Use of the Thiol-specific Derivatizing Agent N-Iodoacetyl-3-[125I]iodotyrosine to Demonstrate Conformational Differences between the Unbound and hsp90-bound Glucocorticoid Receptor Hormone Binding Domain*

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The hormone binding domain (HBD) of the glucocorticoid receptor (GR) contains five cysteine residues, with three of them being spaced close to one another in the steroid binding pocket. The HBD also contains the contact region for the chaperone protein hsp90, which must be bound to the GR for it to have a steroid binding conformation. Binding of hsp90 to the receptor through its HBD inactivates the DNA binding domain (DBD). The DBD contains a number of cysteines essential to its DNA binding activity. Here, we assess the effects of hsp90 binding on the accessibility of cysteine residues in both the HBD and DBD to derivatization by a thiol-specific reagent. We report that N-iodoacetyltirosine (IAT) inactivates steroid binding activity of the immunopurified, untransformed GR in a manner that is prevented by the sulfhydryl reagents cysteine and dithiothreitol but is not reversed by them. The 125I-labeled IAT derivative N-iodoacetyl-3-[125I]iodotyrosine ([125I]IAT) covalently labels the immunopurified, hsp90-bound receptor in a thiol-specific manner. Dissociation of hsp90 leads to a ~2-fold increase in [125I]IAT labeling of the full-length, 100-kDa GR. The increase in thiol labeling is related to the presence of hsp90 because it is blocked by molybdate, which prevents hsp90 dissociation. Cleavage of the [125I]IAT-labeled receptor with trypsin yields a 15-kDa labeled fragment containing the DBD and a 30-kDa labeled fragment containing all of the cysteines in the HBD and the contact region for hsp90. Dissociation of hsp90 from the GR results in a 2.3-fold increase in [125I]IAT labeling of the 15-kDa fragment and a 50% decrease in labeling of the 30-kDa fragment. These data are consistent with the proposal that dissociation of hsp90 from the GR produces a conformational change in the HBD such that some of the thiols that are exposed in the GR-hsp90 complex become buried and are no longer accessible to the [125I]IAT probe. In contrast, binding of the GR to hsp90 restricts access of cysteines in the DBD to this small thiol-derivatizing agent, a restriction that is relieved as a result of unmasking or conformational change accompanying hsp90 dissociation.

The steroid binding site of the glucocorticoid receptor (GR) is located in its COOH-terminal one-third in a region called the hormone binding domain (HBD). The HBD must be properly folded for there to be a high-affinity steroid binding cavity, and the GR must be associated with the 90-kDa heat shock protein (hsp90) component of the protein folding system for there to be an appropriate steroid binding site (see Pratt (1993) for review). It is known that the HBD is both necessary and sufficient for binding of the GR to hsp90 (Pratt et al., 1988; Denis et al., 1988a; Cadepond et al., 1991; Scherrer et al., 1993), and dissociation of hsp90 from the unliganded HBD yields a conformation with either no steroid binding activity or with very low affinity binding activity (Brennock et al., 1989, Nemoto et al., 1990). The HBD can be returned to the high-affinity steroid binding conformation by incubating the GR with rabbit reticulocyte lysate, which contains a protein folding system that restores the receptor to its heterocomplex state with hsp90 (Scherrer et al., 1990; Hutchison et al., 1994a, 1994b). Although the loss of steroid binding activity suggests that a major conformational change occurs within the HBD upon hsp90 dissociation, an analysis of peptides produced by limited proteolysis of the untransformed (hsp90-bound) and the transformed (hsp90-free) GR did not reveal any differences suggestive of conformational change dependent upon hsp90 (Reichman et al., 1984). To date, no studies have been published using chemical reagents to probe the conformation of the hsp90-bound and hsp90-free GR.

