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Hydrogen Peroxide Stabilizes the Steroid-Binding State of Rat Liver Glucocorticoid Receptors by Promoting Disulfide Bond Formation†

Emery H. Bresnick,‡ Edwin R. Sanchez,‡ Robert W. Harrison,§ and William B. Pratt*,‡

Department of Pharmacology, The University of Michigan Medical School, Ann Arbor, Michigan 48109, and Division of Endocrinology and Metabolism, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205

Received October 6, 1987; Revised Manuscript Received December 11, 1987

ABSTRACT: Hydrogen peroxide and diamide inactivate the steroid-binding capacity of unoccupied glucocorticoid receptors in rat liver cytosol at 0 °C, and steroid-binding capacity is reactivated with dithiothreitol. Treatment of cytosol with peroxide or sodium molybdate, but not diamide, inhibits the irreversible inactivation (i.e., inactivation not reversed by dithiothreitol) of steroid-binding capacity that occurs when cytosol is incubated at 25 °C. Pretreatment of cytosol with the thiol derivatizing agent methyl methanethiosulfonate at 0 °C prevents the ability of peroxide, but not molybdate, to stabilize binding capacity at 25 °C. As derivatization of thiol groups prevents peroxide stabilization of steroid-binding capacity and as treatment with dithiothreitol reverses the effect, we propose that peroxide acts by promoting the formation of new disulfide linkages. The receptor in our rat liver cytosol preparations is present as three major degradation products of M_r 40000, 52000, and 72000 in addition to the M_r 94000 intact receptor. Like the intact receptor, these three forms exist in the presence of molybdate as an 8-9S complex, they bind glucocorticoid in a specific manner, and they copurify with the intact M_r 94000 receptor on sequential phosphocellulose and DNA-cellulose chromatography. Despite the existence of receptor cleavage products, it is clear that peroxide does not stabilize steroid-binding capacity by inhibiting receptor cleavage.

Sulfhydryl groups are required for several functions of the glucocorticoid receptor, including its steroid-binding activity, its transformation[1] from an 8-9S to a 4S form, and the binding of transformed receptors to DNA. Early studies by Rees and Bell (1975) demonstrated a requirement for sulfhydryl groups in maintaining an active steroid-binding conformation of the rat thymocyte glucocorticoid receptor. Other workers have since utilized sulfhydryl derivatizing agents, such as *N*-ethylmaleimide (Formstecher et al., 1984), iodoacetamide

† This investigation was supported by Grant DK31573 from the National Institutes of Health.

* Author to whom correspondence should be addressed.
‡ The University of Michigan Medical School.
§ University of Arkansas for Medical Sciences.

[1] In this work, we will use the word "untransformed" to describe the 8-9S form of the receptor that does not bind to DNA and the word "transformed" to describe the 4S, DNA-binding form.

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(Young et al., 1975), methyl methanethiosulfonate[2] (Bodwell et al., 1984a), and *p*-(chloromercuri)benzenesulfonate (Harrison et al., 1983) to study the sulfhydryl group requirement for glucocorticoid binding. With the exception of mercurials (Coty, 1980), sulfhydryl derivatizing reagents inhibit the ability of unliganded receptors to bind glucocorticoids but do not cause the release of prebound glucocorticoid hormone. Studies from our laboratory (Grippo et al., 1983, 1985) suggest that a NADPH-dependent, thioredoxin-mediated, thiol–disulfide exchange system is responsible for maintaining rat liver cytosolic glucocorticoid receptors in a steroid-binding state. It is clear (Bodwell et al., 1984a; Tienrungroj et al., 1987) that sulfur moieties on the receptor must be in a reduced form for the transformed receptor to bind to DNA, and Bodwell et al. (1984b) have provided evidence that the thiols required for DNA binding are different from those required for steroid binding.

Wilson et al. (1986) have shown that the oxidizing agent cupric phenanthroline stabilizes the untransformed androgen receptor in a 10S form in rat Dunning prostate tumor cytosol. In a similar vein, we have reported (Tienrungroj et al., 1987) that hydrogen peroxide treatment of rat liver cytosol containing liganded glucocorticoid receptors inhibits both temperature-mediated conversion of the 9S form of the receptor to 4S and conversion of the receptor from a non-DNA-binding to a DNA-binding state. Both peroxide inhibition of receptor dissociation and peroxide inhibition of conversion to the DNA-binding form are reversed by dithiothreitol (DTT). It was concluded, therefore, that sulfur moieties in the receptor complex must be in a reduced state for transformation to occur. From this work, it was unclear whether peroxide acts by stabilizing a preexisting disulfide bond(s), or, alternatively, if peroxide promotes the formation of new disulfide linkages.

The work of Tienrungroj et al. (1987) showed that peroxide produced all of the effects on the steroid-receptor complex that are produced by molybdate and some other transition metal oxyanions, such as vanadate and tungstate (Pratt, 1987). It is well established that these transition metal anions also stabilize unliganded receptors in a steroid-binding state (Nielsen et al., 1977a,b). In this paper we show that peroxide treatment stabilizes the unliganded rat liver receptor at 25 °C such that, when it is subsequently reduced with dithiothreitol, it is able to bind steroid. We provide evidence that peroxide stabilizes the receptor through the creation of new disulfide linkages.

