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Purification and N-Terminal Amino Acid Sequence of the *Ah* Receptor from the C57BL/6J Mouse

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SUMMARY

The *Ah* receptor is a presumed member of the superfamily of steroid/thyroid hormone receptors, a trace soluble protein present in a wide variety of vertebrate species that mediates the biological effects of halogenated aromatic hydrocarbons. In this paper, we report the purification to homogeneity of this protein (from the liver of C57BL/6J mice) and its N-terminal amino acid sequence. Selective covalent labeling of the *Ah* receptor in hepatic cytosol with the photoaffinity ligands 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin simplified identification and quantitation of the receptor and permitted purification under denaturing conditions. Photoaffinity-labeled hepatic cytosol was applied to a phosphocellulose column at 80 mM NaCl, and the fraction enriched with the *Ah* receptor eluted with 225 mM NaCl. The eluate was diluted to 150 mM NaCl and applied to a DEAE-cellulose column, and the enriched fraction eluted with 300 mM. These two ion exchange chromatography steps usually gave ~100-fold enrichment and 40–50% recovery of *Ah* receptor. The dilute protein in the eluate was precipitated with *n*-propanol/trichloroacetic acid and solubilized in formic acid. The sample was then subjected to three successive rounds of high performance liquid chromatography on C4 reverse phase columns. The final, shallow-gradient chromatography was able to resolve the unlabeled 95-kDa receptor protein from the later eluting ^{125}I-photoaffinity-labeled protein. The pooled high performance liquid chromatography fractions subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide gels contained only the 95-kDa band upon staining with Coomassie blue R250 or silver. Using the above protocol, the *Ah* receptor was purified >150,000-fold, to apparent homogeneity, with an overall yield of 3–5%. The N-terminal amino acid sequence of the purified peptide was determined to be ala/asp-ser-Arg-Lys-arg-Lys-Pro-Val-Gln-Lys-Thr-Val-Lys-Pro-Ile-Pro-Ala-Glu-Gly--Ile-Lys-ser-Asn-Pro-ser-Lys- (where the lowercase indicates a residue determined with less confidence).

The *Ah* receptor, a soluble protein found in a variety of tissues of vertebrates, mediates all or nearly all the biological responses produced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons. These responses include induction of cytochrome P-450 isozymes, a wasting syndrome, thymic involution and immune suppression, proliferation and altered differentiation of a variety of epithelial tissues, and tumor promotion (1). For the most extensively studied response, the induction of cytochrome P_1-450 (P-450IA1), it has been shown that the ligand-receptor complex binds to a specific enhancer sequence (i.e., a "dioxin-responsive element") upstream from the *CYPIA1* gene and initiates its transcription (2). It is not known whether the ligand-Ah receptor complex acts as a direct transcriptional activator for all the genes expressed. At present, there is no known endogenous, i.e., physiological ligand for the *Ah* receptor.

The *Ah* receptor is presumed to be a member of the erb-A superfamily of receptors, which includes the steroid hormone, thyroid hormone, and retinoic acid receptors (3). The *Ah* receptor and steroid hormone receptors share many similarities, which include the following. 1) They bind low molecular weight hydrophobic ligands. 2) They have similar ligand-binding kinetics (4, 5). 3) In their unliganded and unactivated form, these receptors are bound to the 90-kDa HSP and exist in a "heteromeric" complex, which is stabilized by MoO_4^{2-} (6–9). 4) For both the heteromeric and "monomeric" (dissociated) forms of these receptor, hydrodynamic parameters (sedimentation coefficients, Stokes radii, molecular weights, and frictional and axial ratios) are within a similar range (10, 11). 5) Upon binding agonists, the receptor-ligand complexes undergo a temperature-dependent activation, dissociate from the 90-kDa HSP, display an increased affinity for specific enhancer elements 5′ to regulated genes, and activate transcription from adjacent promoters [e.g., *CYPIA1* for the *Ah* receptor (12–14) and the vitellogenin gene for the estrogen receptor (15)].

One of the major obstacles to the study of the *Ah* receptor

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ABBREVIATIONS: HSP, heat shock protein; MOPS, 3-(*N*-morpholino)propanesulfonic acid; RP, reverse phase; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; CAPS, 3-(cyclohexylamino)-propanesulfonic acid; PVDF, polyvinylidinedifluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

has been the inability to purify this protein to a degree that would be useful in antibody generation and amino acid sequencing. In this paper, we describe a method for the purification of the *Ah* receptor to homogeneity and report the N-terminal amino acid sequence of this rare protein.