Steroid binding activity of the GR is inactivated by a variety of sulfhydryl-reactive agents and by redox conditions that promote disulfide bond formation (see Simons and Pratt (1995) for review of GR thiols and steroid binding activity). A series of studies from the Simons laboratory has demonstrated that steroid binding activity is inactivated by the formation of disulfide bonds between cysteine SH groups that are vicinally spaced in the HBD when it is bound to hsp90 (Miller and Simons, 1988; Simons et al., 1990, Chakraborti et al., 1990, 1992). Simons et al. (1989) have shown that the GR can be cleaved with trypsin to a 16-kDa fragment of the HBD that binds glucocorticoids with ~23-fold lower affinity than the intact 98-kDa receptor. This fragment of the HBD (amino acids 537–673 of the rat GR) is bound to hsp90 (Chakraborti and Simons, 1991), and it contains three cysteines (640, 656, and 663) solely to indicate this fact.

The abbreviations used are: GR, glucocorticoid receptor; HBD, hormone binding domain; DBD, DNA binding domain; hsp90, the 90-kDa heat shock protein; IAT, N-iodoacetyltirosine; IAIT, N-iodoacetyl-3-iodotyrosine; DTT, dithiothreitol; MMTS, methyl methanethiosulfonate; PAGE, polyacrylamide gel electrophoresis; TE5, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.
661 in the rat GR, or 628, 644, and 649 in the mouse GR), of which any two can form an intramolecular disulfide (Chakraborti et al., 1992), suggesting that there is a cysteine cluster in this region of the steroid binding site. A variety of observations indicate that a short region of the HBD containing this cysteine cluster directly contacts hsp90 in the untransformed GR heterocomplex (Dalman et al., 1991; Chakraborti and Simons, 1991; Cadepend et al., 1991).

One of the cysteines in the vicinal thiol cluster (Cys-656) is the site that is covalently labeled by the site-specific affinity label dexamethasone 21-mesyolate (Simons et al., 1987), and the steroid binding activity of the GR is abrogated by arsenite at low concentrations where it acts as a vicinal thiol-specific reagent (Chakraborti et al., 1990; Lopez et al., 1990; Simons et al., 1990), reacting specifically with Cys-656 and Cys-661 in the thiol cluster (Chakraborti et al., 1992). We have previously compared the effects of the reversible thiol-reactive agents arsenite and methyl methanethiosulfonate on both the steroid binding activity of the unliganded GR and dissociation of steroids from preformed steroid-receptor complexes (Stancato et al., 1993). Our observations were consistent with the concept that the thiol cluster lies in a portion of the binding pocket that is critical for binding of the D-ring of the steroid, and we proposed that labeling of the GR with a derivatizing agent that reacts preferentially with vicinally spaced dithiols might allow detection of conformational changes likely to occur in a critical region of the HBD on dissociation of hsp90.

Many reagents have been used to label protein thiols, including radioactive iodoacetic acid, iodoacetamide, and N-ethylmaleimide. However, N-iodoacetyl-3-[125I]iodotyrosine ([125I] IAIT) has a much higher reactivity with protein thiols and has the advantage of the [125I] label (Gitter et al., 1994). [125I] IAIT is a thiol-specific reagent used to label protein thiols, and it has the unusual property that its reaction with protein thiols is not affected by up to 10 mM dithiothreitol (DTT) or 2-mercaptoethanol (Gitter et al., 1994). Its reaction with vicinal thiols is blocked by arsenicals, and labeling with [125I] IAIT in the presence and absence of a compound such as arsenite or phenylarsine oxide is a sensitive method of distinguishing between reaction with monothiols and vicinally spaced dithiols. In this work, we have examined the accessibility of thiols to [125I] IAIT derivatization in both the DNA binding domain (DBD) and the HBD of both the hsp90-bound and hsp90-free GR.

EXPERIMENTAL PROCEDURES

Materials

[6,7,14N]Triaminoisonolo acetoni (42.8 Ci/mmol) and [125I] conjugated goat anti-mouse and anti-rabbit IgGs were obtained from DuPont NEN. Two crystalized bovine pancreatic trypsin (Type III), nonimmune mouse IgG, protein A-Sepharose, and goat anti-mouse IgG-horseradish peroxidase conjugate were from Sigma. Donkey anti-rabbit IgG-horse radish peroxidase conjugate was from Pierce. Immobilon-P was from Millipore. The BuGR IgG monoclonal antibody against the GR was from Affinity Bioreagents (Neshanic Station, NJ), and the AC88 monoclonal IgG against hsp90 was from StressGen (Victoria, BC). The aP1 rabbit antisemur raised against amino acids 440–795 of the rat GR has been described previously (Hoeck et al., 1989).