Experimental Procedures

Materials

[6,7-^{3}H]Triamcinolone acetonide (42.8 Ci/mmol) and [6,7-^{3}H(N)]dexamethasone 21-mesylate (48.9 Ci/mmol) were obtained from New England Nuclear, Boston, MA. Sodium molybdate was obtained from J. T. Baker Co., Phillipsburg, NJ. Radioinert dexamethasone, nonimmune mouse IgG, protein A–Sepharose, and goat anti-mouse IgG–horseradish peroxidase conjugate were from Sigma Chemical Co., St. Louis, MO. Nitrocellulose paper (0.45 m) and chemicals for electrophoresis were from Bio-Rad, Richmond, CA.

Methods

Cell Source and Fractionation. Liver was obtained from 150–175-g male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) that had been adrenalectomized and maintained on 0.9% saline for one day prior to sacrifice. Livers were excised immediately upon sacrifice by decapitation, washed extensively with Earle's buffered saline, and homogenized in a Waring blender in 1.5 volumes of 10 mM Hepes buffer (pH 7.35) per gram of wet weight, followed by three strokes with a Dounce homogenizer. The tissue homogenate was centrifuged at 27000*g* for 20 min at 4 °C, and the supernatant was centrifuged at 100000*g* for 2 h at 4 °C. The 100000*g* supernatant (referred to as cytosol) was immediately frozen and stored at −70 °C until use.

Incubation Conditions and Steroid-Binding Assay. Incubations containing cytosol and other agents were prepared as indicated in the figure and table legends. Additions were made from fresh stock solutions concentrated at least 20-fold. Following treatment with appropriate agents, cytosol was bound at 0 °C with 10 nM [^{3}H]triamcinolone acetonide for 12 h with or without a 1000-fold excess of radioinert dexamethasone. Steroid binding was assayed by the charcoal absorbtion method. Specific binding values are the average of duplicate assays, calculated by subtracting the nonspecific binding value from total binding, and are expressed as cpm/0.2 mL of the original undiluted cytosol.

Incubations with Antibodies and Adsorption to Protein A–Sepharose. Aliquots of cytosol were mixed with an equal volume of TEG buffer [10 mM TES, 4 mM EDTA, 10% (w/v) glycerol, 50 mM NaCl, pH 7.6 at 4 °C]. Anti-receptor antibody [the BuGR2 monoclonal antibody prepared against the rat glucocorticoid receptor as described by Gametchu and Harrison (1984)] or nonimmune mouse IgG (1 mg/mL) was added at 2–4% of the final volume. The mixture was incubated for 3 h at 0 °C, and then each sample was added to a 50-μL pellet of protein A–Sepharose (preequilibrated in TEG buffer). Samples were mixed by rotation for 2 h at 4 °C, and protein A–Sepharose pellets were washed 5 times with 1-mL aliquots of TEG buffer.

Gel Electrophoresis and Immunoblotting. SDS–polyacrylamide gel electrophoresis was performed in 7% slab gels according to the method of Laemmli (1970). Gels were cooled to 4 °C during electrophoresis. All samples were extracted from protein A–Sepharose by boiling in SDS sample buffer containing 10% β-mercaptoethanol. 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; ovalbumin, M_r 45 000; and carbonic anhydrase, M_r 29 000.

Immunoblotting was carried out by transferring proteins from acrylamide slab gels to nitrocellulose paper, overnight incubation with 1% BuGR2 antibody against the glucocorticoid receptor, and subsequent reaction with peroxidase-conjugated goat anti-mouse IgG antibody as previously described (Sanchez et al., 1985; Housley et al., 1985). In the experiment of Table II, the transformed proteins were developed with ^{125}I-labeled sheep anti-mouse IgG followed by donkey anti-sheep peroxidase-conjugated IgG. The receptor bands visualized by peroxidase staining were excised and counted for radioactivity to quantify the amount of receptor protein as described by Howe and Hershey (1981).

Receptor Purification. Purification of the rat hepatic glucocorticoid receptor was carried out through the DNA–cel-

[2] Trivial names and abbreviations: dexamethasone, 9α-fluoro-16α-methyl-11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione; dexamethasone 21-mesylate, 9α-fluoro-16α-methyl-11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione 21-methanesulfonate; triamcinolone acetonide (TA), 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetonide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TES, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MMTS, methyl methanethiosulfonate; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; M_r 94 000, 72 000, etc., bands on SDS–PAGE; hsp90, M_r 90 000 heat shock protein; IgG, immunoglobulin G; EDTA, (ethylenedinitrilo)tetraacetic acid.

Table I: H_2O_2 and Diamide Reversibly Inhibit Steroid-Binding Capacity of Rat Liver Cytosol at 0 °C[a]

conditions	specific binding capacity (cpm/0.2 mL)
Hepes buffer alone	14403
H_2O_2	8496
sodium azide, H_2O_2	0
sodium azide, H_2O_2, then DTT	14159
diamide	0
diamide, then DTT	15097

[a] Aliquots of rat liver cytosol were incubated for 10 min at 0 °C in the presence or absence of 10 mM sodium azide followed by incubation for 30 min at 0 °C in the presence of buffer alone, 10 mM H_2O_2, or 10 mM diamide. DTT (20 mM) was then added as indicated, and each sample was incubated with 10 nM [^{3}H]TA for 12 h at 0 °C to determine specific binding capacity as described under Experimental Procedures. Values represent the average of duplicate determinations expressed as cpm of [^{3}H]TA specifically bound per 0.2 mL of undiluted cytosol. A nonspecific binding value of 1270 cpm/0.2 mL has been subtracted to yield each value in the table.