Materials and Methods

Chemicals. Activated charcoal, grade PX-21, was a gift from Amoco Research Corp. (Chicago, IL). Bacto-Gelatin was from Difco Laboratories (Detroit, MI). Glacial acetic acid, trichloroacetic acid, and isopropyl alcohol (all reagent grade) were from Fisher Scientific (Fair Lawn, NJ). Formaldehyde solution, (37%, v/v), stabilized with 10% methanol (v/v), was from Mallincrodt (St. Louis, MO). Silver nitrate was from Amend Drug and Chemical Co. (Irvington, NJ). HPLC-grade acetonitrile, methanol, and *n*-propyl alcohol were from Burdick and Jackson Laboratories, Inc. (Muskegon, MI). Lithium dodecyl sulfate was from Gallard-Schlessinger Industries Inc. (New York). DEAE-cellulose (DE52) was from Whatman (Clifton, NJ). Glycerol and formic acid (88%, v/v) were from J. T. Baker (Phillipsburg, NJ). SDS and ammonium persulfate were from Bethesda Research Laboratories (Gaithsburg, MD). SDS-PAGE molecular weight standards, bromphenol blue, *N,N'*-methylene-bis-acrylamide, and acrylamide (99% pure) were from Bio-Rad (Richmond, CA). Soybean trypsin inhibitor, Coomassie blue-R250, EGTA, EDTA, Tris (free acid and sodium salt), dithiothreitol, β-mercaptoethanol, phosphocellulose (50–150 μm), sodium azide, CAPS (free acid), MOPS (free acid and sodium salt), and Nonidet P-40 were purchased from Sigma Chemical Co. (St. Louis, MO). TFA (99% pure) and dimethyl sulfoxide (anhydrous, 99% pure) were from Aldrich Chemical Co. (Milwaukee, WI). Water used in preparation of buffers was deionized; water used in HPLC and staining of gels was deionized and passed through a Milli-Q reagent water system (Millipore, Bedford, MA).

Buffers. MN represents the stock buffer, which contains 25 mM MOPS and 0.02% sodium azide (w/v), pH 7.5 at 4°. MβENG is the stock buffer plus 10 mM β-mercaptoethanol, 1 mM EDTA, and 10% (v/v) glycerol. Electrophoresis sample buffer was 2% lithium dodecyl sulfate (w/v), 62.5 mM Tris, 12.5% glycerol (v/v), 2 mM EDTA, 0.001% bromphenol blue (w/v), and 20 mM dithiothreitol, pH 6.8 at 4°. CM buffer is 10 mM CAPS and 10% (v/v) methanol, pH 11.0 at 20°.

Synthesis of radioligands. The photoaffinity ligand 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin and the reversible radioligand of the *Ah* receptor 2-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin were synthesized as described previously (15, 16). These radioligands were prepared at specific radioactivities of 2176 Ci/mmol and were essentially pure, as indicated by RP-HPLC.

Animals and cytosol preparation. C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in our laboratory. Adult male and female mice were killed by cervical dislocation and their livers were removed, rinsed with ice-cold KCl (150 mM), homogenized in 9 volumes of MβENG buffer plus 5 mM EGTA, and subjected to centrifugation at 10,000 × *g* for 20 min at 4°. The postmitochondrial supernatant was carefully removed to avoid contamination by the surface lipid layer, and the membrane fraction was pelleted by centrifugation at 105,000 × *g* for 1 hr at 4°. The cytosolic fraction (supernatant) was separated from the surface lipids and microsomal pellet and was stored at −80° until processed further.

Photoaffinity labeling. Cytosol prepared from 200 g of liver (total volume, 2 liters, 8–9 mg of protein/ml) was thawed in a warm water bath (37°) for approximately 1 hr. One twentieth of the cytosol (approximately 100 ml) was then removed and diluted with MENG buffer to 2 mg of protein/ml. The photoaffinity ligand was then added to the diluted cytosolic fraction to a final concentration of 3×10^6 dpm/ml, and the sample was incubated for 30 min at 20°. After incubation, the unbound radioligand was removed by the addition of 10 ml of charcoal/gelatin (final concentration, 1:0.1%, w/v) in MN buffer, followed by mixing with a vortex mixer (5 sec) and incubation at 20° for 10 min. The charcoal was then removed from suspension by centrifugation at 2000 × *g* for 10 min at 4°. The supernatant was then transferred to clean tubes and the remaining fine particulate charcoal was removed by centrifugation at 10,000 × *g* for 10 min at 4°. The supernatant containing the receptor-radioligand complex was transferred to a 150-ml beaker and irradiated at 310 nm, 80 W, at 4 cm, for 1 min, to generate the covalently labeled radioligand-receptor complex. After photolysis, β-mercaptoethanol was added to a final concentration of 10 mM to quench any remaining free radicals. The photoaffinity-labeled fraction was then pooled with the bulk of the cytosol.

Phosphocellulose chromatography. All ion exchange chromatography was performed in a room maintained at 4°. The photoaffinity-labeled pooled cytosol was brought to 80 mM NaCl and loaded onto a phosphocellulose column (10-cm i.d. × 14 cm; column volume, ~1 liter), with a flow rate of 15 cm/hr. After sample loading was complete, the flow rate was increased to 30 cm/hr and the column was washed with MβENG buffer, containing 80 mM NaCl, until the UV absorbance at 280 nm returned to baseline. The *Ah* receptor was then eluted with MβENG buffer plus 225 mM NaCl, with a flow rate of 30 cm/hr. The enriched fraction had a volume of 500 ml.

