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Ligand binding and activation of the Ah receptor

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Abstract

The Ah receptor (AhR) is a ligand-dependent transcription factor that can be activated by structurally diverse synthetic and naturally-occurring chemicals. Although a significant amount of information is available with respect to the planar aromatic hydrocarbon AhR ligands, the actual spectrum of chemicals that can bind to and activate the AhR is only now being elucidated. In addition, the lack of information regarding the actual three-dimensional structure of the AhR ligand binding domain (LBD) has hindered detailed analysis of the molecular mechanisms by which these ligands bind to an active AhR signal transduction. In this review we describe the current state of knowledge with respect to naturally occurring AhR ligands and present and discuss the first theoretical model of the AhR LBD based on crystal structures of homologous PAS family members. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

The Ah receptor (AhR) is a basic helix–loop–helix (bHLH)- and Per–Arnt–Sim (PAS)-containing transcription factor that regulates the expression of genes in a ligand-dependent manner [1–4]. Although recent studies have demonstrated that the AhR can bind and be activated by a structurally diverse range of chemicals [5,6], the best characterized high affinity ligands for the AhR include a wide variety of ubiquitous and hydrophobic environmental contaminants [7,8] such as the halogenated aromatic hydrocarbons (HAHs) and the non-halogenated polycyclic aromatic hydrocarbons (PAHs). Exposure to numerous HAHs, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin), the most potent member of this class of chemicals, produces a wide variety of species- and tissue-specific toxic and biological effects [7–11]. The induction of gene expression is one response observed in all species exposed to TCDD and related chemicals. Induction of expression of cytochrome P4501A1 (CYP1A1) has been used as a model system to define the mechanism of action of HAHs. Biochemical and genetic studies over the past 20 years has revealed that induction of CYP1A1 and other HAH/PAH-responsive genes, as well as the toxicity of TCDD and related HAHs, is mediated by the AhR, a soluble intracellular receptor to which these chemicals bind with high affinity [3,7,8,12]. Mechanistically, the inducing chemical diffuses across the plasma membrane and binds to the AhR which is present in the cytosolic compartment as a multiprotein complex containing two molecules of hsp90 (a heat shock protein of 90 kDa), the X-associated protein 2 (XAP2 [13]) (also referred to as AIP or ara9 [14,15]) and p23 (a co-chaperone protein of 23 kDa [16]). Following ligand binding, the cytosolic ligand:AhR complex is presumed to undergo a conformation change exposing a nuclear localization sequence(s) (NLS(s)). The complex then translocates into the nucleus [17,18], dissociates from the protein complex and binds to a closely related nuclear bHLH–PAS protein called Arnt (AhR nuclear translocator [1]). Formation of the AhR:Arnt heterodimer converts the complex into its high affinity DNA binding form [1,19] and binding of the complex to its specific DNA recognition site, the dioxin responsive element (DRE), upstream of the CYP1A1 gene leads to chromatin and nucleosome disruption, increased promoter accessibility and an increase in transcription of the CYP1A1 gene [12,20–22]. DREs have also been identified in the upstream region of most other TCDD-inducible genes [3] and they also appear to be responsible for conferring TCDD- and AhR-responsiveness upon these genes. The presence of the AhR and AhR signal transduction pathway in a diverse range of species, tissues and cell types [23–27] and its ability to act as a ligand-dependent transcription factor suggests that many of the toxic and biological effects of AhR ligands result from differential alteration of gene expression in susceptible cells. In addition, since the majority of the toxic effects of TCDD/HAHs are not observed until weeks following chemical exposure [7,11], the adverse effects of these chemicals likely result from the continuous and inappropriate expression of specific genes in target cells which ultimately results in the delayed toxic responses. Although significant advances in the field over the past 10 years have clearly defined the role of AhR in the toxic and biological effects of AhR ligands, the exact

biochemical events which lead to the spectrum of species- and tissue-specific toxic responses to these chemicals still remain elusive.

All of the high affinity AhR ligands identified to date (HAHs and PAHs) are planar hydrophobic molecules and are able to induce gene expression in an AhR-dependent manner. In previous reviews [5,6], we detailed the spectrum of chemicals that have been documented in the literature to bind to and active the AhR- and/or induce AhR-dependent gene expression. Accordingly, rather than reiterating what we have described previously, this review will highlight more recent developments in our knowledge about AhR ligands with an emphasis on naturally occurring ligands that activate the AhR and AhR signaling pathway. In addition, we will describe reported differences in AhR ligand binding specificity between species and describe recent structural modeling studies of the AhR ligand binding domain (LBD) itself. For a more in depth description of AhR signal transduction and the effects of AhR ligands, the reader is referred both to additional reviews contained within this special issue of Chemico-Biological Interactions and to the many excellent published reviews [1–3,5,7,11,12,28].

