Redox Manipulation of DNA Binding Activity and BuGR Epitope Reactivity of the Glucocorticoid Receptor*

(Received for publication, September 10, 1990)

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The receptor contains 20 cysteine residues, with containing the DNA-binding fingers, and being located in the central -15-kDa tryptic fragment, resulting in rapid elimination of zinc. Reduction of DNA binding activity is due to disulfide bonds formed within the DNA binding domain or between the DNA binding domain and another region of the receptor. In this paper, we examined the ability of hydrogen peroxide to inactivate the DNA binding activity of the mouse glucocorticoid receptor. We show that inhibition of DNA binding activity caused by hydrogen peroxide can be accounted for entirely by the formation of disulfide bonds between cysteine residues lying within the 15-kDa tryptic fragment containing the DNA binding domain of the receptor. Reversal of the peroxide-induced inactivation of DNA binding activity requires both zinc and a thiol-disulfide exchange reagent, such as dithiothreitol. Peroxide also eliminates recognition of the intact receptor and the 15-kDa tryptic fragment by the BuGR monoclonal antibody, and the reactivity of the BuGR epitope is restored by reduction without a requirement for zinc. Pretreatment of the receptor with methyl methanethiosulfonate inhibits much of the peroxide-mediated inactivation of the BuGR epitope but pretreatment with N-ethylmaleimide does not. Similarly, DNA binding activity of the receptor is inhibited by methyl methanethiosulfonate but not by N-ethylmaleimide. These results are consistent with the proposal that peroxide promotes the formation of disulfide bonds between thiols that lie spatially close to one another in the 15-kDa tryptic fragment, resulting in rapid elimination of zinc. Restoration of the zinc finger structure restores DNA binding activity but restoration of the BuGR epitope requires only reduction without restoration of the zinc fingers.

Primary amino acid sequences have been determined for human (1), mouse (2), and rat (3) glucocorticoid receptors. The receptor contains 20 cysteine residues, with 5 cysteines being located in the ~30-kDa tryptic fragment spanning the hormone-binding domain at the COOH terminus, 11 cysteines being located in the central ~15-kDa tryptic fragment containing the DNA-binding fingers, and 4 being located in the amino-terminal half of the receptor. Both the hormone binding and DNA binding functions of the receptor can be blocked in vitro by both thiol-derivatizing reagents and agents that promote disulfide bond formation (see Pratt (5), for review).

In achieving an understanding of both the structure and function of this receptor, it will be necessary to determine which cysteine residues (if any) are involved in the formation of stable intramolecular disulfide bonds, to determine which cysteines lie close enough to each other in the folded molecule such that disulfide bridges can be promoted with oxidizing agents, and to define those cysteines that must be reduced for specific receptor functions such as hormone binding, subunit dissociation, and DNA binding to occur.

Within the DNA-binding domain, 8 cysteines are involved in the tetrahedral coordination of zinc (6) forming two DNA-binding finger structures (7). A number of studies have shown that treatment of the transformed glucocorticoid receptor with sulphydryl-modifying reagents, such as iodoacetamide or methyl methanethiosulfonate, inactivates the DNA binding activity (8–11). It has also been shown that oxidizing agents that promote disulfide bond formation inactive the DNA binding activity of the receptor (11, 12). Recently, Silva and Cidlowski (12) have provided direct evidence that oxidizing reagents cause the formation of intramolecular disulfide bonds in the glucocorticoid receptor. A major question that has not been answered is whether the disulfide bonds are formed within the DNA-binding domain or whether they are formed between the DNA-binding domain and another region of the receptor. In this work we show that inactivation of the DNA binding activity of the glucocorticoid receptor caused by peroxide-induced disulfide bond formation can be accounted for by disulfide bridging between cysteines lying within the 15-kDa tryptic fragment containing the DNA-binding fingers. The inhibition of DNA binding activity produced by peroxide is reversed by DTT and zinc. Interestingly, peroxide also inactivates the epitope for the BuGR monoclonal anti-receptor antibody, but this effect is reversed by reducing agent without a requirement for zinc.

