; NEM, *N*-ethylmaleimide.

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with arsenite in the rat GR have recently been shown to be Cys-656 and Cys-661 (Cys-644 and Cys-649 in the mouse GR) (Chakraborti et al., 1992).

Simons et al. (1989) have shown that trypsin digestion of steroid-free GR yields a 16-kDa fragment derived from the NH_2-terminal half of the hormone binding domain that binds glucocorticoids with ~23-fold lower affinity than does the intact 98-kDa receptor. This fragment is bound to hsp90 (Chakraborti & Simons, 1991), and it contains three cysteines (640, 656, and 661 in the rat GR). Any two of these three cysteines can form a disulfide after treatment with low concentrations of MMTS (Chakraborti et al., 1992), suggesting that there is a cysteine cluster in this region of the steroid binding site. Chakraborti et al. (1992) have shown that mutant GRs in which any one of these cysteines has been changed to serine or the double mutant C656,661S still bind steroid with wild-type affinity. Thus, the thiols per se are not essential for steroid binding.

However, the steroid binding activity of the GR is eliminated by oxidative conditions (e.g., hydrogen peroxide) that promote disulfide bond formation and can be restored by agents that promote thiol–disulfide interchange (Bresnick et al., 1988). This raises the possibility that steroid binding activity could be altered by physiological conditions that affect the cellular redox activity and thiol–disulfide interchange. For example, Granberg and Ballard (1977) reported that the glucocorticoid binding activity of rat lung cytosol was rapidly inactivated at 0 °C and that either DTT or a heat-stable factor in rat liver cytosol reactivated the steroid binding capacity. While studying a similar rapid inactivation of steroid binding activity in rat thymocyte cytosol, Sando et al. (1977, 1979) identified a similar heat-stable activity in rat liver and mouse L cell cytosols that both activated and stabilized the steroid binding capacity. The heat-stable activity in rat liver cytosol was purified and identified as the thiol–disulfide exchange protein thioredoxin (Grippo et al., 1983, 1985). Thus, it was shown that receptors in cytosols prepared from some tissues undergo rapid oxidation, and the observations suggested that glucocorticoid receptors in stable cytosol preparations (e.g., liver, L cell) are maintained in a reduced, steroid binding state by a NADPH-dependent, thioredoxin-mediated reducing system. In retrospect, it seems likely that these observations are explained by the formation of a disulfide bridge between the vicinally spaced dithiol group in the steroid binding domain under oxidative conditions and that this bond undergoes thiol–disulfide interchange with reduced thioredoxin to reactivate the binding site.

The vicinally spaced dithiols that selectively interact with arsenite appear to lie at one end of a flexible cleft that forms the ligand binding cavity of the receptor (Chakraborti et al., 1992), and one of them (Cys-656) is covalently labeled by the thiol-specific electrophilic affinity label dexamethasone 21-mesylate (Simons et al., 1987). The binding of glucocorticoid to the receptor promotes the dissociation of hsp90 (Sanchez et al., 1987; Meshinchi et al., 1990), which binds to the hormone binding domain of the GR (Pratt et al., 1988; Chakraborti & Simons, 1991). It is thought that a conformational change takes place on dissociation of hsp90 that leaves the receptor unable to reassociate with the heat shock protein (Bresnick et al., 1989) unless the receptor is enzymatically unfolded to permit such reassociation (Scherrer et al., 1990; Hutchison et al., 1992). A study using peptides that contain the vicinally spaced dithiol to compete for GR refolding into a heterocomplex with hsp90 suggests that at least one contact site for hsp90 may lie in this region (Dalman et al., 1991), and it follows that this region may undergo a conformational change driven by the hormone. As they can be derivatized, the vicinally spaced thiols obviously lie near the surface of the receptor in its unliganded, untransformed state, and the vicinally spaced dithiol presents a target for selective derivatization of the untransformed conformation of this region. As part of a long-term aim of studying the conformational change induced in this region by steroid binding, in this work we directly compare the reversible thiol-specific reagents MMTS and arsenite on the steroid binding activity of several states of the GR.

MATERIALS AND METHODS

Materials

[6,7-^{3}H]Triamcinolone acetonide (42.8 Ci/mmol), [6,7-(n)-^{3}H]dexamethasone (41 Ci/mmol), and ^{125}I-conjugated goat anti-mouse and anti-rabbit IgGs were obtained from New England Nuclear, Boston, MA. Rabbit reticulocyte lysate was from Promega Corp., Madison, WI. Radio-inert dexamethasone, nonimmune mouse IgG, protein A–Sepharose, goat anti-mouse IgG–horseradish peroxidase conjugate, and TAPS and HEPES buffers were from Sigma Chemical Co., St. Louis, MO. Immobilon-P was from Millipore, Bedford, MA. Methyl methanethiosulfonate was from Aldrich Chemical (Milwaukee, WI). BuGR2 monoclonal antibody prepared against the rat glucocorticoid receptor (Gametchu & Harrison, 1984) was kindly provided by Dr. Robert W. Harrison, III, and Dr. William Hendry (University of Arkansas for Medical Science), and the rabbit serum used to detect hsp90 (Erhart et al., 1988) was a generous gift from Dr. Ettore Appella (National Cancer Institute).