lulose step as described by Wrange et al. (1986). Briefly, 19 mL of rat hepatic cytosol was incubated for 1 h at 0 °C with 100 nM [^{3}H]triamcinolone acetonide (10.6 Ci/mmol) and was rapidly chromatographed through a column (5 × 3 cm) of phosphocellulose (Whatman P11). Pooled flow-through fractions were diluted with an equal volume of equilibration buffer and transformed by heating for 45 min at 25 °C. Transformed cytosol was applied to a DNA–cellulose column (5 × 1.5 cm) and washed with 1.5 volumes of 90 mM NaCl in equilibration buffer and 1 volume of 115 mM NaCl in equilibration buffer, and glucocorticoid receptor was eluted with 25 mM $MgCl_2$. $MgCl_2$-eluted fractions containing [^{3}H]triamcinolone acetonide bound proteins were pooled and precipitated with 10% trichloroacetic acid and 0.2% sodium deoxycholate, and one-ninth of the resulting proteins were analyzed by Western blotting with BuGR2 as the probe antibody.

Results

Hydrogen Peroxide and Diamide Reversibly Inhibit the Steroid-Binding Capacity of Rat Liver Glucocorticoid Receptors. Incubation of rat liver cytosol with 10 mM H_2O_2 for 30 min at 0 °C inactivates specific steroid-binding capacity by approximately 40%, as shown in Table I. Inclusion of sodium azide (10 mM) to inhibit the peroxide scavenging enzyme catalase results in 100% inactivation of steroid-binding capacity, and 20 mM dithiothreitol (DTT) restores binding capacity. Similarly, the thiol oxidant diamide (10 mM) inactivates steroid-binding capacity by 100%, and DTT (20 mM) completely restores it. These results demonstrate the requirement for reduced thiols in maintaining glucocorticoid receptors in a steroid-binding state, as has been previously reported by several laboratories (Rees & Bell, 1975; Harrison et al., 1983; Grippo et al., 1983, 1985). In addition, the effects of peroxide on unliganded receptors at 0 °C are clearly reversible, suggesting that receptors are not physically damaged by peroxide treatment.

Hydrogen Peroxide Stabilizes Unoccupied Receptors against Thermal Inactivation of Steroid-Binding Capacity. Previous work has demonstrated that the group VIA transition metal oxyanion molybdate stabilizes unliganded glucocorticoid receptors against thermal inactivation of steroid-binding capacity (Nielsen et al., 1977a,b; Leach et al., 1979; Dahmer et al., 1981). We ask here whether peroxide shares with molybdate the ability to stabilize the unliganded rat liver glucocorticoid receptor to irreversible inactivation of steroid-binding capacity caused by incubating cytosol at 25 °C.

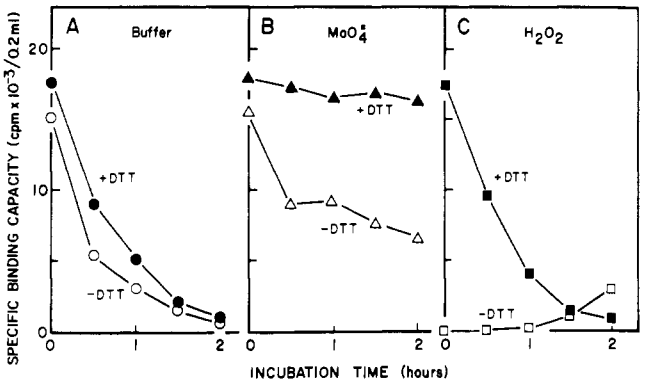

FIGURE 1: Effects of hydrogen peroxide, molybdate, and DTT on the thermal inactivation of steroid-binding capacity in rat liver cytosol. Cytosol was treated for 10 min at 0 °C with sodium azide followed by 30 min at 0 °C with Hepes buffer alone (○), buffer and 20 mM DTT (●), 10 mM sodium molybdate (△), molybdate and DTT (▲), 20 mM hydrogen peroxide (□), or peroxide and DTT (■). Each aliquot was then incubated at 25 °C for the indicated times, and specific binding capacity was assayed with [^{3}H]triamcinolone acetonide, as described under Experimental Procedures. Values represent the average of duplicate determinations expressed as cpm of [^{3}H]steroid specifically bound per 0.2 mL of undiluted cytosol.

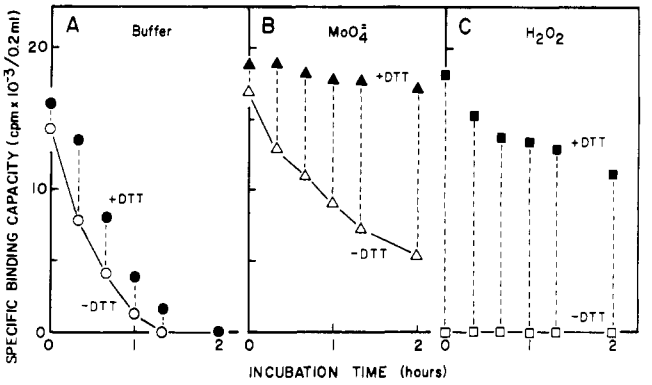

FIGURE 2: Peroxide and molybdate stabilize receptors in rat liver cytosol such that steroid-binding capacity can be reactivated with DTT. Rat liver cytosol was treated at 0 °C with sodium azide followed by buffer alone (○), 10 mM molybdate (△), or 20 mM peroxide (□). Each aliquot was then incubated at 25 °C; at indicated times, buffer (open symbols) or 20 mM DTT (closed symbols) was added, and specific binding capacity was assayed.

