Transformation of the aryl hydrocarbon receptor to a DNA-binding form is accompanied by release of the 90 kDa heat-shock protein and increased affinity for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

Ellen C. HENRY* and Thomas A. GASIEWICZ

Department of Environmental Medicine, Environmental Health Sciences Center, University of Rochester Medical Center, Rochester, NY 14642, U.S.A.

The binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) to the aryl hydrocarbon receptor (AhR) elicits a sequence of poorly defined molecular events that ultimately yield a heteromeric transformed AhR that is active as a transcription factor. We have previously developed a model of the ligand-initiated transformation of the AhR to the DNA-binding state based on characterization of several forms of the AhR with respect to their physicochemical properties and DNA-binding affinities. The present studies were designed to determine whether, and at what stage, this process of transformation alters the receptor's affinity for TCDD. In rat hepatic cytosol, approx. 10% of the TCDD specifically bound to the AhR rapidly dissociated ($t_{1/2} \sim 1$ h), while the remainder was only slowly dissociable ($t_{1/2} \sim 70$ h). The isolated DNA-binding forms of the receptor (monomeric and transformed) bound TCDD very tightly ($t_{1/2} > 100$ h), whereas

TCDD was dissociable from the non-DNA-binding receptor form(s). A lower incubation temperature (0–4 °C) and the presence of molybdate partially stabilized the non-DNA-binding fraction of the TCDD-receptor complex and also enhanced TCDD dissociation in crude cytosol. Immunoprecipitation of the different AhR forms with an anti-AhR antibody and immunoblotting with antibody to the 90 kDa heat-shock protein (hsp90) demonstrated that hsp90 was associated with the unoccupied receptor complex as well as with a fraction of the non-DNA-binding TCDD-receptor complex; isolated DNA-binding forms did not contain detectable hsp90. We conclude that while hsp90 remains associated with the AhR, TCDD is readily dissociable; following release of hsp90, however, TCDD becomes very tightly bound, and remains so upon completion of transformation.

INTRODUCTION

The aryl hydrocarbon receptor (AhR), an intracellular gene regulatory protein found in many tissues and species, mediates the biochemical and toxic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds (Poland and Knutson, 1982; Landers and Bunce, 1991). Our laboratory has previously characterized several forms of this protein in terms of their molecular size, sedimentation coefficient, surface charge and DNA-binding properties (Gasiewicz and Bauman, 1987; Henry et al., 1989; Gasiewicz et al., 1991). The unoccupied form is a multimer which probably comprises the ligand-binding subunit and several other proteins; some evidence exists that, like the steroid hormone receptors, this multimer includes the 90 kDa heat-shock protein (hsp90) (Denis et al., 1988a,b; Wilhelmsson et al., 1990; Landers and Bunce, 1991). TCDD binding appears to initiate a series of as yet poorly defined molecular changes, including release of these other proteins, resulting in the subsequent association of the ligand-bound monomer with at least one other protein to form a heteromeric transformed receptor complex (Elferink et al., 1990; Gasiewicz et al., 1991; Reyes et al., 1992). Since this latter form has the highest affinity for DNA, including specific dioxin-responsive elements (DREs) (Gasiewicz et al., 1991), it is likely to represent the transcriptionally active conformation.

Although TCDD binding is known to elicit conformational or biochemical changes in the receptor which result in modification of its ability to associate with other proteins and recognize specific DNA sequences, it is not clear whether the affinity of the

AhR for TCDD is altered during this transformation process. Although an initial report by Poland et al. (1976) suggested rapid dissociation of TCDD from the AhR in C57BL/6 mouse cytosol, later studies have indicated that this binding may be much tighter, and may include a component from which the ligand is poorly dissociable (Kester, 1987; Farrell and Safe, 1987; Bradfield et al., 1988). Other observations in crude cytosol indicated that changes occurring subsequent to TCDD binding confer on the receptor protein greater stability towards thermal degradation, disruption by thiol reagents and ligand displacement by unlabelled ligand (Kester and Gasiewicz, 1987; Henry et al., 1988; Bunce et al., 1988; Gasiewicz and Rucci, 1991), all of which might accompany changes in ligand affinity. Furthermore, in some species, specific binding of TCDD to the monomeric form is unstable under some assay conditions (Rucci and Gasiewicz, 1988; E. C. Henry, unpublished work). The present studies were undertaken to compare the receptor forms in hepatic cytosol with respect to their affinity for TCDD in order to determine whether, and at what stage of the transformation process, changes in ligand affinity occur.