2. AhR ligands

HAHs (such as the polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans and biphenyls and related chemicals) and the PAHs (such as benzo(*a*)pyrene, 3-methylcholanthrene, benzoflavones, rutacarpine alkaloids, aromatic amines and related chemicals) are the most extensively studied classes of AhR ligands [5,7,8,29–32]. HAHs have a relatively high binding affinity for the AhR (in the pM to nM range) whereas the PAHs have a significantly lower affinity (in the high nM to μM range). Structure–activity relationship studies with a large number of HAH and PAH AhR ligands suggest that the AhR binding pocket can accept planar ligands with maximal dimensions of $14 \times 12 \times 5$ Å. However, high affinity binding appears to be critically dependent upon key thermodynamic and electronic properties of the ligands [29–37]. The overall picture emerging from these studies highlights the role of electrostatic and dispersion-type interactions in ligand–AhR binding. Although the results of these modeling studies have some predictive applications for identification of new high affinity AhR ligands, the constraints of these models are too restrictive, especially given that a large number of chemicals that are reported to bind the AhR have physicochemical and structural properties that deviate significantly from the currently defined structural requirements for AhR ligands (reviewed in Refs. [5,6]). Although the majority of these chemicals are relatively weak inducers or AhR ligands when compared with TCDD, their striking structural diversity is clearly evidenced by comparison of the chemicals for which direct AhR binding has been demonstrated (Fig. 1). While binding remains to be demonstrated for many of these structurally diverse AhR activators [5], their ability to induce CYP1A1 and/or activate the AhR- and AhR-dependent gene expression is consistent with their ability to interact with the AhR. Because most of these chemicals do not fit
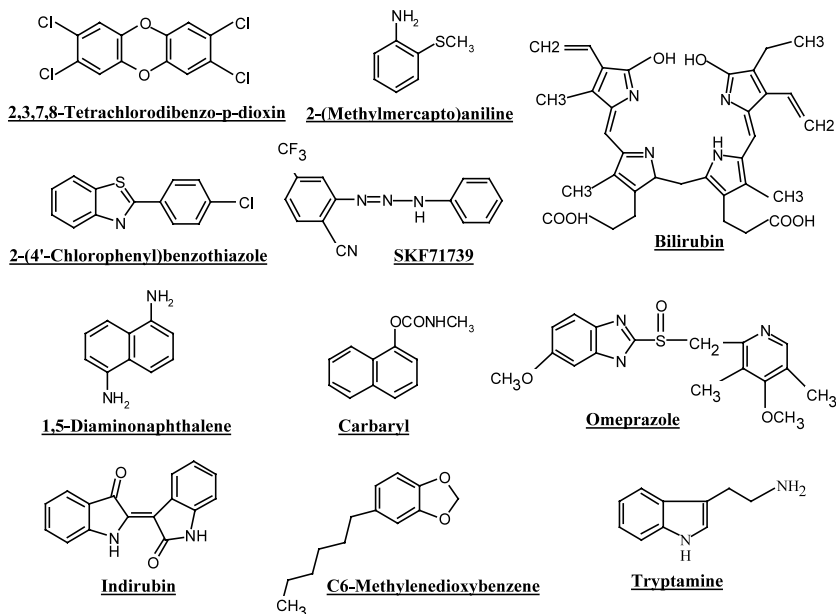
Fig. 1. Structures of selected AhR ligands. See text and Denison et al. [5] for more details.

the established characteristics for known AhR ligands, their identification as ligands supports a reevaluation of the currently accepted view of AhR ligand structure.

For the sake of this review, we have classified AhR ligands in two major categories, synthetic AhR ligands (generally formed from anthropogenic activities) and those that are naturally-occurring (formed in biological systems as a result of natural processes). Within the natural ligand category, there are two subclasses, dietary AhR ligands and endogenous physiological ligands and this review is focused on these classes of AhR ligands. The vast majority of AhR ligands that have been characterized are members of the first category, including the HAHs, and PAHs, and numerous excellent reviews on the physiochemical characteristics and biological/ toxicology potency of these 'synthetic' AhR ligands are available [5–9,30,31,33] and as such, these chemicals will not be discussed here.

2.1. Dietary AhR ligands and inducers

Perhaps our greatest exposure to AhR ligands comes from the food we eat. In fact, since the majority of 'natural' ligands identified to date are dietary or are related to dietary plant products, it has been proposed that the natural ligand(s) for the AhR may be dietary in nature. There are numerous reports of naturally-occurring dietary chemicals (e.g. flavonoids, carotinoids, phenolics) that can activate the AhR signaling pathway, although the majority of these chemicals appear to be relatively weak AhR ligands [38–46]. Recently, the presence of AhR ligands/agonists/ antagonists in extracts of a variety of different vegetables, fruits, herbs and teas

was reported [47,48]. Interestingly, conversion of dietary indoles (including indole-3-carbinol (I3C) and tryptophan (TRP)) in the mammalian digestive tract to significantly more potent AhR ligands/agonists has been demonstrated [38,49]. Indolo-[3,2-b]-carbazole (ICZ), an acidic condensation product formed from I3C (itself a weak AhR ligand), has perhaps the highest affinity of any 'natural' AhR ligand identified to date (~ 0.2–3.6 nM) and it is a potent inducer of AhR-dependent gene expression in cells in culture [29,38]. The formation of relatively potent AhR ligands from precursors that have little or no AhR agonist activity is significant, especially considering that most dietary ligands are themselves relatively weak AhR ligands/agonists. Thus, plant-derived materials appear to commonly contain AhR ligands or products that can be converted into AhR ligands and as such, they represent a major class of 'natural' AhR ligands.