DEAE chromatography. The 225 mM NaCl eluate from the phosphocellulose column was diluted with MβENG buffer, to an ionic strength equal to 165 mM NaCl in MβENG, and loaded onto a DEAE column (5-cm i.d. × 13 cm; column volume, ~250 ml), with a flow rate of 30 cm/hr. The column was washed with MβENG buffer, containing 165 mM NaCl, until the UV absorbance at 280 nm returned to baseline. The receptor was then eluted with MβENG buffer, containing 300 mM NaCl, at a flow rate of 30 cm/hr. The total fraction volume collected, approximately 100 ml, was then stored at −80° until processed further.

RP-HPLC. All RP-HPLC was performed at 56°, using C4 silica-based columns (Vydac 214TP series; The Separations Group, Hesperia,

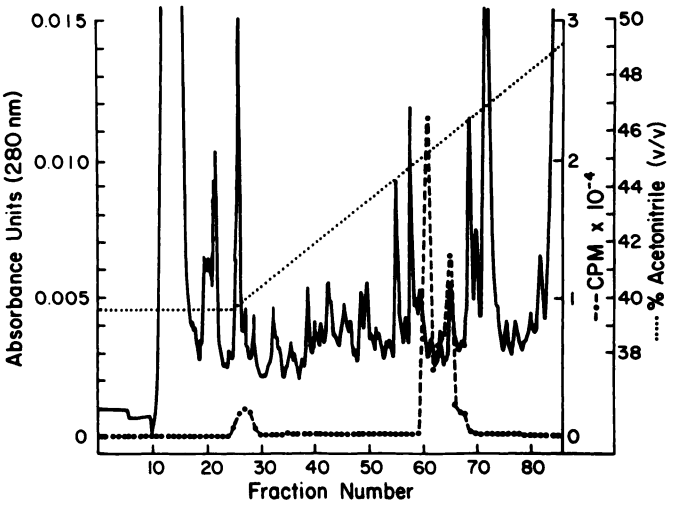

Fig. 1. Resolution of the 95- and 70-kDa receptor species by RP-HPLC. To a fraction of the sample enriched by ion exchange chromatography, containing 1.2 mg of protein, was added lithium dodecyl sulfate and β-mercaptoethanol, each to a final concentration of 2%, and the solution was heated at 56° for 30 min. The sample was then precipitated by addition of *n*-propanol/trichloroacetic acid, to a final concentration of 20:0.1% (v/v), and heating (56°, 2 min). The precipitate was collected by centrifugation at 2,000 × *g* for 20 min, solubilized in formic acid (0.5 ml/mg of protein), diluted with 10 volumes of equilibration solvent (water/acetonitrile/TFA, 60.4:39.5:0.1), and filtered through a 0.45-μm membrane (Duropore; Millipore). The sample was then loaded onto a semi-preparative C4 column (1-cm i.d. × 25 cm, 5-μm particle size), at a flow rate of 2 ml/min, and eluted using a gradient of acetonitrile in water (TFA as ion-pairing agent), with the column temperature held at 56°. The radioactivity in each 2-ml fraction was quantified by γ scintillation counting. (The small peak of radioactivity at fractions 25–29 is not precipitated by acetone and presumably is a radioligand species not bound to protein.) ——, UV absorption 280 nm; – · –, radioactivity (cpm × 10^{-4}).

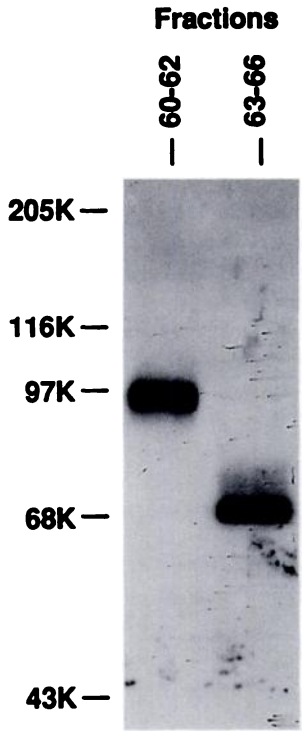

Fig. 2. Analysis of RP-HPLC fractions by autoradiography. The two peaks of radioactivity from the RP-HPLC run depicted in Fig. 1 were analyzed by SDS-PAGE and autoradiography. Five hundred-microliter aliquots of each fraction were pooled (early peak = fractions 60–62; late peak = fractions 63–66), precipitated with 10 ml of ice-cold acetone overnight in the presence of 100 μg of soybean trypsin inhibitor, solubilized in electrophoresis sample buffer, and analyzed by SDS-PAGE and autoradiography (see Materials and Methods).

CA) in line with cartridge precolumns (Hi-Pore Guard C4, 4.6 × 30 mm; Bio-Rad). The HPLC hardware consisted of two model 510 pumps interfaced with a microprocessor gradient control unit (Waters, Milford, MA). For specific conditions, see the legends to Fig. 1, 4, and 5.