Methods

Cell Culture and Fractionation—L929 mouse fibroblasts (L cells) were grown in monolayer culture in Dulbecco's modified Eagle's medium supplemented with 10% iron-supplemented calf serum. The WCL2 line of Chinese hamster ovary cells overexpressing the mouse GR was established by Hirst et al. (1990). WCL2 cells were grown in monolayer culture in Dulbecco's modified Eagle's medium plus 3 mM methotrexate, 40 μg/ml proline, and 10% iron-supplemented calf serum. All cells were harvested by scraping into Earle's balanced saline followed by a second wash and centrifugation at 500 × g. The washed cells were suspended in 1.5 volumes of HE buffer (10 mM HEPES, 1 mM EDTA, pH 7.4) and ruptured by Dounce homogenization. Homogenates were centrifuged for 1 h at 100,000 × g, with the supernatant from this step being the "cytosol" from which the GR was immunoadsorbed.

Receptor Immunoadsorption—Prior to immunoadsorption, the BuGR antibody was prebound to protein A-Sepharose by incubating 40 μl of a 20% slurry of protein A-Sepharose for 1 h at 4 °C with 1 μg (Fig. 1) or 3 μg (all other figures) of BuGR and 150 μl of TEG buffer (10 mM TES, 0.5 M NaCl, 4 mM EDTA, pH 7.6), followed by centrifugation and washing with TEG. GR was immunoadsorbed from 100-μl aliquots of cell cytosol for (for experiments of Fig. 1) or 200 μl of WCL2 cell cytosol (all other experiments) by rotation for 2 h at 4 °C with 8 μl of protein A-Sepharose-bound BuGR.

Treatment with Thiol-reactive Reagents and Steroid Binding Assays—In the immune pellets were incubated in 100 μl of TEG buffer (TEG plus 20 mM sodium molybdate) and various concentrations of IAT for 1 h at 1 °C. Samples were then washed twice in TEG buffer and incubated 2.5 h at 0 °C in 100 μl of TEG buffer and 100 μl [3H] triamido acetoni and, after washing 3 times with 1 μl of TEG buffer, the immune pellets were assayed for radioactivity. To normalize data between separate experiments, the counts/min of [3H] triamido acetoni bound in each treated sample was expressed as a percent of the untreated control, and values from three experiments were averaged.

Synthesis of IAT and Radioliodination—N-Iodoacetyltyrosine (IAIT) was synthesized exactly as described by Gitter et al. (1994) and radioiodinated to [125I] IAIT according to the procedure of Bolton and Hunter (1977) with the acid modified in that acid was added to allow extraction of IAIT into the benzene-methyl formamide. Because of a chemical decay, the compound must be used within 2 to 3 weeks after radiiodination. The parent IAT is stable for years. To label the receptor, immunoadsorbed GR was washed once in 1 ml of TEG buffer with 1 mM DTT and then incubated with 3 μl (−μl μl) of [125I] IAIT in 100 μl of TEG buffer containing 1 mM DTT and 3% dimethyl sulfoxide for 1 h at 4 °C with constant rotation. The final concentration of [125I] IAIT was ~1 μM. The immune pellets were then washed 3 times with 1 ml of TEG buffer plus 0.5 M NaCl and 1% Triton X-100. For samples to be cleaved with trypsin, the immune pellets were washed once with 1 ml of TEG buffer.

The concentration of the immunoadsorbed GR in the labeling mixture is ~20 nM, or ~400 nM with respect to GR sulfhydryls. The concentration of total sulfhydryls in the immune pellet, which includes the sulfhydryls in 3 μg of BuGR antibody, is in considerable excess of the ~1 μM [125I] IAIT used for labeling. It should also be noted that washing the immune pellets with 0.5 M NaCl and 1% Triton X-100 eliminates hsp90 and other receptor-associated proteins.