2.2. Evidence for endogenous physiological AhR ligands and inducers

This class of natural AhR ligands/activators includes chemicals and substances that are formed in vivo. Although the specific compound(s) that activate the AhR at normal physiological concentrations remains one of the major unanswered questions in AhR biology, the promiscuous ligand binding activity of the AhR suggests to us that there are multiple endogenous AhR ligands. The existence of endogenous physiological ligands for the AhR is indirectly supported by numerous observations demonstrating AhR activation and AhR-dependent responses in the absence of exogenous ligand. This indirect evidence includes: (1) the presence of nuclear AhR complexes in tissue slices from untreated animals [50] and in cells in culture [51,52]; (2) induction of CYP1A1 in various cell types following suspension of the cells in methylcellulose [53–55]; (3) evidence that the AhR plays a role in regulating cell cycle in the absence of any exogenous ligand [56–58] and the observation that disruption of AhR expression results in a decrease in mouse blastocyst development [59]. These observations are consistent with the notion that an endogenous AhR ligand is responsible for these events. Perhaps the best evidence for an endogenous AhR ligand in animals comes from studies using AhR knockout mice. The occurrence of a spectrum of developmental defects and physiological changes in AhR($-/-$) mice [60–63] suggest that activation of the AhR is required in a critical developmental stage(s) and it is presumed that this activation is mediated by an endogenous ligand in these specific tissues. However, the identity of the AhR ligand(s) responsible is currently unknown.

2.3. Identification and characterization of endogenous AhR chemicals that bind to and/or activate the AhR

Recently, numerous studies have reported the ability of a variety of distinct endogenous physiological chemicals to bind to the AhR and/or activate AhR-dependent gene expression. Although, the role of these chemicals, if any, in AhR-dependent signaling in vivo remains to be established, these chemicals are present in

many species and tissues. For this discussion, we have focused on three categories of 'endogenous' ligands, indoles, arachidonic acid (AA) metabolites and tetrapyroles.

2.3.1. Indole-containing chemicals

The ability of a variety of indole-containing chemicals to bind to and activate the AhR suggests that endogenous indoles may be AhR ligands. Activation of AhR- and AhR-dependent gene expression in vivo and in cells in culture by UV light has been demonstrated and this effect appears to arise from UV photoproducts of TRP and histidine that can directly bind to and activate the AhR [64–72]. Two of the UV TRP photoproducts were subsequently identified by Rannug et al. [73] and although their formation in vivo remains to be confirmed they are very similar in structure to the potent exogenous indole ICZ [38]. Interestingly, it has been proposed that these TRP photoproducts may be novel chemical messengers of light [72]. Given the ability of light to activate other members of the PAS superfamily [74,75], this hypothesis takes on more potential significance. The ability of TRP and naturally occurring TRP metabolites (tryptamine and indole acetic acid) to directly bind to and activate the AhR and AhR-dependent gene expression in both yeast and mammalian cells in culture has also been reported [76,77]. Although these studies demonstrated the ability of numerous TRP metabolites to active the AhR, the relatively weak affinity and generally low cellular concentration of these chemicals suggest that they are unlikely to be endogenous ligands for the AhR under normal physiological conditions. However, under abnormal conditions, such as in the presence of monoamine oxidase inhibitors, tissue levels of some TRP metabolites (tryptamine) are reported to be elevated to as much as ~ 700 μM [78], a concentration that would be sufficient to activate the AhR.

Indirubin and indigo represent another group of endogenous indole-containing chemicals that can activate the AhR [79]. These chemicals were extracted from human urine and their dose-dependent ability to induce AhR-dependent reporter gene expression was demonstrated using a recently described AhR/Arnt-containing yeast cell gene expression bioassay [79,80]. Both indirubin and indigo were reported to be extremely potent inducers, with indigo being equipotent to TCDD and indirubin 50-times more potent than TCDD in the yeast bioassay. However, these relatively high potency values are somewhat misleading. This conclusion is derived from the observation that the relative potency of TCDD in the yeast bioassays is 900–1500-fold lower than that reported in many mammalian cell cultures (compare the EC_{50} for TCDD of 9 nM in yeast [79] to that of 6–10 pM in mammalian cells [reviewed in ref. [81]]), while that of β-naphthoflavone (a PAH AhR ligand) has a similar affinity in yeast and mammalian cells. This decrease in potency likely results from decreased availability of TCDD to the yeast cells as a result of its extremely poor solubility in aqueous yeast culture media [80]. Although indirubin levels in fetal bovine serum were found to be ~ 0.07 nM [79], a concentration sufficient to activate the AhR in the yeast bioassay system, the concentration of these chemicals in human serum remains to be established.