SDS-PAGE, staining, and autoradiography. The efficiency of photoaffinity labeling and estimation of recoveries and purification factors were determined as follows: 100 μg of soybean trypsin inhibitor, as carrier protein, were mixed with the labeled sample and precipitated with 9 volumes of ice-cold acetone overnight at 4°. The protein pellet was collected by centrifugation (2000 × *g* for 10 min), washed with 1 ml of ice-cold acetone/water (9:1), and dissolved in electrophoresis sample buffer. The samples were then subjected to denaturing electrophoresis on discontinuous slab gels (3% stacking gel, 7.5% separating gel; acrylamide/bisacrylamide ratio = 37.5:1), at 0.7 mA/cm² for 16 hr at 4° (17). The gels were routinely fixed with methanol/acetic acid, stained with Coomassie blue R250 (18) or silver (19), dried, and placed on top of a sheet of preflashed XAR-5 film (Kodak Chemical Co., Rochester, NY) backed by an intensifying screen (Cronex Lightning Plus, E. I. Dupont de Nemours Inc., Wilmington, DE), and the film was exposed for a period of 5 to 24 hr at −60° before developing. The 95- and 70-kDa bands were identified in the dried gels by autoradiography and excised, and the radioactivity was quantified by a γ scintillation counting.

Protein determination. Protein concentrations were determined by the method of Warburg and Christian (20). The protein concentration after electrophoresis and brilliant blue-R staining was quantified by laser scanning densitometry, using phosphorylase *b* from the molecular weight standard mix as reference protein.

Amino acid sequencing. The 95-kDa receptor species that had been electrotransferred to a PVDF membrane (21) (see legend to Fig. 6) was carefully excised and stored at −20°. The protein sample was sequenced directly from PVDF, using an ABI477 liquid pulse sequencer (Applied Biosystems, Foster City, CA). These analyses were performed at the University of Wisconsin Biotechnology Center, using protocols described previously (22).

Results

Calculations. Published data from this laboratory indicate that the *Ah* receptor is present in C57BL/6J hepatic cytosol at a concentration of 100 fmol of ligand binding sites/mg of protein (11) and that, during tissue homogenization, as much as 40% of the parent 95-kDa receptor protein can be proteolyzed to yield a 70-kDa fragment (16). Thus, we estimate there are 5.8 and 2.8 ng/mg of cytosolic protein of the 95- and 70-kDa species, respectively.[1] Therefore, to purify the 95-kDa protein to homogeneity requires an enrichment of 170,000-fold and the 70 kDa fragment requires a factor of 360,000-fold.

Preliminary experiments. We examined the chromatographic behavior of the photoaffinity-labeled *Ah* receptor on ion exchange resins using gradient elution. The *Ah* receptor from C57BL/6J hepatic cytosol eluted from a phosphocellulose column as a single sharp peak at 170 mM NaCl and from a DEAE-cellulose column as a single sharp peak at 225 mM NaCl. Receptor yields were routinely 75% and purification factors were 10–20-fold for each column run independently.

We examined the behavior of the *Ah* receptor on C4 reverse phase HPLC columns. In these experiments, the enriched preparation after ion exchange chromatography was loaded onto a C4 column and eluted with various organic solvents (i.e., acetonitrile, *n*-propanol, methanol, or ethanol) and ion-pairing

[1] Calculations assume that there is one ligand binding site/molecule of receptor.

TABLE 1

Purification of the *Ah* receptor: receptor enrichment by ion exchange chromatography

Values presented are means ± standard deviations from five determinations.

Fraction	Protein	95-kDa band			70-kDa band		
		Specific activity[a]	Recovery[b]	Purification[b]	Specific activity[a]	Recovery[b]	Purification[b]
	mg	*dpm in 95-kDa band/mg of protein*	%	*fold*	*dpm in 70-kDa band/mg of protein*	%	*fold*
Cytosol	16,800 ± 300	320 ± 70[c]	100	1	190 ± 60[c]	100	1
Phosphocellulose	750 ± 270	4,000 ± 1,300	56 ± 8	12 ± 3	2,500 ± 1,000	59 ± 10	13 ± 4
DEAE	75 ± 12	33,700 ± 7,700	47 ± 8	105 ± 14	18,600 ± 6,500	44 ± 5	98 ± 15

[a] The specific activity of a given fraction was estimated after resolution of 200 μg of protein by SDS-PAGE, identification of the radiolabeled bands by autoradiography, excision of the labeled bands, and determination of the dpm present in the 95- and 70-kDa bands by γ scintillation counting. Specific activity has been corrected for radiodecay during purification.

[b] All estimates of recoveries and purification factors were calculated relative to hepatic cytosol.

[c] The specific activity of "cytosolic" 95- and 70-kDa bands after dilution with a 20-fold excess of unlabeled cytosolic protein. The specific activity of the undiluted cytosolic sample was 6400 and 3800 dpm/mg of protein for the 95- and 70-kDa species, respectively.