**EXPERIMENTAL PROCEDURES**

Materials

[6,7-3H]Triamcinolone acetonide (42.8 Ci/mmol), [6,7-3H]dexamethasone 21-mesylate (48.9 Ci/mmol), and [125I]-conjugated goat anti-

1 Dissociation of the heteromeric glucocorticoid receptor- hs p90 complex is accompanied by transformation of the receptor from a non-DNA-binding to a DNA-binding form.

2 The abbreviations and trivial names used are: DTT, dithiothreitol; BuGR, monoclonal antiguocorticoid receptor antibody; hs p90, the 90-kDa heat shock protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TES, 4-(2-hydroxy-1,1-bis-(hydroxymethyl)ethyl)aminoethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLCK, N-p-tosyl-L-lysine chloromethyl ketone; TPCK, tosylphenylalanyl chloromethyl ketone; NEM, N-ethylmaleimide; MMTS, methyl methanethiosulfonate.

* This investigation was supported by Grant DK31578 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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mouse IgG were obtained from Du Pont-New England Nuclear. Radioinactive dexamethasone, nonimmune mouse IgG, protein A-Sepharose, and goat anti-mouse IgG-horseradish peroxidase conjugate were from Sigma. Immobilon-P was from Millipore. BuGR monoclonal antibody prepared against the rat glucocorticoid receptor (13) was kindly provided by Dr. Robert W. Harrison, III.

Methods

Cell Culture and Fractionation—L929 mouse fibroblasts were grown in monolayer culture in Dulbecco's modified Eagle's medium supplemented with 10% bovine or calf serum. Cells were harvested in late log phase growth by scraping into Earl's balanced saline solution 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, 1 mM EDTA, pH 7.35, at 4 °C) and ruptured by Dounce homogenization. Homogenates were centrifuged for 1 h at 100,000 × g. The supernatant from this centrifugation was removed and is referred to as "cytosol." Receptor Transformation and DNA-binding Assay—Cytosol was bound overnight at 0 °C with 50 nM dexamethasone or [3H]dexamethasone 21-mesylate with or without a 1000-fold excess of radiiodinated dexamethasone where appropriate. To transform receptors to the DNA binding state, cytosol containing steroid-bound receptors was incubated for 15 min at 25 °C. Concentrations containing cytosol and other agents were prepared as indicated in the figure and table legends. For assay of DNA binding, 100-μl aliquots of incubation mixture were incubated for 45-60 min at 4 °C with a 200-μl suspension of 12.5% (v/v) DNA-cellulose. In one experiment receptors were transformed by incubating cytosol with 0.4 M KCl for 5 h at 0 °C before addition of 2 volumes of DNA-cellulose suspension. In this case the final concentration of 130 mM KCl does not affect DNA-cellulose binding. The pellets were washed 3 times with 1 ml of buffer and assayed for radioactivity of bound steroid or solubilized with SDS sample buffer and analyzed by SDS-PAGE as described below.

 Cleavage with Chymotrypsin and Trypsin—Cleavage of receptor was performed in whole cytosol after transformation and before other treatments essentially as described previously (14). Freshly prepared TFE-treated trypsin (200 μg/ml final concentration) or TGLR-treated chymotrypsin (1 μg/ml) were added to cytosol and allowed to incubate on ice for 60 min. To stop proteolysis, 0.1 mg/ml TPCK and 0.2 mg/ml TLCK were added to all incubations, which were then incubated for 15 min at 0 °C, followed by the addition of 1 mg/ml soybean trypsin inhibitor for 30 min on ice.

 Immunoabsorption of Receptor—The BuGR antireceptor 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 of aliquots of TEG buffer (10 mM TES, 4 mM EDTA, 10% (w/v) glycerol, 50 mM NaCl, pH 7.6). Aliquots (100-400 μl) of cytosol were mixed with preabsorbed antibody pellets, rotated for 1 h at 4 °C, and the pellets were washed 3 times with 1 ml aliquots of TEG buffer.