2.3.2. AA metabolites

A relationship between AA, TCDD and the AhR signal transduction pathway is supported by results from several laboratories. Not only can TCDD increase the release of AA from membranes as a result of its ability to stimulate membrane lipid oxidation and phospholipase A activity [82–85], but it also induces AA-metabolizing CYPs (such as CYP1A1 [86–88]) and PGSH2 (PGSH2 [89,90]) which converts AA to prostaglandins. These results strongly support an effect of TCDD and the AhR pathway on AA metabolism. Although evidence for a reciprocal effect of AA or AA metabolites on the AhR signaling pathway is lacking, it has been suggested that AA metabolites may play a role in the hydrodynamic shear-stress induction of CYP1A1 in cells in culture [91]. In addition, suspension-mediated induction of CYP1A1 in cells in culture [53,54], is presumed to result from the production or release of an endogenous AhR ligand, possibly released from the cell membrane in response to changes in membrane conformation/structure. Accordingly, it seems reasonable to propose that biological lipids may also be AhR ligands. The identification of lipoxin A4, a lipoxygenase product of AA, and several prostaglandins (most notably PGG_2) as both AhR ligands and activators of AhR-dependent gene expression support this hypothesis [92,93]. Lipoxin A4 transiently induces expression of CYP1A1 and a DRE-dependent reporter gene at 30–60 nM (a concentration near physiological in some situations [94,95]. In contrast, dose–response relationship studies revealed that the prostaglandins, with the exception of PGG_2, are relatively weak AhR agonists that active AhR-dependent gene expression only at concentrations of ≥ 10 μM, much higher than normal blood levels (usually < 1 nM [96]). However, since prostaglandins are local hormones, concentrations may actually reach 5–10 μM in the proximity of hepatocytes due to non-parenchymal liver cells secreting these chemicals into the narrow space of Disse [97]. Thus, it is possible that selected prostaglandins, or a combination of prostaglandins or related chemicals can activate the AhR in vivo. Interestingly, several prostaglandins (most notably PGG_2) induced AhR-dependent gene expression to a level two- to fivefold greater than that produced by a maximally inducing dose of TCDD [93]. These results suggest that these prostaglandins also affected a secondary signal transduction system that augments the AhR-dependent gene expression response. The cellular signaling pathway(s) responsible for this dramatic increase in AhR-dependent gene expression is currently unknown.

2.3.3. Tetrapyroles

The observation of persistent expression of hepatic CYP1A1 gene expression in congenitally-jaundiced Gunn rats [98] suggested the presence of endogenous AhR ligands in the blood of these animals. Studies from two laboratories [99,100] demonstrated that bilirubin (BR), a heme-degradation product present in high levels in the blood of Gunn rats, induced expression of CYP1A1 and a DRE-dependent reporter gene in a dose- and AhR-dependent manner in cultured cells. This induction was observed using physiologically relevant concentrations of BR. Biliverdin (BV), the metabolic precursor of BR, also induced DRE-dependent gene expression in several species, although it was suggested that it did so indirectly by serving as a

precursor to the formation of BR via normal heme degradation pathway [99]. BR and BV stimulated AhR transformation in cells and DRE binding in vitro and competitive inhibition of [^{3}H]TCDD specific binding to the cytosolic AhR confirmed that they were indeed AhR ligands [100]. In addition, the greater inducing potency of these chemicals in intact cells, as compared to the in vitro bioassays [100], suggested that BR and BV might also be metabolically converted to a more potent activator(s) in vivo. Together, these results demonstrate that the heme degradation products BR and BV are AhR ligands, which can regulate the AhR-dependent gene expression pathway.

3. Species differences in AhR ligand binding

Significant differences in the ability of AhR ligands to elicit toxic and biological responses in various species and tissues have been observed for many years [7,101] and these differences can be due to variations in a wide variety of species- and tissue-specific biochemical and physiological characteristics, including, but not limited to: differences in ligand pharmacokinetics, pharmacodynamics and metabolism, AhR functionality, cellular AhR subunit concentrations, the presence or absence of intra-/extracellular ligand binding sites (such as P450IA2), and other species- and tissue-specific regulatory effectors. It has generally been accepted that although the affinity of binding of different chemicals (HAHs and PAHs) for the AhR can vary between species, the rank order potency of the chemicals is the same. However, results from our laboratory and others suggest that the ligand binding specificity of the AhR is not identical between different species. Some of the earliest evidence for species differences in ligand binding specificity and rank order potency comes from studies examining competitive binding of a series of PAHs [23] and single hydroxylated benzo(*a*)pyrene molecules to hepatic cytosolic AhR [102]. More recently, species-specific differences in antagonism of TCDD-inducible, AhR-dependent gene expression by several 'non-AhR' di-ortho PCBs, such as 2,2′,5,5′-tetrachlorobiphenyl were reported [103,104]. In these studies, 2,5,2′,5′-tetrachlorobiphenyl could bind to and completely antagonize the murine AhR while only partially antagonizing the rat AhR; no binding or antagonism the human or guinea pig AhR was observed. Interestingly, we also observed species-specific antagonism of hepatic AhR transformation and DNA binding by α-naphthoflavone, wherein this chemical bound to and antagonized the rat AhR, but failed to antagonize the guinea pig AhR (Denison et al., unpublished observation). The benzimidazole drug omeprazole is reported to be another species-specific ligand, because it activates the AhR present in human but not in mouse cells in culture [105,106]. However, recent evidence suggests that the species difference is actually due to a species/tissue-specific cellular factor other than the AhR [107]. Anderson and coworkers [108,109] recently demonstrated the ability of phenobarbital and gamma amino butyric acid (GABA) to bind to and activate the rainbow trout AhR, yet these chemicals are known not to activate the AhR in mammalian AhR. Additional studies observed that while mono-ortho PCBs were able to active the human AhR, they were generally ineffective in activating