Trypsin Cleavage of the Immunoadsorbed GR—Protein A-Sepharose pellets containing immunoadsorbed [125I] IAIT labeled or unlabeled GR were incubated in 100 μl of TEG buffer containing 1 μg/ml tosylphenylalanyl chloromethyl ketone-treated trypsin. For the untransformed, hsp90-bound GR the incubation in the trypsin was for 1 h at 0 °C and for the transformed, hsp90-free GR the incubation was for 1 h at 20 °C. The supernatants containing the GR fragments released from the immune pellets were treated with SDS sample buffer, and proteins were resolved by SDS-PAGE. The trypsin-treated immune pellets were washed 3 times with 1 ml of TEG buffer containing 0.5 M NaCl and 1% Triton X-100 prior to resolving the BuGR antibody-bound fragments by SDS-PAGE.

Cell Electrophoresis and Western Blotting—Immune pellets and supernatants from trypsin-digested immune pellets were boiled in SDS sample buffer with 10% β-mercaptoethanol, and proteins were resolved in a 12% SDS-polyacrylamide gel as described in Hutchison et al. (1992a). To optimize the transfer to an Immobilon membrane of 15-kDa, 16-kDa, and 30-kDa trypsic fragments of the GR in the experiments of Figs. 4 and 6, proteins were transferred for 16 h at 0.1 V. For the transfer to an Immobilon-P membrane, GR and receptor fragments were probed both with 2 μg/ml BuGR monoclonal IgG and with 0.05% aP1 rabbit anti-GR serum. hsp90 was detected by probing with 1 μg/ml AC88. The immunoblots were then incubated a second time with the appropriate [125I] IAIT labeled or horseradish peroxidase-conjugated acceptor antibody to visualize the immunoreactive bands.

For quantitative Western blotting experiments of Figs. 4 and 6, the intact GR or GR fragments on the immunoblots were identified by developing them first with horseradish peroxidase-conjugated acceptor antibody. Reflect UV photographs were taken of the peroxidase-stained gel to produce the panels labeled immunoblot in the figures. The GR bands identified by color were excised and counted for [125I] IAIT radioactivity, as was an equivalent region from the nonimmune sample. The cut pieces of immunoblot were then counterblotted with [125I] IAIT labeled or horseradish peroxidase-conjugated acceptor antibody against the appropriate [125I] IAIT labeled or horseradish peroxidase-conjugated acceptor antibody to visualize the immunoreactive bands.
munopurified receptor is blocked if 30 mM cysteine or DTT is bound receptor complex (affected by concentrations 100-fold lower than the steroid-teine or DTT (Fig. 1). contrast to the reversible inhibitors MMTS or arsenite, inacti- steroid from the prebound receptor. Fig. 1. shows the inhibition of the steroid binding activity of the unliganded GR at concentrations much lower than those required for release of steroid-freereceptorsweresuspendedin100 μl of TEGM bufferfor1h (Fig. 1A shows the IAT inhibition of steroid binding activity. Aliquots (100 μl) of L cell cytosol containing steroid-free or steroid-bound receptors were immunoadsorbed with BuGR to protein A-Sepharose, the immune pellets were incubated for 1 h at 0 °C with the indicated concentrations of nonradioactive IAT, and binding of [3H]triamcinolone acetonide was assayed as described under “Methods.” ○, immunoadsorbed, steroid-free GR; ●, immunoadsorbed, steroid-bound GR. B, effect of cysteine or DTT on IAT inhibition. Immunoadsorbed, steroid-free receptors were suspended in 100 μl of TEGM buffer for 1 h at 0 °C with 2 mM IAT (black bar), with IAT plus 30 mM l-cysteine or DTT (stippled bars), or with IAT followed after 1 h by 30 mM cysteine or DTT after 1 h (hatched bars). Samples were then washed and assayed for binding of [3H]triamcinolone acetonide. Binding is expressed as percent of untreated control, mean ± S.E. for three experiments.