Methods

Cell Culture and Fractionation. L929 mouse fibroblasts were grown in monolayer culture in Dulbecco's modified Eagle medium supplemented with 10% bovine or calf serum. Cells were harvested in late log-phase growth by scraping into Earle's balanced saline followed by a second wash and centrifugation at 500*g*. The washed cells were resuspended in 1.5 volumes of HEPES buffer (10 mM HEPES and 1 mM EDTA, pH 7.4, at 4 °C) and ruptured by Dounce homogenization. Homogenates were centrifuged for 1 h at 100000*g*. The supernatant from this centrifugation was removed and is referred to as "cytosol". HTC cell cytosol was prepared by freeze–thaw lysis of cells grown in spinner culture in S77 medium supplemented with 5% fetal and 5% newborn bovine sera (Biofluids) and 0.03% glutamine as described by Miller and Simons (1988).

Incubations with Reagents and Steroids. L cell cytosols were incubated at 0 °C (4–12 h) with 50 nM [^{3}H]dexamethasone or [^{3}H]triamcinolone acetonide with or without a 1000-fold excess of radio-inert dexamethasone where appropriate. In some experiments (Figure 2), 30% cytosol incubations at pH ~9.0 were prepared by mixing 3 parts of HTC or L cell cytosol, 2 parts of pH 9.5 TAPS buffer (25 mM TAPS, 1 mM EDTA, 10% glycerol, and 20 mM sodium molybdate), and 5 parts of pH 8.8 TAPS to yield an incubation mixture with a final pH ~9.0 at 0 °C. For 30% cytosol incubations at pH 7.4 (Figure 2B), 3 parts of L cell cytosol were mixed with 7 parts of a HEPES buffer (25 mM HEPES, pH 7.4, 1 mM EDTA, 10% glycerol, and 20 mM sodium molybdate).

To transform receptors to the DNA binding state, cytosol containing steroid-bound receptors was either incubated for

Table I: Effect of MMTS and Arsenite on Steroid Binding by Various States of the Mouse GR at 4 °C

conditions	specific binding (cpm/0.1 mL × 10^{-3})	conditions	specific binding (cpm/0.1 mL × 10^{-3})
	Cytosolic Receptor[a]		
R	109 ± 12	R	65 ± 8
R + MMTS	1 ± 0.5	R + $NaAsO_2$	0 ± 0.5
RS	105 ± 6	RS	86 ± 11
RS + MMTS	55 ± 14	RS + $NaAsO_2$	64 ± 12
RS^t	74 ± 8	RS^t	59 ± 10
RS^t + MMTS	76 ± 10	RS^t + $NaAsO_2$	51 ± 12
(RS^t·DNA)	48 ± 4	(RS^t·DNA)	34 ± 9
(RS^t·DNA) + MMTS	1 ± 0.5	(RS^t·DNA) + $NaAsO_2$	9 ± 1
(RS^t)·DNA	63 ± 3	(RS^t)·DNA	38 ± 2
(RS^t + MMTS)·DNA	4 ± 1	(RS^t + $NaAsO_2$)·DNA	10 ± 3
	Immunopurified Receptor[b]		
R	33 ± 6	R	33 ± 6
R + MMTS	2 ± 1	R + $NaAsO_2$	1 ± 0.5
RS	47 ± 7	RS	47 ± 7
RS + MMTS	23 ± 2	RS + $NaAsO_2$	39 ± 4

[a] Aliquots (100 μL) of L cell cytosol containing unbound receptors (R), untransformed steroid-bound receptors (RS), or salt-transformed steroid-bound receptors (RS^t) were treated for 1 h at 4 °C with 10 mM MMTS or sodium arsenite. At the end of the incubation, unliganded receptors were incubated for 3 h with 50 nM [^{3}H]triamcinolone acetonide. For these first three sets of conditions, steroid binding was assayed by charcoal adsorption. Samples were transformed with 0.45 M KCl for 4 h on ice. For the next to last set of conditions involving receptors prebound to DNA–cellulose (RS^t·DNA), transformed receptors were first diluted with 3 volumes of hypotonic buffer to reduce the salt concentration and then bound to DNA–cellulose, and the pellets were washed three times with buffer containing 5 mM DTT. The DNA–cellulose pellets were then resuspended in wash buffer and treated with 10 mM MMTS or 10 mM sodium arsenite. After being washed three additional times, the DNA–cellulose pellets were counted for tritium. In the last set of conditions, transformed receptors were pretreated with MMTS [(RS^t + MMTS)·DNA] or sodium arsenite and then bound to DNA–cellulose, washed, and counted for tritium as above. The data are expressed as specific binding per 0.1-mL aliquot of cytosol ± SE for three to five separate experiments. [b] Aliquots (100 μL) of L cell cytosol containing unbound receptors (R) or untransformed steroid-bound receptors (RS) were immunoadsorbed to protein A–Sepharose using either 10% BuGR antibody against the GR [sufficient to immunoadsorb ~50% of the receptor (Hutchison et al., 1992)] or nonimmune mouse IgG as described in Methods. The immune pellets were washed (2 × 1 mL) with TEGM buffer containing 5 mM DTT and suspended in 10 mM MMTS or sodium arsenite for 1 h at 0 °C with 50 nM [^{3}H]triamcinolone acetonide, and all immune pellets were washed in TEGM buffer and assayed for steroid binding by scintillation counting. Steroid binding in nonimmune samples, which represented less than 1% of steroid binding in immune samples that were untreated, was subtracted to yield specific binding.