rainbow trout and zebrafish AhRs [110]. Thus, although it can be generalized that AhR ligand binding specificity is similar between species, significant differences in specificity and affinity do exist and these may contribute to some of the differential species responsiveness that have been reported. These observations also put some additional constraints upon the structural specificity and physiochemical characteristics of an endogenous physiological AhR ligand(s), since it would be expected that binding and activation by such ligands would be conserved across species.

4. AhR LBD analysis

In the last 10 years AhR cDNAs from a wide variety of species have been cloned (reviewed in Ref. [27,111]; also see the review by Hahn in this special issue of Chemico-Biological Interactions) and this has led to an extensive and continuing analysis of functionally important domains within the AhR. The currently defined domain structure for the AhR is presented in Fig. 2. The AhR contains a bHLH region that functions in dimerization with ARNT and DNA binding, hsp90 interaction [1–3,112,113] and contains sequences important for both AhR nuclear localization and nuclear export. The AhR PAS domain contains two structural repeats (PAS A and PAS B) which are involved in AhR/Arnt dimerization (PAS A) and AhR ligand and hsp90 binding (PAS B). The C-terminal Q-rich domain contains the transactivation activity of the AhR. Hsp90, a molecular chaperone protein, is thought to be important for correct folding of the AhR LBD and for regulating nuclear localization of the AhR signaling complex [114–117]. Although several studies have demonstrated the absolute hsp90 requirement for formation of fully functional AhR [114,118,119], there is some controversy as to whether the continued presence of bound hsp90 is required to maintain its high affinity ligand binding conformation [114,119]. The reader is directed to several excellent recent reviews ([1–4,28] and references therein) for a more detailed description of the domain structure of the AhR and Arnt proteins.
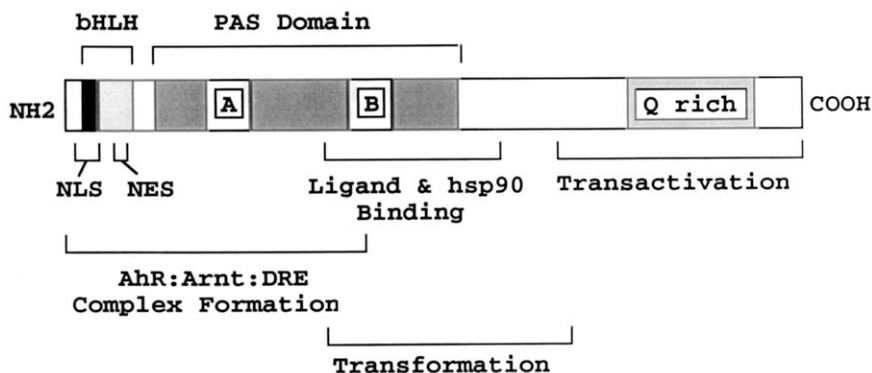

Fig. 2. Domain structure of the AhR.

In contrast to the significant amount of information available regarding AhR ligands, essentially nothing is known about the AhR LBD itself. Full ligand binding activity and specificity of the mouse AhR is reported to be contained within a small fragment (residues 230–421, 24% of the total AhR protein [120]) and several naturally occurring mutations within this region alter AhR ligand binding affinity [121,122]. Deletion of residues 287–421 results in a ligand-independent constitutively active AhR demonstrating the role of the AhR LBD/hsp90 complex in regulating AhR functionality [123].