EXPERIMENTAL

Chemicals

[1,6-³H]TCDD (34 Ci/mmol) was obtained from ChemSyn (Lenexa, KS, U.S.A.), and unlabelled TCDD was purchased from Cambridge Isotopes (Cambridge, MA, U.S.A.). 2,3,7,8-Tetrachlorodibenzofuran (TCDF) was obtained from the National Institute of Environmental Health Sciences (Research

Abbreviations used: AhR, aryl hydrocarbon receptor; DRE, dioxin-responsive element; hsp90, 90 kDa heat-shock protein; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDF, 2,3,7,8-tetrachlorodibenzofuran.

* To whom correspondence should be addressed.

Triangle Park, NC, U.S.A.). Buffer used for all experiments was HEDG [25 mM Hepes, 1.5 mM EDTA, 1 mM dithiothreitol, 10 % (v/v) glycerol, pH 7.6, at 20 °C]; where indicated, 20 mM sodium molybdate was included.

Tissue preparation

Cytosol was prepared from livers of male Sprague–Dawley rats (200–250 g) (Charles River, Wilmington, MA, U.S.A.) as described previously (Gasiewicz and Bauman, 1987), and stored at -80°C until used. Protein concentration was adjusted to $\sim 15\text{ mg/ml}$ prior to incubation with 3 nM [^3H]TCDD or [^3H]TCDD + 300 nM TCDF for 2 h at 20 or 4 °C.

Preparation of partially purified receptor forms by DNA–Sepharose chromatography

Partially purified (approx. 250-fold) forms of the receptor were isolated by DNA–Sepharose chromatography from [^3H]TCDD-pretreated cytosol (2 h, 20 °C) by pooling fractions containing peaks of specific binding corresponding to the flow-through [non-DNA-binding form(s)], Peak 1 (monomeric form, eluting at $\sim 0.15\text{ M NaCl}$) and Peak 2 (transformed form, eluting at $\sim 0.3\text{ M NaCl}$). Conditions and procedures for chromatography were essentially as described (Gasiewicz and Bauman, 1987). BSA (4 mg/ml) was added to Peaks 1 and 2 during collection to minimize loss due to adsorption to collection tubes. We have demonstrated previously that while the ligand-bound monomeric form of the receptor (Peak 1) has some affinity for calf thymus DNA covalently linked to Sepharose, it has no or little affinity for the DRE located upstream of the *P450IA1* gene. The ligand-bound heterodimeric transformed form of the receptor (Peak 2) has high affinity for calf thymus DNA linked to Sepharose, as well as high affinity and specificity for the DRE (Gasiewicz et al., 1991). Nuclear AhR was prepared from [^3H]TCDD-treated ($\sim 10\text{ }\mu\text{g/kg}$) rats as previously described (Rucci and Gasiewicz, 1988), except that nuclei were washed with HEDG + 0.1 M KCl.

Specific binding assays and dissociation studies

The hydroxyapatite assay (Gasiewicz and Neal, 1982) was used to determine specific binding of TCDD to the AhR. Free and loosely bound [^3H]TCDD was removed from crude cytosols with dextran-coated charcoal. Dissociation of [^3H]TCDD was initiated by adding 500–1000-fold excess TCDF; control samples received an equal volume of vehicle (*p*-dioxane; 5 $\mu\text{l/ml}$). Aliquots were removed periodically for determination of specific binding. Kinetic parameters were calculated using the curve-fitting function of Kaleidagraph (Synergy Software, Reading, PA, U.S.A.), which calculates parameter values which achieve the best fit determined by least-squares error. Experiments were performed at least three to four times; representative results are shown.