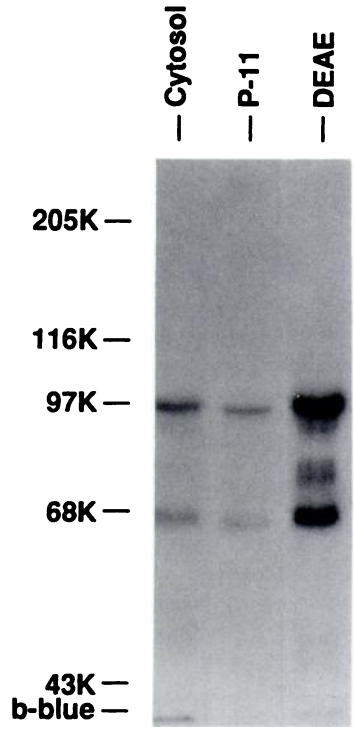

Fig. 3. Analysis of ion-exchange chromatography by autoradiography. Samples from each purification step were precipitated with acetone, dissolved in electrophoresis sample buffer, and subjected to SDS-PAGE. The 95- and 70-kDa species were identified by autoradiography and quantified by γ scintillation counting (see Material and Methods). *Cytosol*, an aliquot photoaffinity labeled before dilution with a 19-fold excess of unlabeled cytosol. *P-11*, the protein fraction that eluted from phosphocellulose between 80 and 225 mM NaCl. *DEAE*, the protein fraction that eluted from DEAE-cellulose between 150 and 300 mM NaCl.

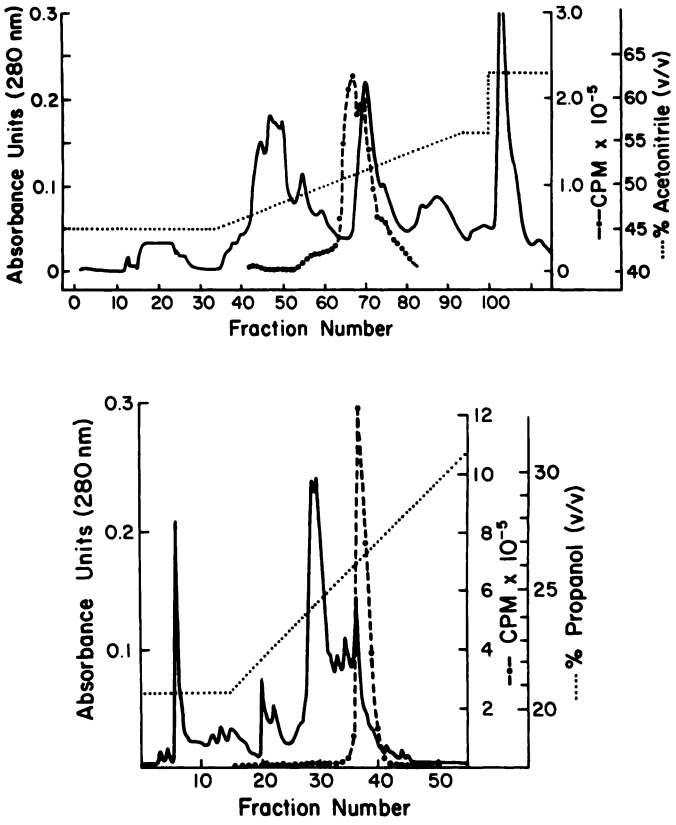

Fig. 4. RP-HPLC purification of the 95-kDa *Ah* receptor. *Upper*, preparative HPLC step. A frozen aliquot of ion exchange-purified receptor, equal to 50 mg of protein, was prepared for RP-HPLC, essentially as described in the legend to Fig. 1. The sample was loaded, at a flow rate of 5 ml/min, onto a preparative C4 column (2.2-cm i.d. × 25 cm, 15–20-μm particle size), and the column was washed with equilibration solvent until the UV absorbance at 280 nm returned to baseline (approximately fraction 30). The 95- and 70-kDa species were eluted with a gradient of acetonitrile in water (0.1% TFA as ion-pairing agent). Fractions corresponding to the 95- (early peak, fractions 63–68) and 70-kDa (late peak, fractions 69–74) receptor species were pooled. *Lower*, second RP-HPLC step. Pooled fractions from two runs of the preparative HPLC column, which contained the 95-kDa species (identified by SDS-PAGE), were diluted with 0.5 volume of column equilibration solvent (water/*n*-propanol/formic acid, 70:21.2:8.8) and loaded, at a flow rate of 3 ml/min, onto a semipreparative C4 column (1-cm i.d. × 25 cm, 5 μm particle size). After loading, the column was washed with equilibration solvent until the UV absorbance at 280 nm returned to baseline (approximately fraction 10). The 95-kDa receptor was eluted, at a flow rate of 2 ml/min, with a gradient of *n*-propanol in water, using 8.8% formic acid (v/v) as ion-pairing agent. The 95-kDa receptor species eluted in fractions 34–41.

agents (i.e., formic acid or TFA). RP-HPLC using water/acetonitrile with TFA resolved two distinct peaks of radioactivity (Fig. 1, fractions 60–62 and 64–66). Analysis of these two peaks by SDS-PAGE and autoradiography revealed that the early eluting peak corresponded to the 95-kDa receptor and that the later peak was the 70-kDa proteolytic product (Fig. 2). A comparison of the elution profiles generated using different solvent systems indicated that water/acetonitrile – with TFA and water/*n*-propanol – with formic acid displayed markedly different elution profiles for the *Ah* receptor, relative to other proteins in the preparation (data not shown).