Currently there are no X-ray or NMR-determined structures of liganded or unliganded AhR. However, three essential questions require detailed three-dimensional structural information: (1) What are the ligand interaction surfaces in the AhR LBD?; (2) At the molecular level, how does ligand binding activate the receptor and trigger nuclear import; (3) What is the structural basis for binding and signaling specificity? Structure determination has been a valuable tool for answering similar questions in analogous but structurally distinct signaling systems such as steroid hormone, thyroid hormone, and retinoic acid receptors, which are highly ligand-specific. In these systems, antagonism often occurs when the ligand is bound with an imprecise fit. In contrast, AhR ligands are diverse and relatively few are antagonists. The challenge is to derive a molecular structure–activity model that accounts for these aspects. However, such a model would be a useful guide for limited mutagenesis experiments to map ligand interaction surfaces inside the LBD as well as those involved in transmitting the signal on the LBD surface. Species sequence variation provides some clues for key interacting residues. Although comparison of the amino acid sequence of this AhR LBD reveals a high degree of identity and homology [27], there are a significant number of variant residues that may contribute to the observed species differences ligand binding specificity and affinity. However, the lack of three-dimensional structure information about the AhR LBD makes determination of the functional significance of these sequence differences difficult to evaluate.

In lieu of an X-ray crystallographic structure of the AhR, we developed a theoretical model for the LBD of the mouse AhR (residues 275–380) by homology modeling techniques [124]. This modeling was based on the knowledge of the crystal structures of homologous proteins belonging to the PAS family that were available: bacterial photoactive yellow protein, PYP (PDB# 2PYP [125]) human potassium channel HERG (PDB# 1BYW [126]) and the heme binding domain of the bacterial O_2 sensing FixL protein (PDB# 1BV5 [127]). These proteins, like most PAS-containing proteins, are involved in signaling processes, yet each has quite different mechanisms to perform its function [74,128]. While the HERG PAS domain does not bind a ligand [126], in FixL, oxygen binding at the heme cofactor controls the activity of a histidine kinase domain [127] and in PYP, a local conformational change occurs when the p-hydroxycinnamyl chromophore photoisomerizes [129].

Despite the low level of sequence similarity, the three PAS structures show highly conserved structural characteristics. All have a five-stranded antiparallel β-sheet with a α-helix at one side and the largest conformational differences between them are localized around this long central helix, the so-called helical connector, which defines

the size of the central cavity. Since it is known that the signal-transducing regions of PYP and FixL are located at the opposite ends of this helix, this has been proposed as the critical regulatory region of the whole family. The multiple alignment on which the homology modeling procedure was based took into account information on the predicted and the observed secondary structures of the target and reference proteins as well as the sequence and structure conservation in their families. The analysis suggested that FixL was the better template for modeling. This choice was motivated by the observations that FixL binds the cofactor non-covalently and that its helical connector is translated away from the β-sheet to allow the accommodation of the heme group, a situation also expected to be present in the AhR.

The mAhR LBD homology model is shown in Fig. 3 along with the FixL PAS domain structure for comparison. This AhR model is consistent with a number of
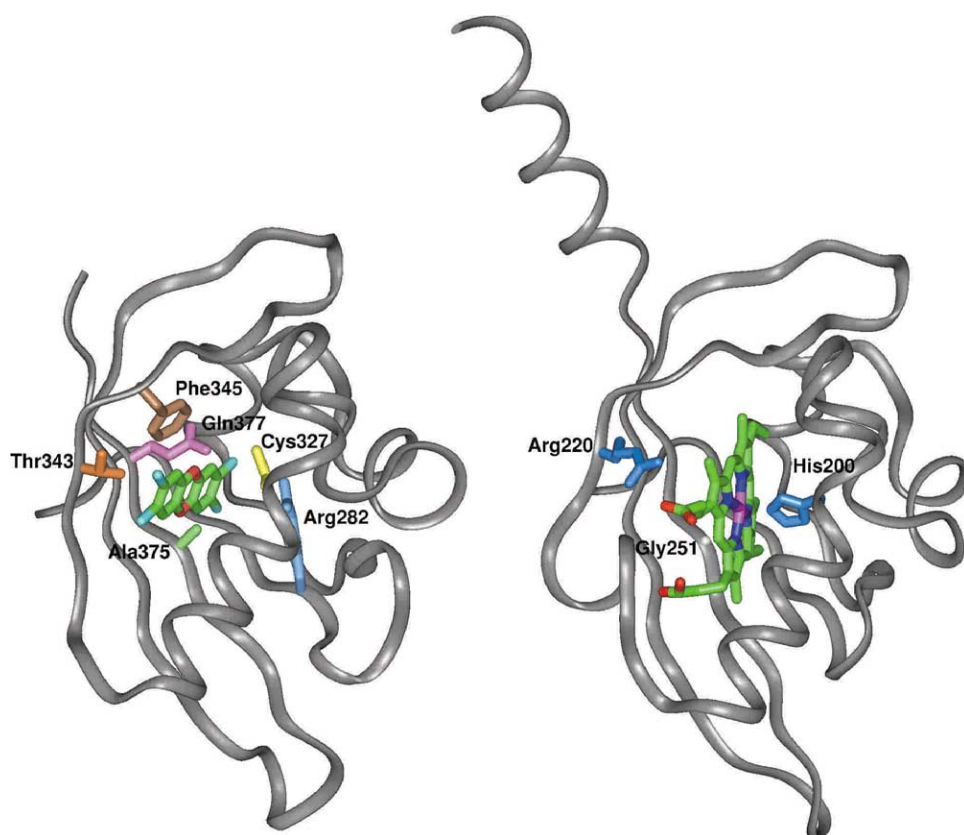