As shown in Figure 1A, rat liver cytosol rapidly loses specific glucocorticoid binding capacity with a $t_{1/2}$ of approximately 30 min at 25 °C. Molybdate reduces the inactivation rate in the absence of DTT, and as previously reported (Sando et al., 1979), inclusion of DTT with molybdate during the 25 °C incubation results in virtually complete stabilization of the binding capacity (Figure 1B). As predicted from the experiments presented in Table I, peroxide-treated cytosol incubated at 25 °C in the presence of sodium azide does not exhibit measurable specific binding capacity (Figure 1C). If DTT is present with peroxide during the 25 °C incubation, steroid-binding capacity is not inactivated at zero time and binding capacity is lost at the same rate as in the cytosol without additions. Thus, peroxide does not stabilize unliganded receptors at 25 °C in the presence of DTT, and in the absence of DTT, binding capacity is not measurable.

In order to determine if peroxide stabilizes receptors, the experimental protocol was revised. Cytosol was incubated with peroxide at 25 °C in the absence of DTT, and at various times, aliquots were removed and steroid-binding capacity was determined at 0 °C in the presence or absence of DTT. The results illustrated in Figure 2 demonstrate that, like molybdate,

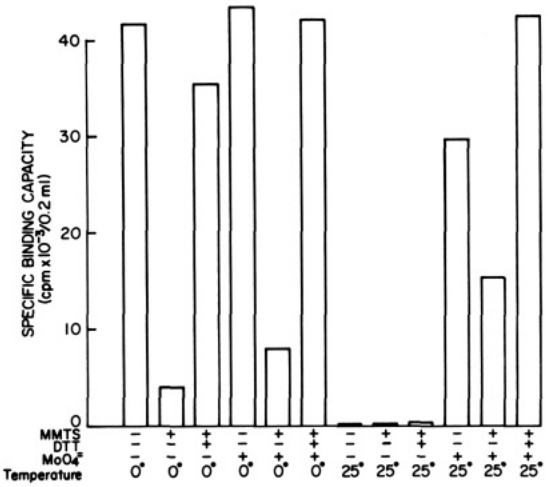

FIGURE 3: Effect of MMTS on rat liver cytosol steroid-binding capacity at 0 and 25 °C. Rat liver cytosol was pretreated at 0 °C with 10 mM sodium azide followed by buffer or 5 mM MMTS for 45 min. The buffer-treated and MMTS-treated cytosols were each divided into two equal aliquots and incubated for 2 h at 0 or 25 °C in the presence or absence of 10 mM molybdate. At the end of the incubation at 0 or 25 °C, 20 mM DTT was added to half of the samples, and specific steroid binding was assayed.

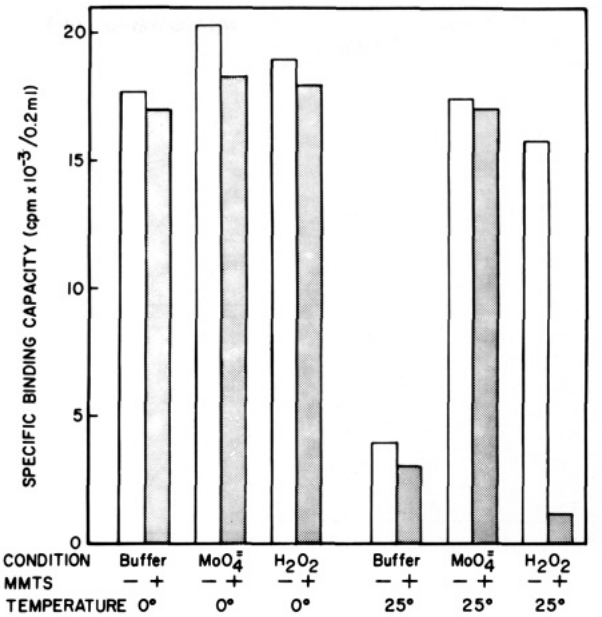

FIGURE 4: At a concentration of 5 mM, MMTS prevents the stabilization of rat liver glucocorticoid receptors by peroxide but not by molybdate. Rat liver cytosol was incubated for 45 min at 0 °C in the presence or absence of 5 mM MMTS. Each portion of cytosol was divided into three equal aliquots, treated for 10 min with 10 mM sodium azide, and incubated for 2 h at 0 or 25 °C with buffer alone, with 10 mM molybdate, or with 20 mM peroxide. At the end of the 25 °C incubation, 30 mM DTT was added to all samples at 0 °C, and specific steroid-binding capacity was assayed.

peroxide treatment stabilizes unoccupied receptors to thermal inactivation. In the absence of any treatment at 25 °C, DTT addition does not significantly restore binding capacity (panel A). In contrast, DTT addition to molybdate-treated cytosol results in reactivation of binding capacity to 100% of the time-zero value (panel B), and peroxide-treated cytosol is reactivated to approximately 70% of the time-zero value (panel C). Under these conditions, 10–12 mM peroxide is required for maximum stabilization of receptors to thermal inactivation. Thus, peroxide shares with molybdate the ability to stabilize unliganded glucocorticoid receptors in a physical state at 25 °C such that DTT reactivates receptor steroid-binding capacity. In contrast to peroxide, the thiol oxidant diamide does not inhibit thermal inactivation of the receptor at 25 °C, suggesting that the thiols that are reversibly inactivated by diamide at 0 °C and are required for steroid-binding activity are distinct from the thiol moieties that mediate receptor stabilization. Alternatively, peroxide and diamide could have different mechanisms of action in the complex cytosolic milieu.