Purification. Photoaffinity labeling of the *Ah* receptor average 6400 dpm/mg of protein for the 95-kDa protein and 3800 dpm/mg for the 70-kDa proteolytic product (approximately 2 fmol of photoaffinity ligand bound to receptor/mg of cytosolic protein). Assuming 100 fmol of receptor/mg of protein (see above), this is equivalent to labeling 2% of total receptor. We routinely labeled a fraction of the cytosolic protein (1/20th) and then added this back to the bulk of the cytosolic protein, to yield a preparation with a specific activity of 320 and 190 dpm/mg for the 95- and 70-kDa proteins, respectively. After phosphocellulose and DEAE-cellulose chromatography, the specific activity was increased 100-fold, with a recovery of 46% (Table 1). A typical autoradiogram for these steps is shown in Fig. 3.

Because attempts at further purification of this 100-fold-enriched fraction using nondenaturing means were unsuccessful, we chose to proceed with the purification using denaturing conditions. To reduce the protein mass, we chromatographed the 100-fold-enriched material on a preparative RP-HPLC column (2.2-cm i.d. × 25 cm) with a large particle size (15–20 μm). Using a linear gradient of acetonitrile in aqueous TFA (rate of change for acetonitrile = 0.18%/cm/min), the 95-kDa receptor species eluted at 51.2% acetonitrile and the 70-kDa species eluted at 52% acetonitrile. Although the resolution was inferior to that obtained with smaller particle size columns (compare the resolution of the two peaks of radioactivity in Figs 1 and 4, *upper*), use of the preparative column reduced protein approximately 20-fold and provided nearly complete resolution of the 95- and 70-kDa species. After multiple runs on the preparative HPLC column, fractions containing the 95-kDa species were pooled and purified further on a semipreparative column (1-cm i.d. × 25 cm) with a particle size of 5 μm

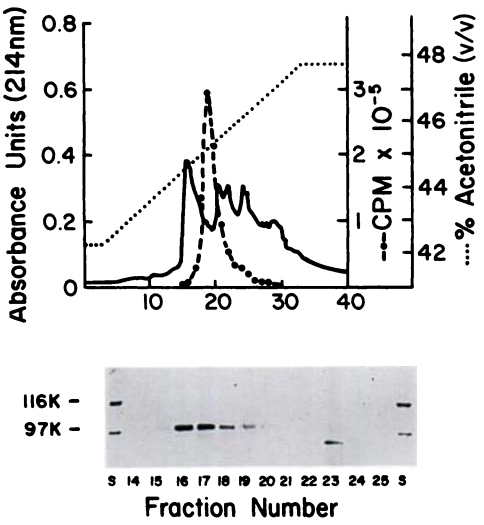

Fig. 5. *Upper*, Final RP-HPLC purification step. Fractions from the semi-preparative HPLC column (Fig. 4, *lower*), which contained the 95-kDa species, were pooled, diluted with 0.5 volume of 0.1% TFA, and loaded, at a flow rate of 1.5 ml/min, onto an analytical C4 column (4.6-mm i.d. × 25 cm, 5-μm particle size) that had been equilibrated in water/acetonitrile/TFA (57.6:42.3:0.1). After the sample had been loaded, the column was washed with equilibration solvent until the UV absorbance at 214 nm returned to baseline (the flow-through is not shown, due to the excessive absorbance of formic acid at 214 nm). The 95-kDa receptor species was eluted with a gradient of acetonitrile in water, using 0.1% TFA as ion-pairing agent. *Lower*, Analysis of final HPLC run by SDS-PAGE. Fifty-microliter aliquots from fractions 14 through 25 were brought to 0.1% (v/v) Nonidet P-40 and dried under vacuum (Speed Vac concentrator; Savant Instruments, Mount Prospect, IL). The sample was then dissolved in 100 μl of electrophoresis sample buffer and subjected to SDS-PAGE, and the gel was stained with silver (see Materials and Methods). *S*, lane in which were loaded 50 ng each of the standards phosphorylase *b* (97 kDa) and β-galactosidase (116 kDa).

(Fig. 4, *lower*). Using a linear gradient of water/*n*-propanol, with formic acid as a modifier (rate of change for *n*-propanol = 0.1%/cm/min), the 95-kDa receptor eluted as a sharp peak at 26.3% *n*-propanol.

The final HPLC step was performed on an analytical column (4.6-mm i.d. × 25 cm) column with a particle size of 5 μm, using a shallow linear gradient of acetonitrile in aqueous TFA (rate of acetonitrile change = 0.06%/cm/min). The elution of the 95-kDa receptor was monitored by counting the radioactivity present in the fractions (Fig. 5, *upper*) and by subjecting an aliquot of each fraction to SDS-PAGE and analysis by silver staining and autoradiography (Fig. 5, *lower*). As seen in Fig. 5, fraction 19 contains the peak of radioactivity, but fraction 16 contains the most intense silver-staining band at 95 kDa. For those fractions that had silver-staining material and significant radioactivity, the autoradiographic signal superimposed exactly over the silver-stained band at 95 kDa (data not shown). Thus, we concluded that we can separate the unliganded receptor from the photoaffinity-labeled *Ah* receptor under the conditions employed in this final chromatography step (see Discussion).