 Gel Electrophoresis and Immunoblotting—SDS-PAGE was performed in 7 or 10% slab gels essentially as described previously (14). 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, standards were: myosin, M, 205,000; β-galactosidase, M, 116,000; phosphorylase b, M, 97,000; bovine serum albumin, M, 66,000; egg albumin, M, 45,000; glyceraldehyde-3-phosphate dehydrogenase, M, 36,000; carbonic anhydrase, M, 29,000; trypsinogen, M, 24,000; soybean trypsin inhibitor, M, 21,100; and α-lactalbumin, M, 14,500.

 Immunoblotting was carried out by transferring proteins from acrylamide slab gels to Immobilon-P membranes under transfer conditions of 2 h × 0.6 A for the intact receptor, 4 h × 0.4 A for the 42-kDa chymotryptic receptor fragment, and 16 h × 0.1 A for the 15-kDa tryptic receptor fragment. Immobilon-P membranes were then incubated with 1% BuGR antibody against the glucocorticoid receptor followed by a second incubation with peroxidase-conjugated goat anti-mouse IgG. In some cases, the amount of receptor was quantitated by incubating with 125I-labeled goat anti-mouse IgG and then exciting receptor bands visualized by peroxidase staining and counting for 125I as described previously (15).

RESULTS

Hydrogen Peroxide Inhibits DNA Binding by the Transformed Glucocorticoid Receptor—In a previous publication (11) we showed that treatment of L cell cytosol containing transformed dexamethasone-receptor complexes with hydrogen peroxide inhibits binding to DNA-cellulose. But in a recent series of experiments (4), we found that similar peroxide treatment of transformed receptor bound with the antagonist dexamethasone 21-mesyate does not inhibit DNA binding. This suggested to us that the transformed antagonist-bound receptor might not be in a conformation that permits peroxide-mediated disulfide bond formation, whereas the transformed agonist-bound receptor has a conformation in which sulfhydryl residues are spatially close enough to each other such that disulfide bond formation is readily promoted by the oxidizing agent. In the experiment of Fig. 1, unbound receptors, dexamethasone-bound receptors, or receptors bound with [3H]dexamethasone 21-mesyate were transformed at 0 °C with salt, and then treated with hydrogen peroxide and bound to DNA-cellulose. It can be seen that peroxide inhibited receptor DNA binding activity regardless of whether the receptor was unbound, agonist-bound, or antagonist-bound. Our previous failure to see an effect of peroxide alone inhibiting DNA binding activity of the antagonist-bound receptor (4) was due to the fact that we had used weaker peroxide. It is important to emphasize that the effects demonstrated in our current work require the use of freshly purchased hydrogen peroxide.

To determine if peroxide affects the DNA-binding domain directly, the transformed receptor was cleaved with trypsin. Trypsin cleaves the receptor just to the NH2-terminal side of the BuGR epitope and just to the COOH-terminal side of the DNA-binding domain (16), yielding a 15-kDa fragment containing both the DNA-binding fingers and the epitope for the BuGR antibody (17). In Fig. 2, the intact receptor or the trypsin-cleaved receptor were treated with buffer or peroxide, bound to DNA-cellulose, and both the DNA-binding intact receptor and the 15-kDa receptor fragment were resolved by Western blotting with 125I-labeled counterantibody. The bands for the intact receptor and the 15-kDa tryptic fragment were excised and counted. It is clear that the concentration of peroxide in 12.5% (v/v) DNA-cellulose. In one experiment receptors were transformed by incubating cytosol with 0.4 M KCl for 5 h at 0 °C before addition of 2 volumes of DNA-cellulose suspension. In this case the final concentration of 130 mM KCl does not affect DNA-binding activity of the antagonist-bound receptor (4) was due to the fact that we had used weaker peroxide. It is important to emphasize that the effects demonstrated in our current work require the use of freshly purchased hydrogen peroxide.