Methyl Methanethiosulfonate (MMTS) Prevents the Stabilization of Unliganded Receptors by Hydrogen Peroxide but Not by Molybdate. The fact that the thiol–disulfide exchange reagent DTT prevents the stabilizing activity of peroxide suggests that the mechanism by which peroxide stabilizes receptors reflects either its ability to promote formation of disulfide bonds from preexisting thiols or, alternatively, its ability to prevent the reduction of preexisting disulfide bonds. To distinguish between these two possibilities, we employed the DTT-reversible thiol derivatizing agent MMTS to block accessible thiols on cytosolic proteins. MMTS reacts selectively with sulfhydryl groups (Bodwell et al., 1984a; Simons, 1987), and MMTS derivatization of receptor thiols should prevent their subsequent oxidation by peroxide. The effect of MMTS on the steroid-binding capacity of unoccupied receptors was determined in the presence or absence of peroxide and molybdate at 0 °C. As shown in Figure 3, MMTS (5 mM) treatment of cytosol for 45 min at 0 °C inhibits steroid-binding capacity in a DTT-reversible manner. MMTS does not affect thermal inactivation of steroid-binding capacity and, at this concentration, it does not alter the ability of molybdate to stabilize the receptor, as long as a stoichiometric amount of DTT is added to the system prior to assaying the steroid-binding capacity. Figure 4 shows that, while 5 mM MMTS does not affect the ability of molybdate to stabilize the receptor, the ability of peroxide to inhibit the thermal inactivation is prevented by MMTS pretreatment. Under these conditions, a concentration of 6–7 mM MMTS is required to inhibit peroxide stabilization of steroid-binding capacity. As MMTS blocks the peroxide effect, we suggest that peroxide may stabilize the receptor by promoting the formation of disulfide bonds between preexisting thiols.

Peroxide Does Not Stabilize Binding Capacity by Inhibiting Cleavage of the Rat Liver Receptor. It is well established that rat liver cytosol preparations contain proteases that cleave the glucocorticoid receptor (Sherman et al., 1983), and it is important to establish that peroxide stabilization does not simply reflect inhibition of receptor cleavage. Figure 5A demonstrates the size heterogeneity of the glucocorticoid receptor in our cytosol preparation. The receptor is demonstrated here by Western blotting using the BuGR2 monoclonal antibody that recognizes an epitope at the amino-terminal end of the DNA binding domain (Eisen et al., 1985; Rusconi & Yamamoto, 1987). The major forms of the receptor detected with BuGR2 have M_r of 40 000, 52 000, 72 000, and 94 000. The M_r 40 000, 52 000, and 72 000 species represent from 70 to 90% of the total immunoreactive receptor in cytosol prepared in this manner. In addition, we recover varying amounts of two minor degradation species migrating at M_r 80 000 and M_r 45 000, respectively.

Analysis of [^{3}H]triamcinolone acetonide bound receptors in rat liver cytosol by centrifugation on molybdate-containing sucrose gradients demonstrates a 9S peak (Tienrungroj et al., 1987), despite the clear presence of multiple receptor forms. This observation is in agreement with that of Mendel et al. (1985), who demonstrated that the untransformed rat thymus receptor does not undergo a measurable reduction in molecular size by nondenaturing agarose gel filtration when the receptor