1 h at 25 °C or treated with 0.45 M KCl for 4 h at 0 °C. Unbound, steroid-bound, or transformed steroid-bound receptors in cytosol were incubated at 0 °C for 1 h with various concentrations of MMTS or sodium arsenite. After treatment with the thiol reagent, unliganded receptors in HTC or L cell cytosols were incubated for 3 h with [^{3}H]dexamethasone or [^{3}H]triamcinolone acetonide. Steroid binding was quantitated by mixing the incubations with 1.5 volumes of a dextran-coated charcoal suspension (1% charcoal and 0.2% dextran in 10 mM HEPES, pH 7.4) for 10 min at 0 °C. The radioactivity remaining in the charcoal supernatant was assayed, and the specific binding was determined by subtracting radioactivity obtained in the presence of competing dexamethasone from that in its absence. For assays of DNA binding, 100-μL aliquots of treated cytosols were incubated for 1 h at 4 °C with a 200-μL suspension of 12.5% (v/v) DNA-cellulose; the pellets were washed 3 times with 1 mL of buffer and assayed for radioactive bound steroid or solubilized with SDS sample buffer and analyzed by SDS–PAGE as described below.

Immunoadsorption of Receptor. The BuGR anti-receptor antibody (hybridoma culture supernatant) or nonimmune mouse IgG (1 mg/mL) was preadsorbed to protein A–Sepharose by rotation with the Sepharose beads for 2 h at 4 °C, and the beads were then washed 3 times with 1-mL aliquots of TEG buffer (10 mM TES, 4 mM EDTA, 10% (w/v) glycerol, and 50 mM NaCl, pH 7.6). Aliquots (100–400 μL) of cytosol were mixed with preadsorbed antibody pellets and rotated for 1 h at 4 °C, and the pellets were washed 3 times with 1-mL aliquots of TEG buffer.

Reconstitution of the GR–hsp90 Complex. Reconstitution of GR–hsp90 complexes in the experiments of Figure 6 was performed essentially as described by Scherrer et al. (1990). GR was immunoadsorbed from 250-μL aliquots of L cell cystol with BuGR antibody and protein A–Sepharose. Immune pellets (6 μL of protein A–Sepharose) were stripped of associated hsp90 by incubation for 2.5 h at 4 °C with 0.5 M NaCl followed by 3 washes with 1 mL of TEG buffer. Stripped immune pellets were washed 2 times with 1 mL of HEPES–DTT buffer (10 mM HEPES, pH 7.4, and 5 mM DTT) and then mixed with 100 μL of rabbit reticulocyte lysate and incubated at 30 °C for 20 min. Following the incubation, the immune pellets were washed 4 times with 1 mL of TEG buffer containing 20 mM sodium molybdate (TEGM). Immunopellets were boiled in SDS sample buffer; proteins were resolved by SDS–PAGE, and hsp90 was detected by immunoblotting with anti-hsp90 serum.

Gel Electrophoresis and Immunoblotting. SDS–PAGE was performed in 7% or 10% slab gels essentially as described previously (Bresnick et al., 1989). Gels were cooled to 4 °C during electrophoresis. All samples were extracted from DNA–cellulose or protein A–Sepharose by boiling in SDS sample buffer with or without 10% β-mercaptoethanol as indicated. M_r standards were myosin, M_r 205 000; β-galactosidase, M_r 116 000; phosphorylase B, M_r 97 000; bovine serum albumin, M_r 66 000; egg albumin, M_r 45 000; and carbonic anhydrase, M_r 29 000. Immunoblotting was carried out by transferring proteins from acrylamide slab gels to Immobilon-P membranes under transfer conditions of 2 × 0.6 A. Immobilon-P membranes were then incubated with 1% BuGR antibody against the glucocorticoid receptor followed by a second incubation with ^{125}I-conjugated goat anti-mouse IgG.

RESULTS AND DISCUSSION

Effects of MMTS and Arsenite on Unliganded and Liganded GR. The effects of 10 mM MMTS on unliganded, liganded, and salt-transformed liganded cytosolic GR are presented in Table I. As reported many years ago with irreversible sulfhydryl-modifying reagents (Koblinski et al., 1972; Rees & Bell, 1975), treatment of the unoccupied receptor inactivates steroid binding activity. However, steroid binding appears to stabilize the receptor in the sense that the sulfhydryl-modifying reagents have less of an effect in causing release of the prebound ligand from the steroid binding stie than they do in preventing binding to a previously unoccupied site. This same differential sensitivity of the unliganded versus liganded receptor is seen when GR immunoadsorbed to protein A–Sepharose is treated with the reagents (Table I, immunopurified receptor), indicating that both MMTS and arsenite act directly on the receptor protein itself. As illustrated in Table I, when cytosolic receptors are transformed by treatment with KCl, about 30% of the bound steroid is lost and the