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RESULTS AND DISCUSSION

Effect of IAT on Steroid Binding Activity of the GR—In a previous report (Stancato et al., 1993), we studied the effects of two thiol-reactive reagents, MMTS and arsenite, on the steroid binding activity of the GR in L cell cytosol. These reagents inactivate steroid binding activity of the unliganded GR at concentrations much lower than those required for release of steroid from the prebound receptor. Fig. 1A shows the IAT inhibition of the steroid binding activity of the immunoadsorbed unliganded GR-hsp90 complex (open circles), which is affected by concentrations 100-fold lower than the steroid-bound receptor complex (closed circles). Inactivation of the immunopurified receptor is blocked if 30 mM cysteine or DTT is added prior to the IAT (Fig. 1B, stippled bars); however, in contrast to the irreversible inhibitors MMTS or arsenite, inactivation by IAT is not reversed by subsequent addition of cysteine or DTT (Fig. 1B, hatched bars).

These data are consistent with IAT inactivation of glucocorticoid binding activity through covalent reaction with the receptor. Thus, we prepared the radiiodinated derivative of IAT and incubated it with the immunoadsorbed GR-hsp90 complex. The GR was resolved by gel electrophoresis, and autoradiogra-

Fig. 2. Tryptic fragments of the [125I]IAT-labeled GR. Aliquots of WCL2 cell cytosol were immunoadsorbed with nonimmune IgG or with BuGR. Immunopellets were labeled with [125I]IAT and washed. One set of immunopellets was incubated at 0 °C for 1 h with 1 μg/ml trypsin as described under “Methods.” Both the polypeptides remaining with the immunopellet and those released into the cleavage buffer were resolved by SDS-PAGE and autoradiography. Lane 1, BuGR immunopellet without trypsin; lane 2, nonimmune pellet without trypsin; lanes 3 and 4, nonimmune and BuGR immunopellets, respectively, digested with trypsin; lanes 5 and 6, supernatants from samples 3 and 4, respectively. The 100-kDa intact GR and the tryptic fragments are diagrammed above the autoradiogram. Cysteines are assigned according to the primary sequence of the mouse GR by Danielsen et al. (1986) and tryptic fragments are according to Simons et al. (1989). The 30-kDa and 15-kDa fragments are derived from cleavage of the 44-kDa fragment shown in the autoradiogram. The hormone binding domain is stippled, the DNA binding domain is hatched, and the black band designates the BuGR epitope. The conserved cysteines involved in the tetrahedral coordination of zinc in the DBD are indicated. The thiol cluster in the HBD is comprised of Cys-628, -644, and -649, hc = antibody heavy chain.

Fig. 3 demonstrates the immunoreactivity of each of the fragments generated by trypsin digestion to confirm their identity. When the immunoblot is probed with BuGR (condition 2), 44-, 42-, and 15-kDa fragments are seen in the immunopellet.
When the immunoblot is probed with aP1 (condition 3), the 44- and 42-kDa species are seen in the immunopellet as well as 30-kDa and 16-kDa fragments that have been released into the supernatant. There is a 30-kDa fragment in the immunopellet migrating a little slower than the light chain of the BuGR antibody that reacts with both the BuGR and aP1 antibodies. This fragment is recovered only occasionally, and it likely represents an uncleaved combination of the 15-kDa and 16-kDa segments. The BuGR monoclonal antibody does not recognize the 30-kDa and 16-kDa fragments released into the supernatant, but the aP1 antiserum does react very faintly with a 15–16-kDa species in the immunopellet (condition 3), which likely represents a trace of 16-kDa fragment remaining in the immunopellet.

125\text{I}]\text{IAIT} Labeling of the Intact hsp90-free GR—In the experiments of Fig. 4, we examined labeling by \(^{125}\text{I}\) IAIT of three states of the GR. In condition 1, the immunoadsorbed GR-hsp90 complex was labeled. In condition 2, the GR was stripped of hsp90 (cf. lanes 2 and 4) by incubating the immunopellet with 0.5 M NaCl. In condition 3, the immunoadsorbed GR was incubated with salt but in the presence of molybdate to inhibit hsp90 dissociation. The relative amount of \(^{125}\text{I}\) \text{IAIT} labeling of the GR under each condition is expressed in the bar graph. It can be seen that the intact 100-kDa GR that is dissociated from hsp90 (condition 2) is labeled by \(^{125}\text{I}\) \text{IAIT} 1.9-fold more with respect to the GR that is associated with hsp90 (condition 1). The increase is related to dissociation of hsp90, because it is blocked by molybdate, which prevents hsp90 dissociation (condition 3). The same increase in labeling is seen if hsp90 is dissociated by heating the immunopellet in buffer at 25°C and after hsp90 dissociation from the dexamethasone-bound receptor (data not shown).