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FIG. 1. Hydrogen peroxide inhibits the DNA binding activity of unbound, agonist-bound, and antagonist-bound glucocorticoid receptor. Aliquots (100 μl) of cytosol were incubated overnight at 0 °C with buffer or with 50 nM dexamethasone, or with 100 nM [3H]dexamethasone 21-mesyate plus or minus a 1000-fold excess of competing dexamethasone. Cytosol was then incubated 5 h with 0.4 M KCl at 0 °C to cause transformation. Transformed receptors were incubated for 1 h with or without 20 mM hydrogen peroxide and bound to DNA-cellulose. The DNA-cellulose pellets were washed with buffer and solubilized in SDS sample buffer containing β-mercaptoethanol. The DNA-bound receptor was resolved by SDS-PAGE and Western blotting with BuGR, followed by incubation with 125I-labeled goat anti-mouse IgG (lanes 1-4) or by autoradiography of the dried polyacrylamide gel (lanes 5-8). Lanes 1 and 2, dexamethasone-bound receptor incubated with buffer or peroxide, respectively; lanes 3 and 4, unbound receptor plus buffer or peroxide; lanes 5 and 7, receptor incubated with [3H]dexamethasone 21-mesyate in the presence of competing dexamethasone followed by treatment with buffer or peroxide, respectively; lanes 6 and 8, [3H]dexamethasone 21-mesyate-bound receptor incubated with buffer or peroxide, respectively.
transformation, the cytosol was divided into two portions and one protease inhibitors as described under "Methods." Aliquots (200 µl) of untreated and trypsin-treated cytosol were incubated for 1 h at 0°C with various concentrations of hydrogen peroxide and then bound to DNA-cellulose. The DNA-bound receptor or receptor fragment was resolved by SDS-PAGE under reducing conditions and Western blotted using BuGR as the probe antibody, followed by 125I-
nanotate-bound receptor incubated with buffer alone; lane 3, incubated with NEM alone; lane 4, H2O2 alone; lane 5, NEM then H2O2. Panels E and F, cytosol containing transformed receptors was cleaved with trypsin and 200-µl aliquots were incubated as above, proteins were resolved by SDS-PAGE and Western blotting using BuGR as the probe antibody and visualizing the receptor with peroxidase-conjugated goat anti-mouse IgG (panels A and B). After photography, the Western blots were sprayed with ENHANCE and the bound [3H]dexamethasone 21-mesyate was visualized by autoradiography (panels C and D). Lane 1, receptor labeled in the presence of a 1000-fold molar excess of nonradioactive dexamethasone; lane 2, [3H]dexamethasone 21-mesyate-bound receptor incubated with buffer alone; lane 3, incubated with NEM alone; lane 4, H2O2 alone; lane 5, NEM then H2O2. Panels E and F, cytosol containing transformed receptors was cleaved with trypsin and 200-µl aliquots were incubated as above, proteins were resolved by SDS-PAGE in the absence (panel E) of [3H]-mercaptoethanol and transferred to Immobilon membranes. The membranes were probed with the BuGR antibody, followed by 125I-conjugated counterantibody. Lane 1, no trypsin; lane 2, trypsin-
nanotate-bound receptor incubated with buffer alone; lane 3, incubated with NEM alone; lane 4, incubated with buffer alone; lane 5, incubated first with NEM then with H2O2.

d and D show autoradiograms of the [3H]dexamethasone 21-mesyate-bound receptor in the same Western blot. It can be seen that the peroxide-treated receptor is not recognized by the BuGR antibody (panel B, lane 4) unless it is reduced (panel A, lane 4). In panels E and F of Fig. 4, the 15-kDa trypsic fragment was treated with peroxide and proteins were resolved under reducing and nonreducing conditions, respectively. Because the same results are obtained with the 15-kDa trypsin fragment as with the intact receptor, it is clear that inactivation of the BuGR epitope involves only residues in the DNA-binding domain. In contrast to the situation with DNA binding, zinc is not required for reversal of peroxide-mediated inhibition of BuGR recognition (Fig. 4, A and E).

The autoradiogram of [3H]dexamethasone 21-mesyate-labeled receptor (Fig. 4D, lane 4) demonstrates that the peroxide-treated receptor migrates somewhat faster under nonreducing conditions than untreated receptor, presumably because intramolecular disulfide bonds cause it to assume a folded conformation as shown by Silva and Cidlowski (12). Derivitization of SH groups with N-ethylmaleimide (NEM) prevents the formation of the more rapidly migrating receptor forms (c.f. lanes 4 and 5 in panel D) and results in sharper protein bands in nonreduced gels. Interestingly, pretreatment with NEM does not inhibit the ability of hydrogen peroxide to cause intramolecular disulfide bonds cause it to assume a folded conformation as shown by Silva and Cidlowski (12).