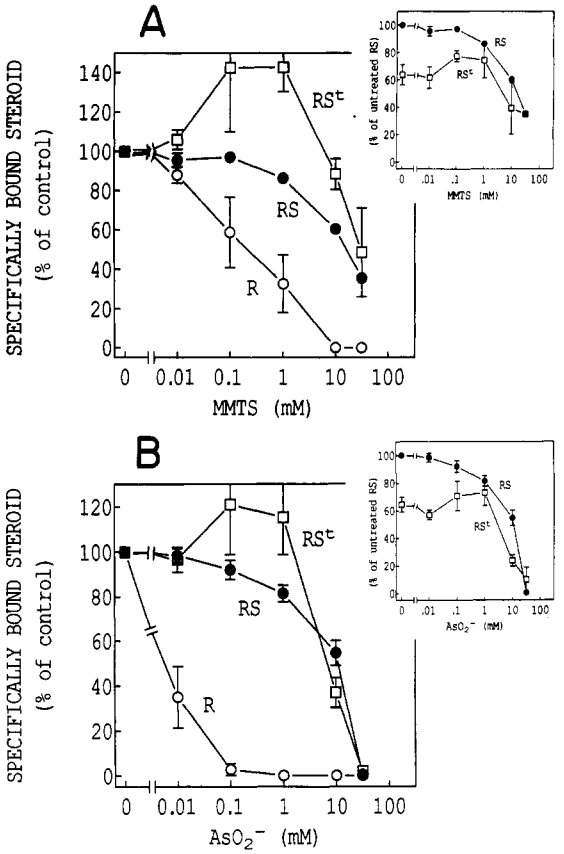

FIGURE 1: Concentration dependence of the effects of MMTS and arsenite on the GR steroid binding activity. Panel A: aliquots (45 μL) of L cell cytosol were incubated with buffer or various concentrations of MMTS for 1 h at 0 °C, either before (R) or after steroid binding (RS) or after salt transformation of the steroid-bound receptor to the DNA binding state (RS^t). The amounts of specifically bound steroid for R, RS, and RS^t were assayed by charcoal adsorption and expressed as a percent of respective untreated values. Panel A, inset, shows the amount of specifically bound steroid for RS^t at each concentration of MMTS, calculated as a percent of the untreated RS value. The data were then plotted along with values for RS as in panel A. Panel B: the procedure was the same as for panel A, except that various concentrations of AsO_2^- were used. Panel B, inset, shows the amount of specifically bound steroid for RS^t at each concentration of AsO_2^-, calculated as a percent of the untreated RS value. The data were then plotted along with values for RS as in panel B. Each value represents the mean ± SE for three separate experiments. Where the error bars are not shown, the error falls within the symbol.

receptors appear to become less sensitive to the steroid releasing effect of either reagent.

When the effects of the reagents are examined over a wide range of concentrations (shown in Figure 1), several observations may be made. Both MMTS and arsenite are effective at inactivating the unliganded receptor at concentrations that are much lower than those required to cause release of triamcinolone acetonide from the prebound receptor. Indeed, arsenite inactivates the unliganded receptor at about 3 orders of magnitude lower concentration than it inactivates the steroid-bound receptor. This effect can be interpreted in several ways. As Simons et al. (1990) have proposed, the vicinally spaced dithiol must be open and accessible to arsenite when the steroid binding cleft is empty and formation of a complex between the vicinally spaced dithiol and arsenite prevents binding. When the steroid binding site is occupied, however, it is not known whether the vicinally spaced dithiol can complex with arsenite or whether that interaction is sterically blocked by the steroid. Presumably, the effect of high concentrations of arsenite on the steroid-bound receptor (Figure 1B) reflects its interaction with one or more of the three thiols in the hormone binding domain other than the vicinally spaced dithiol. It is also possible that steroid binding alters the conformation of the region such that the dithiol (Cys-644 and Cys-649 mouse) is no longer spatially vicinal and its components react with arsenite as two monothiols. Another possibility is that arsenite binds to the vicinally spaced dithiol, but when the steroid has occupied the binding cleft, this interaction does not affect its dissociation rate.

When the data of Figure 1 are plotted using the untreated control for each form of the receptor as the 100% value (main graphs), then it appears that untransformed and transformed receptors may have different sensitivities to the steroid dissociating effect of both thiol-reactive agents. However, when the same data are plotted using the untreated, untransformed, steroid-bound receptor (RS) as the 100% reference (see Figure 1 insets), then it can be seen that the two curves nearly superimpose at concentrations of 1 mM and higher for both MMTS (Figure 1A) and arsenite (Figure 1B). This suggests that transformation of the receptor may not have changed the conformation of the receptor in such a way as to limit access of the reagents to monothiols, nor was the effect of such monothiol reaction on steroid dissociation changed.