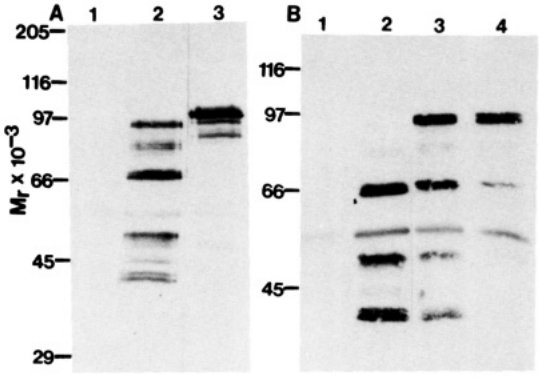

FIGURE 5: Glucocorticoid receptor is present in rat liver cytosol predominantly as cleavage products. (Panel A) Western blot analysis of rat liver and L cell glucocorticoid receptors with the BuGR2 monoclonal antibody. Rat liver cytosol (0.5 mL) or L929 cytosol (0.5 mL) was diluted with an equal volume of TEG buffer and incubated for 2 h at 0 °C with nonimmune mouse IgG (lane 1) or BuGR2 (lanes 2 and 3). Samples were adsorbed to protein A–Sepharose, the Sepharose pellets were washed 4 times with TEG buffer, and the immunoadsorbed proteins were resolved by SDS–PAGE and Western blot analysis using BuGR2 as the probe antibody. Samples applied to each lane of the gel were (lane 1) rat liver cytosol incubated with nonimmune mouse IgG, (lane 2) rat liver cytosol immunoadsorbed with BuGR2, and (lane 3) L cell cytosol immunoadsorbed with BuGR2. (Panel B) Inhibition of glucocorticoid receptor proteolysis by molybdate or by phosphocellulose adsorption during rat liver cytosol preparation. Livers from three adrenalectomized rats were excised and minced. Minced liver was divided into three portions, and cytosol was prepared with the following homogenization buffers: (lanes 1 and 2) 10 mM Hepes; (lane 3) Hepes with 10 mM sodium molybdate. Lane 4 represents cytosol prepared by homogenization in 10 mM Hepes followed by centrifugation at 27000*g* and batch incubation of the supernatant with 0.5 volume of phosphocellulose (equilibrated in 10 mM Hepes) for 5 min at 4 °C to remove protease activity. The phosphocellulose was removed by centrifugation, and the resulting supernatant was centrifuged at 100000*g* for 2 h. The cytosol preparations (0.5 mL) were immunoadsorbed to protein A–Sepharose, and the protein A–Sepharose-bound proteins were resolved by SDS–PAGE and analyzed by Western blotting with BuGR2 as the probe antibody. (Lane 1) Normal cytosol immunoadsorbed with nonimmune IgG; (lane 2) normal cytosol immunoadsorbed with BuGR2; (lane 3) molybdate-containing cytosol immunoadsorbed with BuGR2; (lane 4) phosphocellulose-adsorbed cytosol immunoadsorbed with BuGR2.

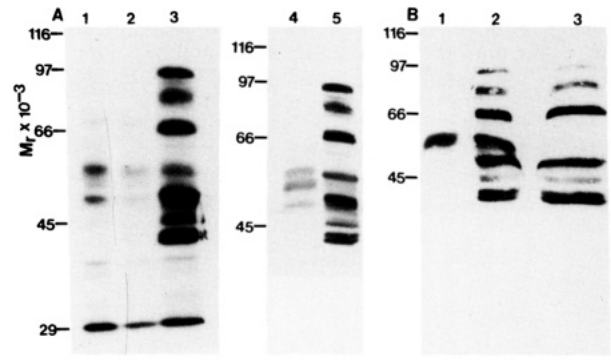

FIGURE 6: Receptor fragments in rat liver cytosol bind steroid, transform, and bind to DNA. (Panel A) [^{3}H]Dexamethasone 21-mesylate affinity labeling of receptor fragments bound to protein A–Sepharose. Aliquots (0.5 mL) of rat liver cytosol prepared with 10 mM molybdate were incubated with nonimmune mouse IgG or BuGR2 and adsorbed to protein A–Sepharose. Pellets were washed 4 times with 1-mL aliquots of TEG buffer containing 10 mM molybdate and then incubated for 2 h at 0 °C with 50 nM [^{3}H]dexamethasone 21-mesylate in the presence or absence of a 1000-fold excess of radioinert dexamethasone. Proteins were resolved by 7% SDS–PAGE, and autoradiography was performed (lanes 1–3). Additional unlabeled pellets were resolved on the same gel, and Western blotting was performed with BuGR2 as the probe antibody (lanes 4 and 5). Samples applied to each lane of the gel were (lane 1) rat liver cytosol incubated with nonimmune mouse IgG and labeled with [^{3}H]dexamethasone 21-mesylate, (lane 2) cytosol immunoadsorbed with BuGR2 and labeled with [^{3}H]dexamethasone 21-mesylate in the presence of a 1000-fold excess of radioinert dexamethasone, (lane 3) cytosol immunoadsorbed with BuGR2 and labeled with [^{3}H]dexamethasone 21-mesylate, (lane 4) cytosol incubated with nonimmune mouse IgG and Western probed with BuGR2, and (lane 5) cytosol immunoadsorbed with BuGR2 and probed with BuGR2. (Panel B) Copurification of receptor degradation products on sequential phosphocellulose and DNA–cellulose chromatography. Rat liver cytosol (19 mL) was labeled with 50 nM [^{3}H]triamcinolone acetonide and rapidly passed through a column of phosphocellulose. Flow-through fractions were diluted with an equal volume of 10 mM Hepes, heated for 45 min at 25 °C to transform receptors, and chromatographed on a DNA–cellulose column as described under Experimental Procedures. Proteins eluted with 25 mM $MgCl_2$ were precipitated with trichloroacetic acid and sodium deoxycholate and dissolved in SDS–PAGE sample buffer, and one-ninth of the resulting proteins were resolved by SDS–PAGE. The proteins were developed by Western blotting with BuGR2 as the probe antibody. Samples applied to each lane of the gel were (lane 1) rat liver cytosol (0.5 mL) incubated with nonimmune mouse IgG, (lane 2) cytosol (0.5 mL) immunoadsorbed with BuGR2, and (lane 3) protein eluted from the DNA–cellulose column with $MgCl_2$.

is cleaved to a M_r 50000 species. In rat liver cytosol prepared by our method, addition of a mixture of protease inhibitors (2 mM PMSF, 1 mM leupeptin, 0.2 mM antipain, 2 mM EDTA) does not affect the extent of receptor proteolysis. As shown in lanes 3 and 4 of Figure 5B, however, either inclusion of molybdate in the homogenization buffer or reducing protease activity by batch absorption of the 27000*g* rat liver supernatant with phosphocellulose results in significant enrichment of the M_r 94000 intact receptor, with reduced amounts of the M_r 40000, 52000, and 72000 species.