Fig. 3. Comparison between the mAhR LBD model (left) and the FixL PAS domain structure (right). The coordinate set used for FixL corresponds to entry 1DRQ [127] of the PDB protein data bank [145]. Key residues within the binding cavities are highlighted (see text for details). Coloring scheme for residues: red: acidic; blue: basic; purple: polar; yellow: Cys; brown: aromatic; green: hydrophobic; orange: Ser, Thr; grey: Pro; white: Gly. TCDD and heme cofactor atoms are colored green (carbon), red (oxygen), blue (nitrogen), magenta (iron) and cyan (chlorine). The figure was generated by Insight II [146].

experimental data [124]. The cavity size, determined by the helical connector position, is intermediate between those observed in HERG and in FixL, in agreement with the different ligand requirements. While HERG lacks any binding activity, mAhR binds with high affinity PCDDs and the other 'classical' ligands and FixL binds the larger heme cofactor. The side-chain volume of some residues within the cavity shows a good complementarity with the size of the ligands: while the latter decreases in going from FixL to AhR to HERG, the side-chain volume increases. For example, in the position corresponding to Ala375 in mAhR, a glycine (Gly251) and a leucine are observed in FixL and in HERG, respectively. Interestingly, the human AhR shows low binding affinity for PCDDs and has bigger side-chains at this position. These observations are also consistent with site-directed mutagenesis results that identified the side-chain size in the position corresponding to the Ala375 of mAhR as critical for the ligand binding activity [121]. Finally, it should be noted that the arginine residue at the entrance of the FixL cavity that binds a heme propionate group (Arg220), is replaced by a threonine in mAhR (Thr343) and by an isoleucine in the human AhR. The side-chain length of the Ile residue could partially block the entrance of the binding cavity. However, static access to the cavity may not be essential for binding. Binding of aromatic compounds to engineered cavities in the T4 lysozyme hydrophobic core [130–132] is facile and rapid, even when crystal structures show no clear path to the protein surface [133]. Thus, dynamic protein flexibility may allow small aromatic compounds to enter an obscured binding site, provided that it is large enough to accommodate the ligand.

The LBD model has also led to preliminary hypotheses on the residues that may be involved in ligand binding within the cavity [124]. Due to their high binding affinities, PCDDs were selected as reference ligands for this analysis. Previous structure–activity relationship studies highlighted some molecular requirements for high affinities. In particular, the analysis of the molecular electrostatic potential (MEP) [33,34] and the molecular polarizability [35] suggested that the concentration of negative MEP values at the extremes of the PCDD's long molecular axis may lead to favorable interactions with electrophilic residues of the receptor in these positions. Moreover, a depleted charge above and below the aromatic rings indicated the possibility of stabilizing interactions between a nucleophilic region of the AhR and the central part of the ligand molecules. Some residues that reside within the binding cavity in our LBD model may interact with the PCDDs that fit well with the above electronic characteristics. This can be shown by placing TCDD in the modeled cavity, in a similar position as that observed for the heme group in FixL, and analyzing the residues around it (Fig. 3). Where in FixL, a histidine residue (His200) coordinates the ferric heme ion, all AhRs with high affinity for PCDDs contain a cysteine residue (Cys327 in mAhR). The side chain of this residue can act as an electron-donor group to provide favorable interactions with the electrophilic central region of the TCDD. At the same time, the positively charged arginine residue pointing to the TCDD chlorinated side (Arg282) may contribute to the binding by electrostatic interactions with this electron-rich region of the ligand. Moreover, the phenylalanine residue (Phe345) may play a role in a stabilizing stacking interaction with the aromatic rings of the TCDD, while the polar glutamine (Gln377) may

stabilize the binding by interacting with the chlorine atom lateral region. Although these interactions may be important for binding of TCDD and related high affinity HAHs, the role for these interactions in the binding of other ligands, which lack these determinants remains to be elucidated.

However approximate the model is at this time, the observed consistency of our AhR LBD model with the experimental and theoretical data available constitutes an initial framework for analyzing both the ligand-binding mechanism and the ligand-induced effects on structure and functions of the AhR. What clearly emerges from the structural comparison of the modeled binding cavity with the known ligands is that a good steric complementarity exists for PCDDs and closely related HAHs. However, many of the recently identified AhR ligands have structures that are not easily accommodated into the modeled cavity and could suggest that additional key residues for binding of these ligands my also reside at positions in close to the entrance of the proposed binding cavity. Further refinement and testing of the model should allow us to examine this in greater detail. A key question remaining is how ligand binding results in signal transduction. Two possibilities seem reasonable: (1) flexation of the LBD from ligand binding disrupts the LBD–Hsp90 interaction and induces the conformational change that exposes the NLS; or (2) ligand binding induces a refolding event, perhaps destabilizing the unbound conformation while stabilizing the ligand-bound one, which exposes the NLS. The latter hypothesis seems more likely given recent evidence that indicates ligand-dependent nuclear translocation of the intact AhR:hsp90 complex [134,135].