Dependence of peroxide inhibition of DNA binding activity is the same for the 15-kDa trypsin fragment as for the intact receptor. As shown in Fig. 3, the peroxide-induced inhibition of DNA binding activity is reversed by the combination of DTT and zinc. Peroxide inhibition is shown in lane 6 and the very small reversal seen with DTT alone is shown in lanes 7 and 11. Addition of zinc alone after peroxide has no effect (not shown). Addition of DTT and zinc, however, produced a modest increase in DNA binding activity with 1 h of incubation (lane 8) and a large increase with 24 h of incubation (lane 12).

Hydrogen Peroxide Prevents Recognition of the Receptor by BuGR on Western Blots—In the experiment shown in Fig. 4, receptors labeled with [3H]dexamethasone 21-mesyate were incubated with buffer or peroxide and then submitted to SDS-PAGE under reducing (panels A and C) or nonreducing (panels B and D) conditions and transferred to Immobilon-P membranes. Panels A and B show Western blots of the reduced and nonreduced samples, respectively, and panels C

**Fig. 2.** Concentration dependence of hydrogen peroxide-mediated inhibition of DNA binding activity by intact receptor and the 15-kDa tryptic fragment. Cytosol containing dexamethasone-bound receptors was heated at 25°C to permit receptor transformation, the cytosol was divided into two portions and one portion was incubated at 0°C with trypsin followed by addition of protease inhibitors as described under "Methods." Aliquots (200 µl) of untreated and trypsin-treated cytosol were incubated for 1 h at 0°C with various concentrations of hydrogen peroxide and then bound to DNA-cellulose. The DNA-bound receptor or receptor fragment was resolved by SDS-PAGE under reducing conditions and Western blotted using BuGR as the probe antibody, followed by 125I-

**Fig. 3.** Reversal of peroxide-mediated inhibition of DNA binding activity by zinc and DTT. Transformed receptors were cleaved with trypsin and replicate aliquots (200 µl) of cytosol were incubated for 1 h at 0°C with buffer, 30 mM DTT, 1 mM ZnCl2, DTT plus ZnCl2, or 20 mM hydrogen peroxide. All samples were incubated a second time for 1 h (or 24 h as noted below) with buffer, DTT, or DTT plus ZnCl2. The 15-kDa receptor fragments were then bound to DNA-cellulose and the DNA-bound protein was resolved by SDS-PAGE and Western blotting. The lanes on the autoradiogram are: lane 1, no trypsin control; lane 2, fragment incubated with buffer alone; lane 3, DTT then buffer; lane 4, ZnCl2 then buffer; lane 5, DTT plus ZnCl2 then buffer; lane 6, H2O2 then buffer; lane 7, ZnCl2 then DTT; lane 8, H2O2 then DTT plus ZnCl2; lane 9, buffer alone (24 h); lane 10, H2O2 then buffer (24 h); lane 11, H2O2 then DTT (24 h); lane 12, H2O2 then DTT plus ZnCl2 (24 h).

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**Hydrogen Peroxide Prevents Recognition of the Receptor by BuGR on Western Blots**—In the experiment shown in Fig. 4, receptors labeled with [3H]dexamethasone 21-mesyate were incubated with buffer or peroxide and then submitted to SDS-PAGE under reducing (panels A and C) or nonreducing (panels B and D) conditions and transferred to Immobilon-P membranes. Panels A and B show Western blots of the reduced and nonreduced samples, respectively, and panels C