HPLC fractions that contained the peak of the 95-kDa protein (as determined by silver staining) were pooled, subjected to SDS-PAGE, and electrotransferred to a PVDF membrane. The 95-kDa band was visualized on the membrane by staining with Coomassie blue R250, and the quantity of this protein was estimated by a comparison of staining intensities with known quantities of phosphorylase *b* (Fig. 6). A typical experiment yielded 3–5 μg of the 95-kDa receptor from 10 g of cytosolic protein (Table 2). Final recoveries and purification factors were calculated by estimation of the protein in the 95-kDa Coomassie-stained band using laser densitometry. This method indicated a purification factor of 180,000-fold, with an overall recovery of 5%. This purification factor is identical to that estimated theoretically (see above).

Amino acid sequencing. Three separate samples of purified receptor, which ranged in mass from 2.5–5 μg (25–50 pmol), were submitted for direct amino acid sequencing by Edman degradation chemistry on a liquid phase sequencing apparatus. Each sample yielded an N-terminal residue signal that was within 30% of the quantity of receptor estimated by Coomassie blue staining and laser densitometry (e.g., a sample that yielded a 35-pmol alanine signal at cycle 1 was judged to be 50 pmol, or 5 μg, by densitometry). The consensus sequence from three separate sequencing runs is presented in Fig. 7.

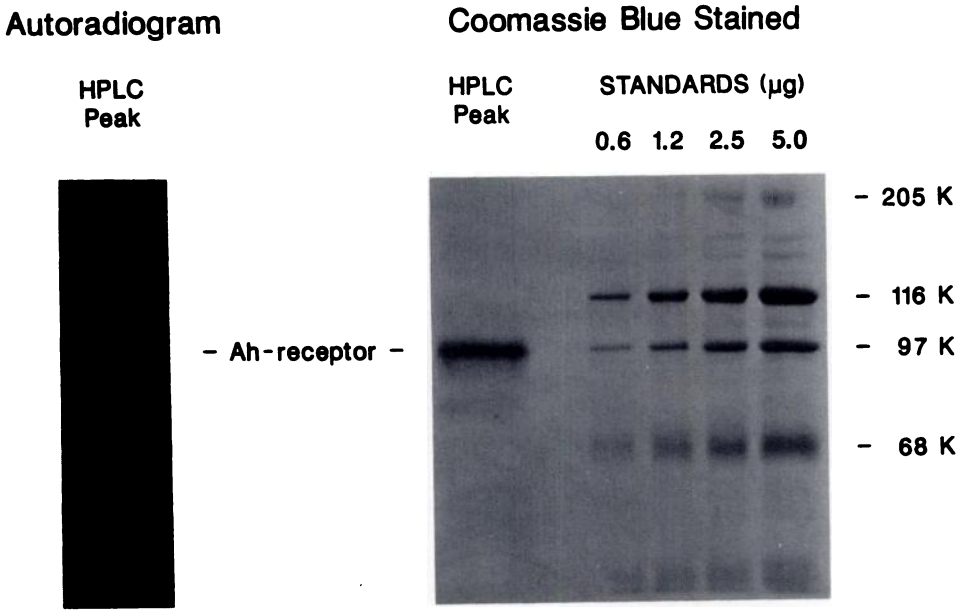

Fig. 6. Electroblot of the purified *Ah* receptor on PVDF. The *Ah* receptor from HPLC fractions 16–19 (Fig. 5) was pooled, brought to 0.1% Nonidet P-40, dried under vacuum, and solubilized in 200 μl of electrophoresis sample buffer. The sample was subjected to SDS-PAGE and then transferred from the polyacrylamide gels by the method of Matsudaira (21), using a TRANS-BLOT electrophoretic transfer cell (Bio-Rad). Electrotransfer onto PVDF membranes was performed at room temperature for 3 hr in CM buffer, with a current of 300 mA. Following transfer, the gel was washed in water for 5 min, and the protein was stained with 0.1% (w/v) Coomassie blue-R250 and 50% methanol (v/v) for a period of 5 min. The gel was then destained in 50% methanol (v/v), 10% acetic acid (v/v), for 10 min and washed in water for an additional 10 min. The autoradiogram was obtained by exposure of the PVDF membrane to film for a period of 16 hr (see Materials and Methods).

TABLE 2

Purification of the 95-kDa *Ah* receptor: final purification by RP-HPLC, SDS-PAGE, and electrotransfer to a PVDF membrane

Fraction	Protein	Specific activity[a]	Recovery[b]	Purification[b]
	mg	*dpm in 95-kDa band/mg of protein*	%	*fold*
DEAE[c]	105	3×10^4	51	120
Preparative/Semipreparative HPLC	ND[d]	ND	23	ND
Analytical HPLC	ND	ND	6	ND
SDS-PAGE & Transfer to PVDF	0.005	2.7×10^7	3–5	1.8×10^5

[a] The specific activity of the ion exchange-purified material was estimated as described in the legend to Table 1. The specific activity of the purified receptor was determined by quantitation of the radioactivity present in the 95-kDa protein blotted onto PVDF. This specific activity value was then corrected to account for the fraction of radioactivity that was removed by RP-HPLC (typically by multiplication by a factor of 1.4 to account for the removal of 30% of the radioactivity by HPLC).