As shown in Figure 6A, all of the cleavage products recognized by the BuGR2 antibody have steroid-binding activity. This is demonstrated by their ability to bind [^{3}H]dexamethasone 21-mesylate in a specific manner after immunoadsorption to protein A–Sepharose. The receptor species were labeled in the immune pellets in order to eliminate the labeling of nonreceptor proteins in rat liver cytosol that has been described previously by others (Eisen et al., 1981; Simons et al., 1983). Panel B of Figure 6 shows that all of the receptor species identified by reaction with BuGR2 and labeling with [^{3}H]dexamethasone mesylate bind to DNA–cellulose after thermal transformation.

In the experiments of Table II, the amount of receptor protein in each species on the Western blot was quantified with ^{125}I-labeled counter antibody and compared to steroid-binding capacity in aliquots of the same cytosol. These data show that the degree of receptor inactivation is much greater than the loss of immunodetectable receptor and that peroxide-mediated stabilization of steroid-binding capacity is not due to inhibition of receptor cleavage.

Discussion

In a previous paper we demonstrated that treatment of cytosol with H_2O_2 inhibits temperature-mediated transformation of glucocorticoid-bound rat liver receptors to the DNA-binding state (Tienrungroj et al., 1987). It is clear from the experiments presented here that H_2O_2 also stabilizes unbound receptors to loss of steroid-binding capacity. Taken together, the observations of the two papers show that H_2O_2 can produce essentially the same effects on the glucocorticoid receptor as molybdate.

Because pretreatment with MMTS blocks H_2O_2 stabilization of steroid-binding capacity and treatment with DTT reverses the effect, we propose that peroxide acts by promoting the formation of new disulfide linkages. At this time, we do not know where the sulfur moieties affected by peroxide are

Table II: Peroxide Does Not Stabilize Steroid-Binding Capacity in Rat Liver Cytosol by Inhibiting Receptor Proteolysis[a]

conditions	specific binding capacity (% of 0 time control)	immunoreactive glucocorticoid receptor (% of 0 time control)
0 °C, zero time	100	100
0 °C, 2 h	105 ± 4	87 ± 8
25 °C, 2 h	16 ± 1	71 ± 5
25 °C, 2 h + molybdate	105 ± 0	93 ± 5
25 °C, 2 h + peroxide	52 ± 2	63 ± 5

[a] Rat liver cytosol was treated for 10 min with 10 mM sodium azide followed by buffer alone, 10 mM molybdate, or 20 mM peroxide for 10 min at 0 °C. Each aliquot was then incubated for 2 h at 0 or 25 °C; then 125 μL of the cytosol was diluted to 250 μL with buffer, and steroid-binding capacity was assayed after addition of 30 mM DTT. Values represent the average of duplicate determinations expressed as percent of the zero-time specific binding value. The remainder of each aliquot of cytosol (375 μL) was diluted with an equal volume of TEG buffer and incubated for 2 h at 0 °C with nonimmune mouse IgG or BuGR2. Samples were adsorbed to protein A–Sepharose, the Sepharose pellets were washed 5 times with TEG buffer, and the immunoadsorbed proteins were resolved by SDS–PAGE and Western blot analysis using BuGR2 as the probe antibody. Blots were developed by first incubating with an ^{125}I-labeled counter antibody, followed by a horseradish peroxidase conjugated third antibody. Five receptor species of M_r 94 000, 80 000, 72 000, 52 000, and 40 000 were visualized by peroxidase staining and excised from the blot, and ^{125}I cpm was determined by liquid scintillation spectroscopy. Values represent single determinations expressed as percent of the zero-time total receptor value. Zero-time total receptor values were 231 000 and 172 000 cpm for experiments 1 and 2, respectively. Control samples immunoadsorbed with nonimmune IgG demonstrated that 98% of the ^{125}I radioactivity in the excised bands is specific for receptor. The values in the table represent an average of two experiments plus or minus the variation.

located. Disulfide bonds could be formed within the steroid-binding protein, between the steroid-binding protein and other components of the receptor complex, or possibly in some other cytosolic compound that affects steroid-binding capacity.

Although it is not yet clear how disulfide bond formation stabilizes the receptor, it seems clear that it neither renders the receptor unable to be cleaved by endogenous cytosolic proteases nor inactivates the proteases themselves. The receptors in our rat cytosol are cleaved (Figures 5 and 6), but the data of Table II demonstrate that inactivation of steroid-binding capacity is much greater than the extent of receptor cleavage and the cleavage that occurs is not affected by peroxide. Taken together, these observations suggest that H_2O_2 inhibits another mechanism of receptor inactivation.

There is now substantial evidence that the untransformed glucocorticoid receptor exists in cytosolic preparations as an 8–9S (M_r approximately 300 000) heteromeric complex consisting of a single 4S (M_r 94 000–100 000) steroid-binding protein (Okret et al., 1985; Gehring & Arndt, 1985) and at least one molecule of the M_r 90 000 heat shock protein, hsp90 (Sanchez et al., 1985, 1987; Mendel et al., 1986). Both molybdate and peroxide stabilize the receptor in this heteromeric structure (Tienrungroj et al., 1987), and it is possible that this stabilization accounts for the stabilization of steroid-binding capacity. It is important to note that conditions which transform steroid-bound receptors to the DNA-binding state (e.g., heating of cytosol, dilution, and increasing ionic strength or pH) inactivate the steroid-binding capacity of unliganded receptors (Leach et al., 1979; Dahmer et al., 1981). These are all conditions that promote dissociation of the heteromeric 9S complex, and it is possible that glucocorticoid receptors must be associated with another component, or components, of the complex for there to be a competent steroid-binding site. This could explain why we find that unliganded glucocorticoid receptors that have been treated with salt and recovered in the 4S form on density gradient centrifugation do not bind steroid, whereas non-salt-treated receptors recovered in the 8S peak do bind steroid (data not shown).