Assessment of specific DNA binding

An electrophoretic gel mobility assay, using an oligonucleotide containing the DRE, was used to assess sequence-specific binding of the AhR to DNA. The gel retardation assay was performed using a synthetic oligonucleotide containing a single consensus

sequence: 5'-TTGCGTG-3' sequence: 3'-AACGCAC-5'. The complete 27-base sequences of

the oligonucleotides are given in Gasiewicz et al. (1991). Cytosol ($\sim 90\text{ }\mu\text{g}$ of protein) was mixed with poly(dI,dC), adjusted to a final concentration of 0.08 M NaCl, and incubated with

$(2-3) \times 10^4\text{ c.p.m.}$ of ^{32}P -labelled oligonucleotide. Samples were then analysed by non-denaturing PAGE and autoradiography, as described in Denison et al. (1988). AhR·DRE complex was quantified for each sample by cutting out the TCDD-dependent band in each lane, digesting the gel piece in tissue solubilizer, and counting ^{32}P .

Immunoprecipitation and immunoblotting

Different receptor forms, adjusted to 0.12–0.15 M NaCl and 20 mM molybdate, were incubated for 2–3 h at 4 °C with antibody raised in rabbits against the N-terminal peptide of the AhR (Poland et al., 1991). These complexes were precipitated with Protein A–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) for 1 h at 4 °C, and pellets were washed with 40 mM Tris (pH 7.5), 0.4 M NaCl, 20 mM molybdate and 0.05 % Triton X-100. Samples were then boiled for 5 min in electrophoresis sample buffer for analysis by SDS/PAGE. Proteins were transferred to Immobilon-P (Millipore, Bedford, MA, U.S.A.) using the Semi-Phor apparatus (Hoefler, San Francisco, CA, U.S.A.), and the membrane was blocked with 5 % non-fat dry milk in 50 mM Tris (pH 7.5), 150 mM NaCl and 0.2 % Tween 20. Duplicate halves of the membrane were blotted with either anti-AhR (kindly provided by A. Poland, Madison, WI, U.S.A.) or anti-hsp90 (AC88, Stressgen, Victoria, BC, Canada). Bands were visualized by means of alkaline phosphatase-labelled secondary antibodies (Sigma Chemical Co., St. Louis, MO, U.S.A.; Jackson ImmunoResearch, Westgrove, PA, U.S.A.).

RESULTS

Dissociation of TCDD from the AhR in crude cytosol

Initially we examined the dissociation kinetics of [^3H]TCDD from [^3H]TCDD·AhR complexes preformed (2 h, 20 °C) in crude rat hepatic cytosol. Dissociation data were fitted ($r = 0.99$) to a two-compartment exponential equation, and the resultant $t_{1/2}$ values were approx. 0.8 h and 30 h (Figure 1). Receptor loss and/or degradation (monitored in samples receiving vehicle

Figure 1 Dissociation of TCDD from the AhR in rat hepatic cytosol after a 2 h preincubation at 20 °C with [^3H]TCDD in the presence or absence of TCDF

Free and loosely bound [^3H]TCDD was removed with dextran-treated charcoal, and the sample was divided into two aliquots; 2 μM TCDF was added to one (●) and *p*-dioxane vehicle was added to the other (○; degradation control). Incubation was continued at 20 °C. Specific binding was measured periodically using the hydroxyapatite assay. Points are from one representative experiment. Lines are computer-generated fits; $r = 0.996$ for TCDF and 0.82 for control.