Tryptic Fragments Derived from hsp90-bound and hsp90-free \(^{125}\text{I}\) \text{IAIT}-labeled GR—The hsp90-free GR is less sensitive to trypsin digestion than the hsp90-bound GR. As shown in Fig. 5 (condition 2), when the salt-stripped, hsp90-free GR is incubated for 1 h at 0°C with 1 μg/ml trypsin, less 15-kDa fragment and 30-kDa fragment is obtained than after an identical digestion of the hsp90-bound GR (cf. with condition 1) and no 16-kDa fragment is recovered. The best way we have found to get substantial amounts of the 15-kDa and 30-kDa fragments is to incubate the immunoadsorbed salt-stripped GR with the same concentration of trypsin for 1 h at 20°C (condition 3). As reported in 1984 by Reichman et al. for the transformed and untransformed GR, we find that the same tryptic fragments are derived with the hsp90-free as the hsp90-bound GR. However, the hsp90-free GR requires more intense cleavage conditions to generate the fragments. This is true regardless of whether the immunoadsorbed GR was stripped of hsp90 by salt (as in Fig. 5) or by incubation in buffer at 25°C (data not shown).
In the experiments summarized in Fig. 6, [125I]IAIT-labeled GR-hsp90 complex (condition 1) or hsp90-free GR (condition 2) was cleaved with trypsin and the radioactivity in the 15-kDa and 30-kDa fragments was assayed. Fig. 6A shows an immunoblot and an autoradiogram of [125I]IAIT radioactivity from the same immunoblot. Fig. 6B presents a bar graph of the relative amount of [125I]IAIT labeling of the fragments under each condition after averaging data from several experiments. We find that the 15-kDa fragment containing the DNA binding domain from the hsp90-free GR is labeled 2.3-fold more by [125I]IAIT than the 15-kDa fragment from the hsp90-bound GR. In contrast, the 30-kDa fragment containing the HBD of the hsp90-free GR is labeled only half as much as the 30-kDa fragment from the hsp90-bound GR. It should be emphasized that 1 mM DTT was present during labeling with [125I]IAIT; thus, differential oxidation is not responsible for differences in incorporation of label. These data are consistent with the proposal that dissociation of the hsp90 from the GR produces a conformational change that allows increased access of the IAIT probe to thiols of the DBD and decreased access to thiols in the HBD.

We had hoped to examine specifically the [125I]IAIT labeling of the three cysteines in the thiol cluster of the 16-kDa fragment. This proved not to be possible for two reasons. First, the recovery of sufficient 16-kDa fragment from the hsp90-bound GR to visualize [125I]IAIT labeling as in Fig. 2 is erratic (cf. Figs. 2 and 6A). Second, it has been known for some time that dissociation of hsp90 from the GR is accompanied by acquisition of DNA binding activity (Sanchez et al., 1987; Denis et al., 1988b), with the rates of the two processes being identical (Meshinchi et al., 1990). It is not known whether hsp90 abrogates DNA binding activity solely by physically masking the DBD or whether the DBD of the hsp90-bound GR is in a different conformation from the hsp90-free GR. The [125I]IAIT probe used here is small, and it can access and derivatize cysteine thiols in the DBD even when the GR is bound to hsp90. Yet, dissociation of hsp90 allows a 2.3-fold increase in labeling of the 15-kDa, DBD-containing GR fragment (Fig. 6). In the event hsp90 inhibits the DNA binding activity of the GR solely by physically blocking access of DNA to the DBD, we infer from the fact that hsp90 also impedes the access of the very small IAIT reagent to DBD thiols that there must be rather close association of hsp90 with the DBD.