**Fig. 4.** Peroxide inactivates the BuGR epitope in a manner that is reversed by β-mercaptoethanol. Panels A–D, aliquots of cytosol containing transformed receptors bound with [3H]dexamethasone 21-mesyate were incubated for 1 h at 0°C with buffer or 20 mM NEM, followed by an additional 1-h incubation with buffer or 20 mM H2O2. Samples were divided in half and one portion was boiled in SDS sample buffer containing 10% β-mercaptoethanol (panels A and C) and the other portion was boiled in sample buffer without reducing agents (panels B and D). Proteins were resolved by SDS-PAGE and Western blotting using BuGR as the probe antibody and visualizing the receptor with peroxidase-conjugated goat anti-mouse IgG (panels A and B). After photography, the Western blots were sprayed with ENHANCE and the bound [3H]dexamethasone 21-mesyate was visualized by autoradiography (panels C and D). Lane 1, receptor labeled in the presence of a 1000-fold molar excess of nonradioactive dexamethasone; lane 2, [3H]dexamethasone 21-mesyate-bound receptor incubated with buffer alone; lane 3, incubated with NEM alone; lane 4, H2O2 alone; lane 5, NEM then H2O2. Panels E and F, cytosol containing transformed receptors was cleaved with trypsin and 200-µl aliquots were incubated as above, proteins were resolved by SDS-PAGE in the absence (panel E) or presence (panel F) of β-mercaptoethanol and transferred to Immobilon membranes. The membranes were probed with the BuGR antibody, followed by 125I-conjugated counterantibody. Lane 1, no trypsin; lane 2, trypsin-

and D show autoradiograms of the [3H]dexamethasone 21-mesyate-bound receptor in the same Western blot. It can be seen that the peroxide-treated receptor is not recognized by the BuGR antibody (panel B, lane 4) unless it is reduced (panel A, lane 4). In panels E and F of Fig. 4, the 15-kDa trypsic fragment was treated with peroxide and proteins were resolved under reducing and nonreducing conditions, respectively. Because the same results are obtained with the 15-kDa trypsin fragment as with the intact receptor, it is clear that inactivation of the BuGR epitope involves only residues in the DNA-binding region. In contrast to the situation with DNA binding, zinc is not required for reversal of peroxide-mediated inhibition of BuGR recognition (Fig. 4, A and E).

The autoradiogram of [3H]dexamethasone 21-mesyate-labeled receptor (Fig. 4D, lane 4) demonstrates that the peroxide-treated receptor migrates somewhat faster under nonreducing conditions than untreated receptor, presumably because intramolecular disulfide bonds cause it to assume a folded conformation as shown by Silva and Cidlowski (12). Derivitization of SH groups with N-ethylmaleimide (NEM) prevents the formation of the more rapidly migrating receptor forms (c.f. lanes 4 and 5 in panel D) and results in sharper protein bands in nonreduced gels. Interestingly, pretreatment with NEM does not inhibit the ability of hydrogen peroxide
to block recognition of the receptor by BuGR (lane 5 in Fig. 4, B and F).

As we are proposing that peroxide is inactivating the BuGR epitope by promoting disulfide bond formation within the 15-kDa DNA-binding tryptic fragment, it was surprising that pretreatment with the sulfhydryl derivatizing agent NEM did not prevent the inactivation. It would seem, however, that NEM either cannot get to the sulfhydryls in the DNA binding region of the receptor or for some reason cannot react with them. Consistent with this proposal is the fact that, as shown in Fig. 5, NEM does not inhibit the DNA binding activity of the transformed receptor in L cell cytosol, whereas another sulfhydryl-modifying agent MMTS inactivates most but not all of the DNA binding activity. It should be mentioned that NEM does not affect the ability of peroxide to inhibit DNA binding activity (data not shown).

In the experiment shown in Fig. 6, the receptor was preincubated with MMTS prior to treatment with peroxide and resolution under reducing and nonreducing conditions. It is clear that pretreatment with MMTS prevents much but not all of the peroxide-mediated inactivation of the BuGR epitope (c.f. lanes 3 and 4 in top panel, Fig. 6).

**DISCUSSION**

Because peroxide-induced inhibition of DNA binding can be both prevented by DTT and reversed by DTT in the presence of zinc (Fig. 3), it seems likely that disulfide bond formation is responsible for the inhibition. As the DNA binding activity of the 15-kDa tryptic fragment is inhibited by peroxide with the same sensitivity as that of the intact receptor (Fig. 2), it seems that the inhibition is due to disulfide-bridging within the DNA-binding domain. As reactivation of the DNA-binding activity requires zinc (Fig. 3), it is likely that the cysteines involved in the tetrahedral coordination of zinc are the cysteines that become bridged by disulfide bonds in the presence of peroxide. At this time, there is no way to speculate as to whether only vicinal thiols on the same side of each DNA-binding finger are linked to each other or whether disulfide bridges form between one side of a finger and the other.