Two-Phase Effect of MMTS. Miller and Simons (1988) have reported that the steroid binding activity of rat GR in HTC cell cytosol undergoes a two-phase response when unliganded receptors are exposed to a range of MMTS concentrations. At low concentrations of MMTS, there is an inactivation of steroid binding capacity, reaching a nadir at 10^{-4} M, followed by a rise to ~50% of control levels between 10^{-3} and 10^{-2} M and a subsequent decline. The initial dip in this bimodal dose–response curve is thought to reflect reaction of MMTS with one SH group in the dithiol, followed by attack by the other thiol, with simultaneous elimination of the thiomethyl group and formation of a disulfide bridge. At higher concentrations of MMTS, however, both thiol groups are converted to thiomethyl derivatives and the steroid can enter the binding cleft as it does when the vicinally-spaced dithiol is not disulfide bridged. This bimodal effect of MMTS on the steroid binding capacity of the unliganded receptor is not evident in Figure 1A. In the experiments in Figure 1A, cytosol was preincubated for 1 h with MMTS, whereas Miller and Simons (1988) preincubated for 2.5 h, but we have tested preincubation times up to 5 h without seeing the two-phase response.

The HTC cell cytosol of Miller and Simons (1988) was prepared quite differently from the L cell cytosol employed here, in that cytosols were diluted to 30% concentration by mixing HTC cell cytosol with TAPS buffer to yield a final pH of ~9.0. Also, 20 mM molybdate was present during MMTS treatment, and steroid binding was assayed with [^{3}H]-dexamethasone instead of [^{3}H]triamcinolone acetonide. To replicate these conditions exactly, HTC cell cytosol was prepared in the NIH lab and sent to Ann Arbor, where it was diluted into TAPS at pH ~9.0 and treated with MMTS. As shown in Figure 2A, MMTS treatment of the unliganded rat GR in HTC cytosol produced the expected bimodal effect. When cytosol from L929 cells was diluted in the same way at high pH, MMTS also had a bimodal effect on the mouse GR.

The presence or absence of molybdate and the steroid used for assaying binding capacity are not important for demonstrating the bimodal MMTS effect, but cytosol pH is important. Figure 2B shows concentration curves for the effect of MMTS on steroid binding activity in L cell cytosol that was diluted in buffers to yield a final pH of 7.4 or ~9.0. The

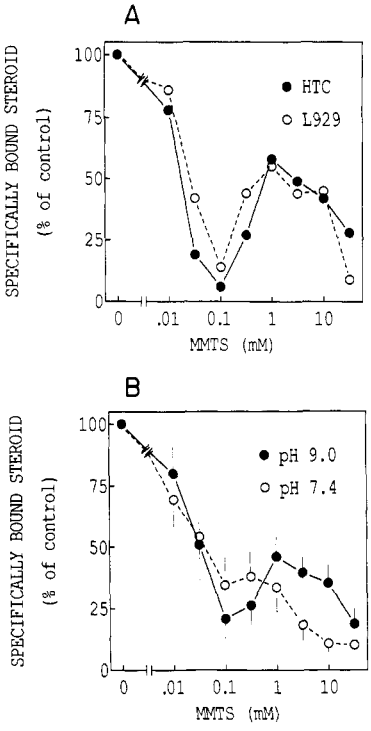

FIGURE 2: Bimodal effect of MMTS on steroid binding capacity of the unliganded GR. Panel A: cytosols prepared from HTC cells or L cells were diluted to 30% concentration in high-pH TAPS buffer as described under Methods. Aliquots (50 μL) were then incubated for 2.5 h with various concentrations of MMTS, and steroid binding capacity was assayed with [^{3}H]dexamethasone. (●) Rat GR in HTC cell cytosol; (○) mouse GR in L cell cytosol. Panel B: L cell cytosol was diluted to 30% in high-pH TAPS buffer (final pH ~9.0, ●) or in low-pH HEPES buffer (final pH 7.4, ○), treated 2.5 h with MMTS, and assayed for steroid binding capacity. Each point is the average of four separate experiments, and the SE is indicated by the line extending above or below each point.

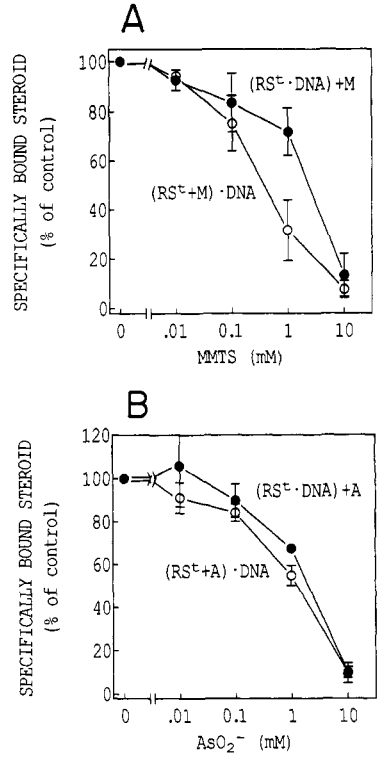

FIGURE 3: Effect of MMTS and arsenite on transformed receptors before and after binding to DNA–cellulose. Receptors in L cell cytosol were bound with [^{3}H]triamcinolone acetonide, and the receptors were then transformed by heating for 1 h at 25 °C. The cytosol was then divided into replicate aliquots, and one-half of them was added to packed pellets of DNA–cellulose and rotated to permit binding to DNA. All samples were then incubated for 1 h at 0 °C with various concentrations of MMTS (panel A) or arsenite (panel B). The half of the samples that had not been bound to DNA–cellulose was then added to a packed pellet of DNA–cellulose and rotated at 0 °C. After the DNA-cellulose pellets were washed three times with buffer, the tritium radioactivity in each DNA pellet was assayed. Each value represents the mean and standard error from three separate experiments. Conditions: (A) (●) [(RS^t·DNA) + M], DNA-bound transformed receptor treated with MMTS; (○) [(RS^t + M)·DNA], transformed receptor treated with MMTS and then bound to DNA; (B) as in panel A except treated with arsenite (A).