Investigations into the mechanism of ligand-dependent AhR activation may follow two different directions. The first concerns the validation of the hypothesis on PCDD binding formulated on the basis of the LBD model, by using experimental and theoretical methods. Site-directed mutagenesis experiments on the residues proposed to mediate the AhR–PCDD interaction in the binding cavity may give information about their contributions to the formation and stabilization of the ligand–receptor complex. These studies are in progress. In addition, molecular docking calculations may define the orientation of different ligands in the binding cavity on the basis of calculated physicochemical complementarities, thus providing more detailed information about the intermolecular forces involved in the binding process as well as the specificity of this interaction. The second direction requires formulation of some hypotheses about the signal transmission mechanism by the AhR and the possibility that a ligand-dependent activation may occur also without the complete fitting of the ligand in the binding cavity.

In addition to the observed structural and functional similarities between AhR and FixL that constituted the basis for the homology model, the signal-transducing mechanism that was proposed for FixL may be used as a reference mechanism. This was derived from the experimental knowledge of the inactive and active signaling states of FixL [136,137]. The binding of oxygen to the ferrous heme ion is suggested to relay a signal by transducing the increased planarity of the porphyrin ring into conformational changes within the loop immediately following the helical connector. Although the conformation of the corresponding loop in the mAhR model is altered when compared to FixL, some key residues involved in the FixL signal transmission

are well conserved in equivalent structural positions of the mAhR. Interestingly, these residues are also conserved in the same positions in other AhRs and absent in other PAS proteins. Mutagenesis experiments on the AhR helical connector and the following loop may be helpful for testing the idea that the AhR and FixL utilize similar external surfaces to transduce events that occur within the cavity. This work is in progress. Further, since homology models and crystal structures are static, theoretical calculations derived from long time-scale molecular dynamics simulations of the target and the reference structures will be a crucial tool to elucidate and compare the changes in conformation and dynamic properties that accompany ligand binding.

5. Concluding remarks

The AhR, unlike most ligand-dependent receptors can be bound and activated by structurally diverse ligands (reviewed in Ref. [5]). This diversity is clearly evidenced by comparison of the natural AhR ligands described in this review to that of TCDD and other synthetic AhR ligands. Although the existence of a high affinity endogenous ligand(s) for the AhR remains to be confirmed, it is possible that they exist. However, the promiscuous nature of AhR ligand binding combined with the diversity of relative weak endogenous ligands previously identified support the existence of multiple endogenous ligands that can bind to and activate the AhR with varying affinities and efficacies. It is possible that distinct endogenous ligands present in different cell types could activate AhR to a similar degree and thus induce expression of gene products important for a desired biological activity in a cell-specific manner. We envision that the majority of endogenous physiological AhR ligands have relatively weak affinity, compared to TCDD, and are rapidly degraded by the coordinately induced detoxification enzymes and as such, they would act as transient inducers. In fact, experiments suggesting that inhibition of CYP1A1 activity results in accumulation of an endogenous AhR ligand [138] are compatible with this hypothesis. The availability of a variety of sensitive and high throughput AhR-based bioassay systems [100,104,139–141] provide an avenue in which to identify, isolate and characterize other endogenous AhR ligands from biological extracts. Similar approaches have been successfully used to isolate and characterize endogenous ligands for orphan nuclear receptors [142]. These approaches will not only provide us with greater insight into the spectrum of chemicals that can bind to AhR, but subsequent structure–activity analysis should provide clues pertaining to the identification of endogenous AhR ligands.

The availability of a theoretical structure for the AhR LBD provides a model in which to examine both AhR ligand binding and specificity. Site directed mutagenesis coupled with ligand binding experiments will not only allow identification of the key residues involved in ligand binding, but subsequent comparisons of LBDs between species may provide insights into those residues important in species-specific differences in ligand binding. Recently, the X-ray structure of the phototropin module of *Adiantum* PHY3, belonging to the LOV domain subgroup of the PAS

superfamily, was published [143] and a crystal structure determination of the PAS domain of the *A. Vinelandii* NifL protein is in progress [144]. Incorporation of structural information from these PAS proteins will allow further refinement and validation of our theoretical model for the mAhR LBD. Finally, the observation that a mere key-lock complementarity of the ligand within the cavity can not explain the structural diversity of activating ligands suggests the importance of modeling the whole signal transmission mechanism. In fact, the remarkable similarities evidenced by the comparative analysis of the PAS-family proteins suggest a deeper computational study of the dynamic behavior of this domain, targeting the functional similarities.

Further analysis of both endogenous and exogenous AhR ligands, determination of the three-dimensional structure of the AhR and its functional domains, analysis of species differences within the AhR LBD as well as analysis of the molecular mechanisms of ligand-dependent AhR signal transduction are important areas for future research.

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