The requirement for zinc in reversal of the peroxide inhibition of DNA binding suggests that peroxide treatment eliminates tetrahedrally coordinated zinc from the finger structure. Previously, Freedman et al. (6) found it necessary to reduce the pH of the buffer to 2.5 in order to facilitate dissociation of zinc from a peptide containing the glucocorticoid receptor DNA-binding region. Metal rebinding was then carried out after the buffer pH was returned to 7.9. It is possible that peroxide treatment followed by reduction may prove to be a useful procedure to employ in such metal elimination and reconstitution studies.

It is perhaps not surprising that the distortion in structure resulting from disulfide bonding in the DNA-binding domain can inactivate (Figs. 4 and 6) the BuGR epitope (residues 395-411) (18), which lies close to the vicinal cysteines at the amino-terminal side of the first DNA-binding finger (Cys-428, Cys-431). Inactivation of the BuGR epitope is reversed by reducing agent without the requirement of zinc (Figs. 4 and 6). Thus, reduction of disulfide bonds is sufficient to restore the appropriate conformation of the BuGR epitope, and the finger structure does not have to be intact for BuGR recognition.

The fact that NEM does not affect peroxide inactivation of the BuGR epitope (Figs. 4 and 6), and that it does not inhibit DNA binding (Fig. 5) or prevent peroxide inhibition of DNA-binding activity (data not shown), suggests either that NEM cannot get to the important cysteine thiols in the DNA-binding domain or that it cannot react with the metal-coordinated thiols. MMTS inhibits both DNA binding activity (Fig. 5) and peroxide inactivation of the BuGR epitope (Fig. 6). MMTS is known to react in a highly specific manner with protein thiols to derivatize them by adding a thiomethyl group through the formation of a mixed disulfide (19). It is important to note, however, that both MMTS inhibition of DNA binding activity and its inhibition of peroxide-mediated inactivation of the BuGR epitope is partial. We do not know why the MMTS effect is not complete. But, at this time the failure to achieve a complete block with MMTS means that we cannot rule out the possibility that peroxide inactivation of the BuGR epitope occurs via two mechanisms, one of which does not involve thiol groups but is nevertheless reversed by reducing agent.

It is clear that NEM reacts with the receptor and it is reasonable to suggest that it inhibits the formation of internal disulfide bonds (c.f. lanes 4 and 5 of Fig. 4D) as proposed by Silva and Cidlowski (12). In our experiments, we see that NEM-treated receptors migrate slower on nonreduced gels than do untreated receptors (c.f. lanes 2 and 3 in panels B and D of Fig. 4), suggesting that disulfide bridges are forming in

FIG. 5. Effect of sulfhydryl modifying agents on DNA-binding activity of the intact receptor. Cytosol containing dexamethasone-bound transformed receptors (200 μl) was incubated with buffer or increasing concentrations of NEM (●) or MMTS (□) for 1 h at 0°C, then bound to DNA-cellulose. The DNA-cellulose-bound receptors were solubilized with sample buffer containing β-mercaptoethanol, and resolved by SDS-PAGE and Western blotting with BuGR, followed by incubation with 125I-labeled goat anti-mouse IgG. Receptor bands were visualized by autoradiography, excised, and counted. Results are expressed as a percent of the DNA binding of a buffer-treated control.

FIG. 6. Pretreatment with MMTS inhibits peroxide-mediated inactivation of the BuGR epitope. Aliquots of cytosol containing transformed receptors were preincubated for 1 h at 0°C with buffer or 20 mM MMTS or 20 mM NEM and then treated for an additional hour with 20 mM hydrogen peroxide where indicated. Receptors were then resolved by SDS-PAGE under reducing or nonreducing conditions and Western blotted. Lane 1, buffer alone; lane 2, treated with MMTS; lane 3, hydrogen peroxide; lane 4, MMTS followed by hydrogen peroxide; lane 5, MMTS then DTT followed by hydrogen peroxide; lane 6, NEM then hydrogen peroxide.