Table 1 Kinetic parameters of TCDD dissociation from the AhR

Data for cytosol samples were fitted to a two-compartment exponential of the form: total specific binding = $Ae^{-k_1t} + Be^{-k_2t}$, where $k = 0.693/t_{1/2}$. A represents the proportion of specific binding which dissociates with a rate constant k_1 , and B is the proportion of specific binding dissociating with a rate constant k_2 . Tabulated values are corrected for concurrent degradation of the receptor (single exponential). Values represent the means $\pm$ S.D. of n experiments. Cytosols were incubated with [3 H]TCDD in the presence or absence of TCDF for 2 h at 20 °C prior to the initiation of dissociation, and all dissociation studies were carried out at 20 °C unless otherwise indicated. Because of the observed variability among preparations, experiments reported using 'cytosol' and 'cytosol + molybdate' were performed using matched cytosols (prepared simultaneously and from the same animals). Partially purified monomeric and transformed forms of the AhR were obtained by DNA–Sephacryl chromatography of rat cytosols (see the Experimental section). For studies at 4 °C, cytosols were incubated with [3 H]TCDD in the presence or absence of TCDF for 2 h at 4 °C prior to the initiation of dissociation, and incubation was continued at 0–4 °C. Since specific binding in cytosols exposed only to dioxane under these conditions continues to increase during the experimental period, these values are not corrected for degradation. The experiments at 4 °C were performed with aliquots of the same cytosols prepared concurrently with and without molybdate, but these were different batches than used for the experiments at 20 °C.

Receptor form (n)	A (%)	$t_{1/2}$ (h)	B (%)	$t_{1/2}$ (h)
Cytosol (3)	11 $\pm$ 3	0.8 $\pm$ 0.2	89 $\pm$ 3	69 $\pm$ 24
Partially purified monomeric (5)	100	> 100		
Partially purified transformed (5)	100	> 100		
Nuclear (2)	100	> 100		
Cytosol + molybdate (3)	17 $\pm$ 1	0.3 $\pm$ 0.08	83 $\pm$ 1	30 $\pm$ 6
Partially purified transformed + molybdate (1)	100	> 100		
Cytosol (4 °C) (3)	23 $\pm$ 4	0.32 $\pm$ 0.17	77 $\pm$ 4	43*
Cytosol + molybdate (4 °C) (3)	34 $\pm$ 1	0.36 $\pm$ 0.17	66 $\pm$ 1	18 $\pm$ 8

* Based on $n = 2$ experiments. In the third experiment, k_2 was not different from 0.

only) was fitted by a single exponential, with a $t_{1/2}$ of 66 $\pm$ 4 h. Correction of dissociation data for this concurrent degradation indicated that TCDD dissociated from approx. 11 % of total receptor·TCDD complexes with $t_{1/2}$ of 0.8 h, but dissociated only slowly from the remainder ($t_{1/2} \sim 70$ h) (Table 1). Similar results were observed when cytosol was pre-incubated with [3 H]TCDD for 16 h at 4 °C (not shown). It should be noted that among different cytosol preparations we observed quantitative variability in half-lives of dissociation. The data presented above and in Table 1 were obtained using the same batches of cytosol used for the molybdate experiments discussed later; with some other batches of cytosol, the larger compartment had even slower TCDD dissociation (e.g. k_2 not different from 0, when corrected for degradation). Despite this quantitative variability, the results were qualitatively consistent among cytosols.

Dissociation of TCDD from partially purified monomeric and heterodimeric receptor forms

[3 H]TCDD-bound monomeric and heteromeric transformed AhRs were partially purified from rat hepatic cytosol by DNA–Sephacryl chromatography (see the Experimental section). As described for crude cytosol, dissociation was monitored by periodically measuring specific binding following addition of excess TCDF (compared with control samples receiving an equal volume of *p*-dioxane). Over a 4 h period at 20 °C there was essentially no dissociation of [3 H]TCDD from either the mono-

Figure 2 Lack of dissociation of TCDD from isolated forms of the AhR

Monomeric (a) and transformed (b) forms of the AhR were isolated from rat hepatic cytosol by DNA–Sephacryl chromatography, and nuclear extracts (c) were prepared from [3 H]TCDD-treated rats, as described in the Experimental section. Excess TCDF (■) or *p*-dioxane (□) was added and specific binding was measured with hydroxyapatite during incubation at 20 °C. Points represent means $\pm$ S.D., $n = 4$, in (a) and (b).