Models of Folding Changes Occurring upon hsp90 Dissociation—It has been known for some time that dissociation of hsp90 from the GR is accompanied by acquisition of DNA binding activity (Sanchez et al., 1987; Denis et al., 1988b), with the rates of the two processes being identical (Meshinchi et al., 1990). It is not known whether hsp90 abrogates DNA binding activity solely by physically masking the DBD or whether the DBD of the hsp90-bound GR is in a different conformation from the hsp90-free GR. The [125I]IAIT probe used here is small, and it can access and derivatize cysteine thiols in the DBD even when the GR is bound to hsp90. Yet, dissociation of hsp90 allows a 2.3-fold increase in labeling of the 15-kDa, DBD-containing GR fragment (Fig. 6). In the event hsp90 inhibits the DNA binding activity of the GR solely by physically blocking access of DNA to the DBD, we infer from the fact that hsp90 also impedes the access of the very small IAIT reagent to DBD thiols that there must be rather close association of hsp90 with the DBD.

It has been proposed that binding of hsp90 to the HBD causes the polypeptide as a whole to assume a partially “unfolded” conformation that is reversed on hsp90 dissociation (Picard et al., 1988). It seems unlikely to us that hsp90 causes the DBD to have a different conformation via a folding mechanism. Spanjaard and Chin (1993) have demonstrated reconstitution of hormone-mediated activity by expressing as individual proteins an amino-terminal fragment of the GR containing the trans-activation and DNA binding domains and a fragment containing the COOH-terminal HBD, with each fragment being fused to either a c-jun or c-Fos leucine zipper. As each fragment was translated and folded independently, this observation argues strongly against a model in which hsp90 binding to the HBD causes the DBD to assume an unfolded conformation.

At this time, we do not know which of the cysteines in the 15-kDa fragment are derivatized after hsp90 dissociates. As shown in Fig. 2, the 15-kDa fragment contains 8 cysteines in a vicinal thiol arrangement coordinating two atoms of zinc, and there are 2 or 3 additional cysteines, depending on the amino-terminal trypsin cleavage site. Given that half of the cysteines of the GR are in the 15-kDa fragment, the 2.3-fold increase in labeling of this fragment occurring with hsp90 dissociation (Fig. 6) may account for the overall 1.9-fold increase in labeling that is observed in the intact GR (Fig. 4) despite the 50% reduction in labeling of the 30-kDa fragment of the HBD which
contains 5 cysteines (Fig. 6). However, as with arsenite, the presence of zinc should prevent cysteine labeling. In this event, increased labeling of the 15-kDa fragment may reflect increased availability of only Cys-438 and -448 to [125I]IAIT. Because [125I]IAIT labeling of the hsp90-bound receptor is reduced about 70% by 1 mM arsenite (data not shown), it is likely that cysteine SH groups are the predominant labeled moiety in the intact 100-kDa receptor. However, it is at least theoretically possible that [125I]IAIT could react with non-thiol groups in the DBD of the hsp90-bound GR.

We know that hsp90 is a component of a multiprotein chaperone system that folds the HBD of the GR into a high affinity steroid binding conformation (for review, see Pratt (1993)). It is thought that the steroid receptors have evolved a tight interaction with hsp90 and that the heterocomplex probably represents a normal transition state in a general folding process. The hsp90 can be conceived as trapping the HBD and disruption of its complex with hsp90 (Hutchison et al., 1992b). In essence, process from the folding intermediate (i.e. the GR-hsp90 complex) has been brought under hormonal control.

In this model, the hsp90 binding region of the HBD assumes a folded conformation upon dissociation of the hsp. Because [125I]IAIT is small and specifically derivatizes thiol moieties, we can show that dissociation of hsp90 is accompanied by decreased accessibility of thiols in the HBD to derivatization by the reagent (Fig. 6). This is the first demonstration that specific sites that were previously accessible in the HBD become inaccessible upon receptor transformation. We have worked here with the steroid-free receptor because we wanted to eliminate the possibility that the presence of steroid in the hormone binding site could sterically block access of the reagent to thiols lying in the binding pocket. The fact that the change in the HBD is from accessibility to reagent in the hsp90-bound state to less accessibility when hsp90 is not bound is consistent with an internalization of thiol moieties occurring as the unliganded receptor HBD assumes a more folded conformation. In this folded conformation, there is no steroid binding activity until the receptor is reassociated with the hsp by the hsp90/hsp70-based chaperone system.

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