The numbering of amino acids in this paper is according to the primary sequence of the mouse glucocorticoid receptor published by Danielson et al. (2).
the sample buffer and after application of samples to the gel.

It has not been determined if the glucocorticoid receptor contains transient or stable internal disulfide bonds in its normal functional state. In determining the normal folding state of the glucocorticoid receptor it will be critical to know whether stable disulfide bridges exist. The fact that the receptor can be cleaved to the 15-kDa tryptic fragment or to the 42-kDa chymotryptic fragment (DNA-binding domain plus hormone-binding domain) in the absence of reducing agents and that these fragments can be resolved on nonreducing gels (Fig. 4, data not shown) strongly suggests that no stable disulfide bonds connect the DNA-binding domain to the hormone-binding domain or connect either of these domains to the amino-terminal half of the receptor. We cannot rule out the possibility that proteolytic cleavage opens up the molecules such that internal disulfides are cleaved by endogenous thiol-disulfide exchange agents, like thioredoxin or glutaredoxin. With this reservation, however, the weight of the evidence indicates that, if there are any intramolecular disulfide bonds that stabilize an active conformation of the receptor, they must bridge cysteines within each domain.

When we combine the observation that there are no stable interdomain disulfide bridges with a number of pieces of information published by several other laboratories, we can make some useful predictions about the maximum possible number of disulfide bridges and the cysteines that could be involved. Nothing is known about the oxidation state of the 4 cysteine residues in the amino-terminal one-half of the receptor, and this region could contain one or two stable disulfide bridges. The 11 cysteines in the 15-kDa tryptic fragment from Cys-375 to Cys-488 can be ruled out as being involved in stable disulfide bridging. Eight of these cysteines are ruled out on the basis that they are involved in the tetrahedral coordination of zinc (6, 7). Cys-438 and Cys-488 are ruled out on the basis that Severne et al. (20) have shown that either residue can be converted to a serine without loss of transactivating activity. Cys-375 then becomes ruled out by elimination in that it is the only remaining cysteine residue in the fragment. It should be mentioned that the amino-terminal end of the 15-kDa fragment has not been precisely determined. From fragment size on SDS-PAGE, we have calculated the trypsin cleavage site to be at Lys-374 (14), which places Cys-375 in the 15-kDa fragment. It is possible that this cleavage site is at Arg-374, which would place Cys-375 in the amino-terminal one-half of the receptor.

It is clear that the hormone-binding domain contains a number of reduced cysteines (4), and it can be deduced that only one stable disulfide bridge could exist between the 5 cysteine residues within the domain. Cys-644 must be reduced because it is the site of attack by the covalent affinity ligand dexamethasone 21-mesylate (21, 22). Similarly, it is highly likely that Cys-672 is reduced because it becomes covalently bound to triaminolone acetonide upon UV irradiation of the steroid-bound receptor (23). Simons and his co-workers (24–26) have shown that 2 thiols that lie close to each other in the appropriate tertiary conformation of the hormone-binding domain must be in the reduced (as opposed to disulfide bonded) form for steroid binding to occur. One of these cysteines is almost certainly Cys-644 and the other is most likely Cys-649 (although Cys-68) could possibly be the other cysteine that is inactivated by arsenite under vicinal thiol-specific conditions (26). Thus, there appear to be only 2 cysteines within the hormone-binding domain (Cys-628 or possibly Cys-649 and Cys-671) that could be involved in stable disulfide bond formation in an appropriately folded and fully functional glucocorticoid receptor.

Although this analysis predicts that no more than 3 stable internal disulfide bonds can exist in an appropriately folded glucocorticoid receptor, it should be emphasized that there is, as yet, no evidence for the existence of any stable disulfide bridges within the receptor and there may very well be none. Now that systems for receptor overexpression are available, it would be most useful to directly determine the number of reduced cysteine residues per receptor.

Acknowledgment—We very much thank Dr. Robert Harrison for providing us with the BUGR monoclonal antibody against the glucocorticoid receptor.

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