meric or transformed forms of the AhR (Figure 2), although the fully transformed form was considerably more stable than the monomeric form. The analogous receptor forms prepared from guinea pig hepatic cytosol showed similar resistance to ligand dissociation; additionally, the monomeric form from this species was as stable as the fully transformed receptor (results not shown). To determine whether some cytosolic factor that is eliminated during isolation of these forms could account for the observed difference in dissociation between crude cytosol and isolated receptor forms, crude hepatic cytosol was added back to isolated forms prior to initiation of dissociation with TCDF. This treatment did not enhance TCDD dissociation from either

Figure 3 Effect of molybdate on DNA-Sepharose profile

Rat hepatic cytosol (~ 15 mg of protein/ml) was prepared with (a) or without (b) 20 mM Na_2MoO_4 , and incubated with [^3H]TCDD in the presence or absence of TCDF for 2 h at 20 °C, prior to DNA-Sepharose chromatography (see the Experimental section). Molybdate was also present in elution buffers for (a). The same amount of protein was loaded on to the columns in each case. Profiles are representative of 4–5 experiments; only specific binding is shown for each fraction. Note that molybdate in elution buffers contributes to the anionic charge which disrupts the receptor interaction with DNA, and hence it shifts the [NaCl] at which both peaks of TCDD-AhR elute.

rat or guinea pig partially purified receptor forms (results not shown).

We have previously shown that nuclear extracts from [^3H]TCDD-treated rats contain only the transformed form of the AhR, as determined by DNA-Sepharose chromatography (Henry et al., 1989). Consistent with the above observations, we observed very slow dissociation of [^3H]TCDD from AhR contained in these nuclear extracts (Figure 2c; Table 1).

From these results we conclude that the TCDD-initiated conformational changes associated with AhR transformation cause TCDD to become very tightly bound, and that the monomeric and heterodimeric forms of the receptor probably account for the very slowly reversible component of specific binding observed in crude cytosol.

Dissociation of TCDD from non-DNA-binding receptor form(s)

We have previously found that the flow-through fractions from DNA-Sepharose chromatography of rat hepatic cytosol pre-incubated with [^3H]TCDD for 2 h at 20 °C contain 20–30 % of the specifically bound radioactivity eluting from the column (Henry et al., 1989). The dissociation of [^3H]TCDD at 20 °C in such non-DNA-binding fractions was observed to be similar to

that in crude cytosol, i.e. comprising two components, of which the more slowly dissociating one was dominant. Because of the substantial quantitative variability among cytosol preparations, and since the experiments with flow-through fractions were not performed using the same cytosols as in Table 1, these data are not directly comparable quantitatively. However, using two different cytosols and four separate preparations of flow-through fractions, the half-lives of TCDD dissociation observed were 0.3 ± 0.16 h and 33 ± 17 h, with 80–90 % of the total specific binding having the slower dissociation rate. Upon rechromatography on DNA-Sepharose, flow-through preparations have been shown to contain some transformed and monomeric receptor (> 70 % of eluted specific binding), presumably in equilibrium with receptor bound to the column during the initial chromatography (Gasiewicz et al., 1991); this is likely to account for at least some of the more slowly dissociating component. It is also possible that, during chromatography, transformation of the receptor contained in crude preparations may be enhanced, as has also been suggested by Hannah et al. (1986). Nonetheless, these observations imply that the faster-dissociating component of specifically bound TCDD in crude cytosol may represent non-DNA-binding receptor form(s) that have a lower affinity for ligand compared with the monomeric and heterodimeric transformed receptor forms.