Registry No. H_2O_2, 7722-84-1; MO_4^{2-}, 14259-85-9; diamide, 10465-78-8; triamcinolone acetonide, 76-25-5.

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Effects of 3′ End Deletions from the *Vibrio harveyi luxB* Gene on Luciferase Subunit Folding and Enzyme Assembly: Generation of Temperature-Sensitive Polypeptide Folding Mutants†

Jun Sugihara‡ and Thomas O. Baldwin*
Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843
Received September 2, 1987; Revised Manuscript Received December 18, 1987

ABSTRACT: Ten recombinant plasmids have been constructed by deletion of specific regions from the plasmid pTB7 that carries the *luxA* and *luxB* genes, encoding the α and β subunits of luciferase from *Vibrio harveyi*, such that luciferases with normal α subunits and variant β subunits were produced in *Escherichia coli* cells carrying the recombinant plasmids. The original plasmid, which conferred bioluminescence (upon addition of exogenous aldehyde substrate) on *E. coli* carrying it, was constructed by insertion of a 4.0-kb *Hin*dIII fragment of *V. harveyi* DNA into the *Hin*dIII site of plasmid pBR322 [Baldwin, T. O., Berends, T., Bunch, T. A., Holzman, T. F., Rausch, S. K., Shamansky, L., Treat, M. L., & Ziegler, M. M. (1984) *Biochemistry 23*, 3663–3667]. Deletion mutants in the 3′ region of *luxB* were divided into three groups: (A) those with deletions in the 3′ untranslated region that left the coding sequences intact, (B) those that left the 3′ untranslated sequences intact but deleted short stretches of the 3′ coding region of the β subunit, and (C) those for which the 3′ deletions extended from the untranslated region into the coding sequences. Analysis of the expression of luciferase from these variant plasmids has demonstrated two points concerning the synthesis of luciferase subunits and the assembly of those subunits into active luciferase in *E. coli*. First, deletion of DNA sequences 3′ to the translational open reading frame of the β subunit that contain a potential stem and loop structure resulted in dramatic reduction in the level of accumulation of active luciferase in cells carrying the variant plasmids, even though the *luxAB* coding regions remained intact. Second, the C-terminal ca. 10–15 residues of the β subunit appeared to have little to do with the structure or stability of the active heterodimeric form of the luciferase, but deletion of amino acid residues from this region resulted in greatly reduced levels of accumulation of active heterodimeric luciferase, especially at higher temperatures. The active luciferase that did form from the truncated β subunit (β^t) constructions had essentially normal activity and stability but impaired ability to refold from urea. As with the apparent temperature sensitivity of accumulation of active luciferase in vivo, these variants showed temperature sensitivity in refolding from urea in vitro. We conclude that the carboxyl-terminal region of the β subunit has little to do with the bioluminescence reaction or stability of the dimeric structure per se, but it does appear to play a critical function in proper folding and/or assembly into the active dimeric structure.

Many proteins exhibit structural domains that appear to fold independently within the constraints imposed by the covalent continuity of the peptide chain. That is, individual domains appear in many cases to contain the necessary information to fold properly, independent of the remainder of the protein. Extension of these ideas to a multisubunit protein would suggest that the individual subunits should fold independently, thereby generating interacting surfaces that lead to subunit interfaces in much the same way that structural domains of a single polypeptide interact (Wetlaufer, 1981). One of the more exciting developments over the past decade in the protein folding field comes from the work of King and his colleagues (Smith et al., 1980; Goldenberg & King, 1982; Yu & King, 1984) with the temperature-sensitive folding mutants of the phage P22 tail spike protein, a homotrimer that forms first as an unstable "protrimer" that can be trapped in the cold prior to rearrangements which lead to the highly stabilized trimeric tail spike. The temperature-sensitive folding mutants fail to form the normal yield of the stable trimeric structure at the nonpermissive temperature but do form the mature tail spike structures having normal stability if the cells are grown at the permissive temperature. These temperature-sensitive folding mutants are distinctly different from the classic temperature-sensitive mutant for which the protein formed at the permissive temperature is thermally labile. The temperature-sensitive folding mutants that have been inves-

† Supported by grants from the National Science Foundation (NSF DMB 85-10784) and the Robert A. Welch Foundation (A865). The generous support of the State of Texas through the Texas Advanced Technology Research Program is gratefully acknowledged. The fermentation facilities used in these studies were obtained from NIH Biomedical Research Support Instrumentation Grant SO1 RR01712 and DOD Instrumentation Grant P-20862-LS-RI.
* Address correspondence to this author.
‡ Recipient of postdoctoral research fellowships from the Upjohn Co. and from the Robert A. Welch Foundation. Present address: Kurosaki-Koseinenkin Hospital, Kitakyushu City, Fukuoka, Japan.

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