bimodal effect is barely evident at pH 7.4. It is reasonable that the inhibition of steroid binding activity seen at low concentrations of MMTS should be pronounced at the high pH where the thiolate anion form is favored, providing a greater likelihood of disulfide bond formation when only one of the moieties in the vicinally spaced dithiol has been thiomethylated.

Effects of MMTS and Arsenite on Steroid Dissociation from DNA-Bound GR. In a study of the effect of MMTS on the binding of transformed GR to DNA–cellulose, Tienrungroj et al. (1987) reported that 10 mM MMTS, which has only a partial effect on steroid release from the transformed steroid–receptor complex (e.g., Table I, Figure 1), caused complete release of steroid from DNA-bound receptors (provided that DTT was present to prevent intramolecular disulfide bridges from forming during the washing of DNA–cellulose prior to MMTS treatment). The fact that MMTS caused rapid and complete release of steroid from the DNA-bound receptor but not from transformed receptor in cytosol prompted the speculation (Tienrungroj et al., 1987) that occupancy of the DNA binding domain and/or other more general surface charge interactions that occur with DNA binding may affect the conformation of thiols that are located in (or affect the conformation of) the steroid binding domain.

Here we show that GR binding to DNA does not result in an increased sensitivity to an effect of either MMTS or arsenite on steroid binding. The two thiol-reactive reagents have the same ability to release steroid from the transformed GR (both free in cytosol and DNA-bound), but in contrast to MMTS, arsenite does not affect receptor thiols required for DNA binding. As shown in ranks seven to ten of Table I, 10 mM MMTS causes an apparent complete loss and arsenite causes a substantial (74%) loss of steroid from the DNA-bound steroid–receptor complex. If the transformed steroid–receptor complex is treated with MMTS or arsenite prior to DNA binding, then there is a similar decrease in specifically bound steroid subsequently associated with the DNA–cellulose pellet. As shown in Figure 3, the amount of specifically bound steroid associated with the DNA–cellulose pellet decreases with the same concentration dependency, regardless of whether the receptor is being exposed to arsenite before or after it is bound to DNA. The half-maximal effect of arsenite is at ~1 mM (Figure 3B), rather than at <0.01 mM as seen in Figure 1B with the arsenite effect on the vicinally spaced dithiol in the unliganded GR.

Figure 4 shows the effect of MMTS (panel A) or arsenite (panel B) on DNA binding of transformed receptors as assayed by Western blotting the receptor protein. It is clear from the data of lanes 1–4 of both panels in Figure 4 that neither MMTS nor arsenite promote significant dissociation of receptor from DNA at a concentration (10 mM) that in each case causes complete loss of steroid from the DNA-bound receptor (Figure 3). As shown in lanes 5 and 7 of Figure 4A, MMTS treatment of transformed GR in cytosol inactivates most but not all DNA binding. In contrast, arsenite has no effect on the DNA

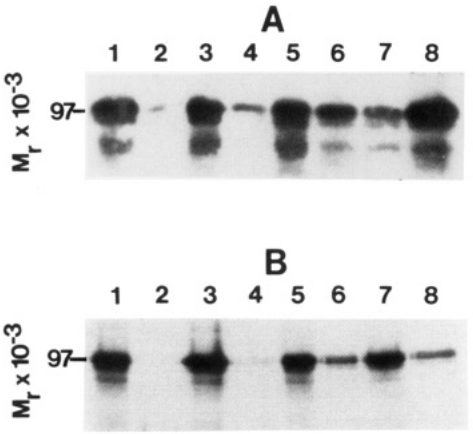

FIGURE 4: Differential effects of MMTS and arsenite on binding of the transformed GR to DNA as assayed by Western blot. [^{3}H]-Triamcinolone acetonide-bound glucocorticoid receptors in aliquots of cytosol (200 μL) (lanes 1–4, panels A and B) were transformed to the DNA binding state by heating for 1 h at 25 °C, bound to DNA–cellulose, and washed in buffer plus 5 mM DTT, and then the pellets were treated with 10 mM MMTS (panel A) or AsO_2^- (panel B). Soluble glucocorticoid receptors were immunoadsorbed from the supernatants of this incubation using BuGR and protein A–Sepharose. Other samples (lanes 5–8) were pretreated with MMTS (panel A) or AsO_2^- (panel B) after transformation to the DNA binding state and then bound to DNA–cellulose. Receptors that did not bind to DNA were immunoadsorbed from the supernatant of the DNA–cellulose binding assay with BuGR and protein A–Sepharose. GR bound to the DNA–cellulose pellet and GR immunoadsorbed to the protein A–Sepharose pellet were solubilized in sample buffer, resolved on SDS–PAGE, and detected by Western blotting. Lane 1, GR remaining bound to DNA–cellulose after incubation with buffer; lane 2, GR released from DNA–cellulose after incubation with buffer; lane 3, GR remaining bound to DNA–cellulose after incubation with MMTS or AsO_2^-; lane 4, GR released from DNA–cellulose after incubation with MMTS or AsO_2^- lane 5, GR that binds to DNA–cellulose after preincubation with buffer; lane 6, GR that does not bind to DNA–cellulose after preincubation with buffer; lane 7, GR that binds to DNA–cellulose after preincubation with MMTS or AsO_2^-; lane 8, GR that does not bind to DNA–cellulose after preincubation with MMTS or AsO_2^-.