One approach to investigating the role of the non-DNA-binding receptor in determining the overall dissociation kinetics observed in crude cytosol is to compare preparations which contain differing proportions of TCDD-receptor complex remaining in the DNA-Sepharose flow-through. Since molybdate is well known as an inhibitor of steroid receptor transformation to a DNA-binding form (Pratt, 1990; Cadepond et al., 1991), we examined its effect on Ah receptor transformation as assessed by DNA-Sepharose chromatography. When cytosol was prepared with 20 mM sodium molybdate, a greater amount of specific binding was not adsorbed to the DNA-Sepharose matrix: 68 ± 3 % was found in flow-through fractions, compared with 29 ± 5 % in matched control cytosols (Figure 3). When the dissociation kinetics at 20 °C were examined, two components of dissociation of [^3H]TCDD were observed in cytosol containing molybdate (Figure 4). The $t_{1/2}$ values for both components (0.3 and 30 h, corrected for degradation; Table 1) were similar to those observed in cytosol without molybdate; the magnitude of the shorter $t_{1/2}$ component in molybdate-treated cytosol was slightly, but consistently, larger than in matched control cytosol (17 % versus 11 % respectively; Table 1). Receptor degradation in the presence of molybdate ($t_{1/2} \sim 115$ h) was slower than in its absence. These differences were due primarily to the presence of molybdate during transformation, since molybdate added to control cytosol following transformation for 2 h at 20 °C had no effect on the dissociation kinetics of [^3H]TCDD or the stability of the receptor (results not shown). Transformed receptor isolated by DNA-Sepharose chromatography from molybdate-treated cytosol showed the same stability and resistance to TCDD dissociation as did control transformed receptor preparations (see Figure 2).

The inconclusive results obtained with molybdate could be due to the inability of molybdate to completely block further transformation of the AhR to a DNA-binding state with higher affinity for TCDD. Denison et al. (1986) have shown that molybdate was much less effective in stabilizing the AhR compared with its effects on steroid receptors, although only thermal degradation and sedimentation properties were examined. To further examine the effect of molybdate on transformation, we followed the time course of transformation of AhR contained in TCDD-treated cytosol prepared in the presence

Figure 4 Effect of molybdate on TCDD dissociation kinetics in hepatic cytosol

Conditions were identical to those described in Figure 1, except that 20 mM molybdate was added to buffers for cytosol preparation as well as incubation with ligands. ●, + TCDF; ○, control. Points are from one representative experiment. A computer-generated fit is shown ($r = 0.998$ for TCDF and 0.75 for control).

Figure 5 Effect of molybdate on the time course of transformation of the AhR to a DRE-binding form

Hepatic cytosol prepared with or without 20 mM molybdate was incubated for the indicated times at 20 °C with 3 nM TCDD. Times of addition of TCDD were staggered so that incubations ended together, and then all samples were placed on ice, incubated with poly(dI,dC) and 32 P-labelled DRE, and analysed by PAGE (all at 4 °C). The TCDD-dependent band corresponding to the AhR·DRE complex in each lane (run in duplicate) was cut out, digested in tissue solubilizer and 32 P activity was counted.

or absence of molybdate, as measured by the ability to bind to the DRE. Molybdate did not prevent but only slightly delayed the transformation process at 20 °C (Figure 5). This demonstrates that, under these conditions, molybdate was not effective in usefully stabilizing the non-DNA-binding form of the receptor.

In a further attempt to maximize the presence of the non-DNA-binding form(s), additional studies were performed utilizing incubation and dissociation at 4 °C with and without molybdate, since we had previously shown that incubation at 4 °C could also delay, though not prevent, AhR transformation to a DNA-binding state (Gasiewicz and Bauman, 1987; Henry et al., 1989). Cytosol in the presence or absence of molybdate was incubated with 3 H]TCDD alone or plus TCDF at 4 °C for 2 h

Figure 6 Effect of molybdate on TCDD dissociation kinetics at 0–4 °C in hepatic cytosol

Cytosol was prepared without (●, ○) or with (■, □) 20 mM molybdate and preincubated for 2 h at 4 °C with 3 H]TCDD in the presence or absence of TCDF. To initiate dissociation, 500-fold excess TCDF (●, ■) was added and incubation was continued at 4 °C; degradation controls received *p*-dioxane (○, □). Specific binding was measured using hydroxyapatite. Data shown are representative of three separate experiments.

prior to the addition of 500-fold excess TCDF and continued incubation at 4 °C. Under these conditions without molybdate, approx. 20 % of the specific binding was associated with the faster-dissociating component; the presence of molybdate enhanced the total percentage of this component to approx. 35 % of the total specific binding (Figure 6, Table 1). These observations indicate that both molybdate and lower temperature allow only relative stabilization of the non-DNA-binding form(s) of the receptor.