[b] An estimate of recovery was determined by quantitation of the receptor by Coomassie blue staining of the protein blotted onto PVDF.

[c] This preparation is a subset of the values presented in Table 1 and, thus, the purification factors and recoveries are not identical to the mean values from five determinations presented in Table 1.

[d] ND, not determined.

ala/asp-ser-Arg-Lys-arg-arg-Lys-Pro-Val-Gln-Lys-Thr-Val-
Lys-Pro-Ile-Pro-Ala-Glu-Gly-Ile-Lys-ser-Asn-Pro-ser-Lys

Fig. 7. N-Terminal amino acid sequence of the *Ah* receptor from C57BL/6J mouse hepatic cytosol. This sequence was the consensus of three independent experiments. The residues determined with lower confidence are presented in lower case.

Discussion

In this paper, we describe the purification and N-terminal amino acid sequence of the *Ah* receptor from C57BL/6J mouse liver. Purification of this trace protein was made possible by covalent labeling with the ^{125}I-photoaffinity ligand. Starting with hepatic cytosol, the entire purification scheme was routinely completed in 5 working days. The yield of purified *Ah* receptor was 3 to 5%. The preparation appeared homogeneous upon analysis by SDS-PAGE (silver staining, Coomassie blue staining, and autoradiography) and by direct sequencing of its N-terminus by Edman degradation chemistry.

An earlier report from this laboratory described the partial purification of the *Ah* receptor, as well as the utility of both photoaffinity labeling and RP-HPLC in receptor isolation (23). Unfortunately, receptor generated by this earlier protocol was not of sufficient purity for amino acid sequencing studies. Additionally, use of this crude preparation as an immunogen did not result in the production of antibodies specific for the *Ah* receptor, but rather for the 90-kDa HSP (6).

One obstacle to receptor purification has been that the *Ah* receptor elutes from most ion exchange chromatography media as multiple species. This chromatographic heterogeneity, which has been well documented using *Ah* receptor prepared from rat hepatic cytosol (24, 25), may be the result of the existence of both free receptor and receptor complexed with other proteins. In contrast to *Ah* receptor prepared from many rodent models, we found that the *Ah* receptor from the C57BL/6 mouse eluted from a variety of ion exchange resins as a single sharp peak, in high yield. The ability to perform high yield purification of the *Ah* receptor using ion exchange chromatography allowed us to generate and stockpile large quantities of 100-fold-purified receptor for use in subsequent purification steps.

Further purification of the *Ah* receptor was accomplished under denaturing conditions, using RP-HPLC. As seen in Fig. 1, the 95-kDa receptor was easily resolved from its 70-kDa proteolytic products on a C4 RP column. Remarkably, using an extremely shallow acetonitrile gradient, the bulk unlabeled 95-kDa protein could be partially resolved from the later eluting ^{125}I-photoaffinity-labeled 95-kDa receptor (0.1% of total, as seen in Fig. 5). Several experiments confirm this capacity of RP-HPLC to resolve the unliganded and liganded species. Repeating the chromatography with *Ah* receptor from C3H/HeJ mouse liver (104-kDa protein) and from Sprague Dawley rat liver (105-kDa protein), we observed the unlabeled protein eluting before the photoaffinity-labeled species, which had the same mobilities on SDS-PAGE. We raised polyclonal antisera against a synthetic peptide hapten corresponding to the N-terminal amino acid sequence obtained for the bulk unlabeled 95-kDa peptide. Immunoaffinity-purified antibodies immunochemically stain, on Western blots, the ^{125}I-photoaffinity-labeled *Ah* receptor from a variety of vertebrate species (26). It should be noted that a number of reports in the literature describe the capacity of RP-HPLC to resolve proteins with minor structural differences. For example, using columns and eluants similar to those reported here, hemoglobin and interleukin-2 variants differing by a single amino acid are completely resolved (27, 28). Even a modification as subtle as the oxidation of a single methionine in interleukin-2 can alter column retention time.

To minimize the possibility that low molecular weight contaminants were present in the receptor preparation, we routinely subjected the RP-HPLC-purified sample to a final purification on SDS-PAGE before amino acid sequencing.[2] Following electrophoresis, the receptor was electroblotted onto PVDF membranes, localized with Coomassie blue stain, and then sequenced directly from this membrane. Confirmation that the N-terminal sequence presented in Fig. 7 is in fact that of the *Ah* receptor comes from the observation that polyclonal antiserum raised against a synthetic peptide corresponding to residues 3 through 22 of this sequence (Arg–Lys, Fig. 7) cross-reacts with the *Ah* receptor from a wide variety of experimental animals and immunoprecipitates these receptors from solution. A complete description of the development of this antiserum and its characterization is described in the accompanying paper (26).

[2] Recently, we have found that purification by SDS-PAGE is not required before sequencing.

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