binding activity of the transformed receptor (Figure 4B, lanes 5 and 7). Thus, the effect of arsenite in Figure 3 is only to cause dissociation of steroid, regardless of whether the receptor is bound to DNA or not. In Figure 3A, MMTS treatment of the DNA-bound GR (solid circles) reflects only steroid release, but the effect on the transformed GR in cytosol (open circles) reflects an apparently more sensitive effect on sulfhydryls involved in DNA binding activity.

Differential Effects of MMTS and Arsenite on Dissociation of Dexamethasone versus Triamcinolone Acetonide. In the experiments shown in Figure 1 the effects of MMTS and arsenite were tested on [^{3}H]triamcinolone acetonide-bound receptors. Curiously, the effect of the two reagents on [^{3}H]-dexamethasone-bound receptors is not the same. The differential effect of arsenite versus MMTS on dissociation of the two steroids is shown in Figure 5, where arsenite causes more rapid dissociation of dexamethasone than triamcinolone acetonide (Figure 5A), and with MMTS dissociation of triamcinolone acetonide is more rapid (Figure 5B). Similar results were obtained after incubating dexamethasone- and triamcinolone acetonide-bound HTC cell receptors in pH 8.8 TAPS buffer with MMTS or arsenite for 2 h at 0 °C (data not shown). Thus, the differential effect of arsenite versus MMTS is not pH-dependent or solely a property of mouse GR in L cell cytosol.

It is not at all clear why the dissociation of the two steroids should be affected differentially. Triamcinolone acetonide

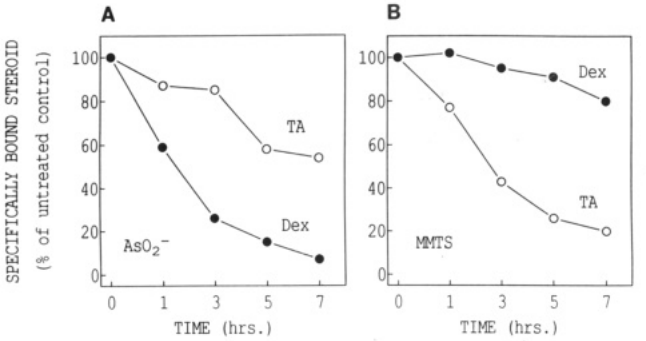

FIGURE 5: Time course of the effects of MMTS and arsenite on triamcinolone acetonide-bound and dexamethasone-bound glucocorticoid receptors. Panel A: replicate aliquots (45 μL) of L cell cytosol containing either [^{3}H]triamcinolone acetonide-bound or [^{3}H]-dexamethasone-bound glucocorticoid receptors were incubated with buffer or 10 mM AsO_2^- at 0 °C. At the indicated times, the amount of specifically bound steroid was assayed by charcoal adsorption and expressed as a percent of the untreated zero time control value. Panel B: the procedure was the same as for panel A, except that 10 mM MMTS was used.

and dexamethasone are both synthetic 9α-fluoro compounds which differ only in their substitutions on the 16 and 17 carbons of the D ring, with dexamethasone being 16α-methyl and 17α-hydroxyl and triamcinolone acetonide having its 16α- and 17α-hydroxyls linked as an acetonide. Given that dexamethasone 21-mesylate reacts with one of the sulfurs in the vicinally spaced dithiol (Simons et al., 1987), it is clear that these cysteines must lie very near the portion of the steroid binding pocket that interacts with the D-ring end of the steroid (Chakraborti et al., 1992). It is possible that monothiol reactions of arsenite and MMTS in this region of the steroid binding pocket produce differential effects on dissociation of the prebound steroids that reflect differential effects on the functional groups at the 16 and 17 carbons. In this respect, it should be noted that substitution of Cys-656 with either serine or glycine yields mutant receptors that have a higher affinity for glucocorticoids and a decreased relative affinity for cross-reacting steroids such as progesterone and aldosterone (Chakraborti et al., 1991), again indicating that this region of the receptor is important for both affinity and specificity of steroid binding.