Association of AhR with hsp90

Association of hsp90 with a 9 S form of the AhR has been reported previously (Denis et al., 1988a,b; Perdew, 1988; Wilhelmsson et al., 1990). Additional evidence suggested that hsp90 serves a role in repressing the DNA-binding activity of the AhR while stabilizing a form that can bind ligand (Wilhelmsson et al., 1990; Pongratz et al., 1992). This evidence and consideration of our present results suggests that the AhR·hsp90 complex may be the form which binds TCDD more loosely; release of hsp90 (and possibly other proteins) from the receptor during transformation enables a more stable interaction with TCDD. We therefore examined the different receptor preparations from rat hepatic cytosol for the presence of hsp90 in order to delineate the dynamics of this association. To verify that hsp90 was actually complexed with AhR rather than just co-eluting, the receptor was immunoprecipitated using an anti-AhR antibody (Poland et al., 1991). Receptor-antibody complexes were precipitated with Protein A-Sepharose and analysed by SDS/PAGE, followed by immunoblotting with antibodies against the AhR and hsp90 (AC88). Both receptor and hsp90 were detectable when unoccupied receptor complexes contained in crude cytosol were immunoprecipitated with anti-AhR, but not using a nonspecific anti-IgG following the same protocol (Figure 7, lanes 1, 2, 6 and 7). Both AhR and hsp90 were also detected in immunoprecipitated flow-through fractions isolated from DNA-Sepharose chromatography of rat cytosol treated with saturating concentrations of ligand (3 nM 3 H]TCDD + 300 nM TCDF) (lanes 3 and 8). Isolated DNA-binding fractions

Figure 7 Association of hsp90 with the AhR

The indicated receptor preparations were immunoprecipitated with an antibody to the AhR (except lanes 2 and 7, for which the antibody was a nonspecific anti-IgG) and Protein A-Sepharose prior to analysis by SDS/PAGE and Western blotting (see the Experimental section). Lanes 1–5 were probed with AC88 (anti-hsp90), and lanes 6–10 with anti-AhR. Lanes 1, 2, 6 and 7, cytosol; lanes 3 and 8, flow-through fraction; lanes 4 and 9, isolated monomeric receptor; lanes 5 and 10, isolated transformed receptor.

obtained from rat cytosol were immunoprecipitated to yield detectable receptor bands but no hsp90 (lanes 4, 5, 9 and 10). These results indicate that hsp90 is associated with AhR forms that are unoccupied or ligand-occupied but in a non-DNA-binding state; hsp90 dissociates from the receptor prior to conversion to the ligand-bound monomeric form or the DRE-binding form.

DISCUSSION

The results that we have obtained are consistent with a model, previously presented by us and others, in which hsp90 is associated with the unoccupied AhR and with an occupied form(s) lacking DNA-binding ability. In addition, the present results provide evidence indicating that TCDD binding to a non-DNA-binding form(s) of the receptor is a readily reversible process. Utilizing chemical cross-linkers, Perdew (1992) has recently suggested that the unoccupied receptor may exist as a tetrameric species consisting of the 97 kDa AhR protein and three additional proteins of 46, 88 and 96 kDa. It is not known if these 88 and 96 kDa species represent two molecules of hsp90. As suggested by Pongratz et al. (1992), and consistent with known interactions of hsp90 with steroid hormone receptors (reviewed by Dahlman et al., 1991), hsp90 probably serves a role in maintaining the receptor in a non-functional, i.e. non-DNA-binding, conformation that is able to bind ligand. The roles and dynamics of other proteins that may be associated with this AhR complex remain to be clarified.

Although the molecular events initiated by ligand binding and leading to the disruption of the multimeric complex are not yet defined, it is clear from our results that the dissociation of hsp90 (and any other associated proteins) from the AhR is followed by increased affinity of the receptor for the ligand. The results which demonstrate that a large fraction of the [3 H]TCDD is only very slowly dissociable from the transformed receptor are consistent with those of Bradfield et al. (1988) and Farrell and Safe (1987). The latter investigators noted that within 1 h TCDD had displaced only 10% of radiolabelled ligand from rat hepatic cytosol pre-incubated for 2 h at 20 °C with [3 H]TCDD. While they suggested that this binding was 'irreversible', later studies, some by the same group, indicated that without u.v.-induced covalent linkage, radiolabelled ligands readily dissociated from the receptor under conditions of protein denaturation (Poland et al.,

1986; Landers et al., 1989; Gasiewicz et al., 1991). Thus transformation of the receptor elicits a protein conformation that does not allow the ligand to readily dissociate. However, these data seem to be in conflict with recent information from Pongratz et al. (1992), who observed that high salt concentrations and increased temperature induced dissociation of the unoccupied receptor from hsp90, resulting in a receptor conformation that bound DNA but was unable to bind ligand. These results are compatible with ours if one considers the possibility that, following release of hsp90, the ligand-binding site becomes inaccessible. Thus a ligand already bound becomes less dissociable, while a free ligand is unable to bind to a site that is not yet occupied. At this time we can only speculate that these events, if occurring, may be involved in compartmentalizing receptor functions, i.e. ligand versus DNA binding.

Interpretation of events leading to the fully transformed receptor is also complicated by the fact that we know of no conditions that adequately stabilize the non-DNA-binding TCDD·AhR complex. For example, the presence of molybdate significantly increased the specific binding eluted in the flow-through fractions during DNA-Sepharose chromatography performed at 4 °C (Figure 3); however, a much smaller effect of molybdate was apparent when TCDD dissociation kinetics were examined at 20 °C. It is likely that rapid transformation occurs following warming of a non-DNA-binding form to 20 °C even in the presence of molybdate, as further suggested by the data in Figure 5. Even at 4 °C transformation proceeds, albeit more slowly than at 20 °C. This characteristic of the AhR is likely to account for at least some of the variability observed in TCDD dissociation kinetics in crude cytosol and isolated non-DNA-binding fractions. Since receptor transformation is only delayed, but not prevented, by these conditions, only a qualitative characterization of TCDD dissociation from the non-DNA-binding form(s) is possible at this time without a further understanding of what multimeric complexes these forms represent and how the dissociation of these proteins from the AhR proceeds. For example, we cannot completely rule out the existence of an intermediate hsp90·AhR complex which exhibits slow dissociation of TCDD typical of the non-hsp-bound receptor. However, it is clear that hsp90 is only detectable in immunoprecipitates of receptor preparations in which a fast-dissociating component is present (crude cytosol and non-DNA-binding forms), whereas AhR conformations (monomeric and heteromeric) which bind DNA have very high affinity for TCDD and no longer include hsp90.

It is noteworthy that the increased affinity of the AhR for TCDD does not appear to depend on the dimerization of the receptor that is necessary for the formation of the DRE-binding complex (Gasiewicz et al., 1991; Reyes et al., 1992). This is indicated by the finding that [3 H]TCDD is bound as tightly by the monomeric receptor as by the heterodimeric transformed form (Figure 2), even though this monomeric form can be distinguished as an intermediate form by its lower molecular mass (Henry et al., 1989), its lower affinity for DNA-Sepharose and DRE (Gasiewicz et al., 1991), and its reconstitution by cytosolic factor(s) to form the fully transformed receptor (Henry and Gasiewicz, 1991). We interpret these and other (Pongratz et al., 1992) studies, together with the present results, to suggest that hsp90 may not play a direct role in the dimerization process, but merely maintains a conformation of the AhR that is able to bind ligand. The ligand-elicited conformational changes in the AhR that release hsp90 and impart higher affinity for ligand also appear to expose a domain on the receptor that is able to interact with the complementary protein to form the active, i.e. DNA-binding, heterodimeric complex. Thus it appears that the dis-

sociation of hsp90 from the AhR and the concomitant changes in conformation that enhance the receptor's affinity for both ligand and an additional protein are key regulatory steps in the process of transformation of the AhR to its DNA-binding state.

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