MMTS and Arsenite Do Not Affect Binding of GR to hsp90. A critical consideration in developing a radiolabeled reagent that specifically interacts with vicinally spaced dithiols as a probe for conformational changes in the hormone binding domain occurring with GR transformation is that the probe itself should not cause transformation. Bodwell et al. (1984) have noted that treatment of the GR with MMTS followed by reduction with dithioerythritol results in a slow conversion of the receptor to the DNA binding form at 0 °C. We have treated both unliganded and steroid-bound GR in cytosol with 10 mM MMTS or arsenite (0 °C, 1 h) and then immunoadsorbed the receptors and assayed for receptor-associated hsp90 by a standard Western blotting procedure used previously by Sanchez et al. (1987) and Meshinchi et al. (1990). We find that neither thiol-specific reagent causes dissociation of hsp90 (data not shown). This confirms the observation of Chakraborti et al. (1992) that arsenite does not cause dissociation of hsp90. One disadvantage of MMTS and arsenite in these studies is that the covalent linkage to receptors can be readily reversed by added thiols (Miller & Simons, 1988; Simons et al., 1990). In order to have irreversibly modified SH groups, we examined the effects of iodoacetamide and NEM, which give stable adducts in the presence of high thiol concentrations. The experiments of Figure 6 show that treatment of unli-

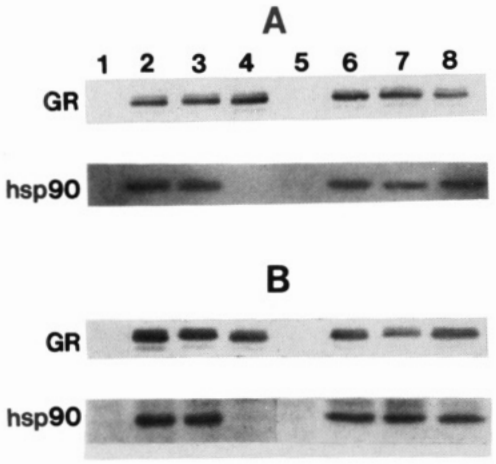

FIGURE 6: Treatment of the untransformed GR with NEM or iodoacetamide does not inhibit subsequent refolding of the GR into a complex with hsp90. Unliganded receptors from L cell cytosol were immunoadsorbed to protein A–Sepharose with BuGR or nonimmune IgG, and hsp90 was stripped from some of the immunopellets by incubating with salt as described in Methods. The indicated immunopellets were incubated at 0 °C for 20 min with 20 mM iodoacetamide (panel A) or 20 mM NEM (panel B), and hsp90 was salt-stripped from the sample in lane 7. Immunopellets in lanes 5–8 were then washed twice with HEPES–DTT buffer and incubated with reticulocyte lysate as described in Methods. The immunopellets were washed again and assayed for hsp90 by Western blotting. Lanes 1 and 2, samples immunoadsorbed with nonimmune IgG (lane 1) or BuGR (lane 2) to demonstrate the native GR-associated hsp90; lane 3, unstripped immunopellet with native hsp90 treated with 20 mM reagent; lane 4, stripped immune pellet; lane 5, stripped nonimmune pellet incubated with lysate; lane 6, stripped immune pellet incubated with lysate; lane 7, immune pellet treated with 20 mM reagent, stripped and incubated with lysate; lane 8, stripped immune pellet, treated with 20 mM reagent and incubated with lysate.

ganded, untransformed GR with the irreversible reagents iodoacetamide or NEM also does not cause hsp90 release (cf. lanes 2 and 3).

In pursuing development of a vicinal dithiol probe for examining conformational changes occurring in the GR with transformation, it would be optimal if GR that has been reacted with a vicinal dithiol probe after transformation were able to be refolded into the heterocomplex with hsp90. To determine if reaction with thiol-modifying reagents affects GR refolding, we have used the irreversible reagents iodoacetamide and NEM, because the effects of MMTS and arsenite are reversed by subsequent exposure to the retiuclocyte lysate, which contains high concentrations of DTT and endogenous thiols. In the experiments shown in Figure 6, receptors, were treated with 20 mM iodoacetamide (panel A) or 20 mM NEM (panel B) before (lane 7) or after (lane 8) they were exposed to salt to strip off the hsp90. After the unreacted reagent was washed away (and hsp90 was stripped off the sample of lane 7), receptors were incubated with reticulocyte lysate to refold them into the heterocomplex with hsp90. We found that 20 mM iodoacetamide or NEM completely eliminated the ability of the untransformed (i.e., hsp90-bound) receptor to bind steroid (data not shown). It is clear from Figure 6 that the lysate reassociated hsp90 with the GR (cf. lanes 4 and 6) and that treatment with the thiol-modifying reagents did not affect receptor refolding so long as any free reagent was eliminated prior to incubation with the lysate (cf. lanes 7 and 8 with lane 6). This supports the conclusion of Chakraborti et al. (1992) from data with mutant GRs that thiol groups are not required for hsp90 association. These results suggest that if a conformational change takes place during receptor transformation that renders the vicinally spaced dithiol group inaccessible to attack by an agent that selectively derivatizes vicinally spaced dithiols, then it should be possible to unfold the receptor, reversing the conformational change and rendering the vicinally spaced dithiol again subject to attack. In this respect it should be mentioned that it has already been shown that GR that is stripped of hsp90 and does not have a high-affinity steroid binding conformation is returned to a normal steroid binding conformation when it is refolded into a heterocomplex by reticulocyte lysate (Scherrer et al., 1990; Hutchison et al., 1991).

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