Partial Purification of a Binding Protein for Polychlorinated Biphenyls from Rat Lung Cytosol: Physicochemical and Immunochemical Characterization†

Johan Lund,‡ Lena Nordlund, and Jan-Åke Gustafsson*
Department of Medical Nutrition, Karolinska Institute, Huddinge University Hospital F69, S-141 86 Huddinge, Sweden
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ABSTRACT: A binding protein for certain methyl sulfone metabolites of polychlorinated biphenyls (PCB) was partially purified from lung cytosol of untreated female rats. The protein has an M_r of 13 000 and a p*I* of 5.3 in the absence of reducing agents. In the presence of dithioerythreitol or β-mercaptoethanol, the protein is split into subunits with a more basic p*I*. The 13-kDa protein was electroeluted from SDS–polyacrylamide gels, and an antiserum against the protein was raised in rabbit. The immunoglobulin fraction was shown to contain monospecific antibodies against the 13-kDa protein as determined by Western immunoblots. The antibodies retained partially purified binding protein labeled with radioactive ligand when subjected to protein A–Sepharose chromatography and caused a shift in the elution of the labeled protein from Sephadex G-75 and a shift in its sedimentation behavior on sucrose gradients. Due to striking similarities in physicochemical characteristics of the 13-kDa protein and a protein purified from rabbit lung and uterus, uteroglobin, the anti 13-kDa protein antibodies were tested for cross-reactivity. As judged by Western immunoblots, the anti 13-kDa protein antibodies did not cross-react with uteroglobin and the two proteins, although similar, do not seem to be identical. The 13-kDa protein is proposed to be responsible for the accumulation of certain methylsulfonyl-PCBs in lung tissue of rats. Monospecific antibodies against the 13-kDa protein should constitute immunochemical probes of great value in attempts to elucidate the physiological role of the protein as well as its possible role in PCB-induced respiratory toxicity.

Polychlorinated biphenyls (PCBs) are industrial chemicals that, due to their chemical and environmental stability as well as their extensive use, have come to contaminate virtually the whole ecosystem [for a review, see Kimbrough (1980)]. The metabolism of the individual isomers of the PCB mixtures proceeds via arene oxide intermediates (Preston et al., 1984) and results in hydroxylated products [reviewed in Safe (1984)] as well as sulfur-containing metabolites such as methyl sulfides and methyl sulfones (Mio et al., 1976; Jensen & Jansson, 1976). In the latter case, the PCB arene oxide reacts with glutathione and during enterohepatic circulation the conjugate is sequentially transformed to PCB thiols that can be reabsorbed, methylated, and oxidized to the corresponding PCB methyl sulfones (Bakke et al., 1982; Bakke & Gustafsson, 1984). This metabolic transformation requires the presence of intact intestinal microflora containing C–S lyase activity (Bakke & Gustafsson, 1984). Depending on the positions of the chlorine atoms and the methylsulfonyl moieties, certain PCB methyl sulfones may accumulate in the lung and kidney of rats and mice (Brandt & Bergman, 1981).

Recently, 4,4′-bis([^{3}H]methylsulfonyl)-2,2′,5,5′-tetrachlorobiphenyl [([^{3}H]$MeSO_2$)$_2$TCB][1] was shown to selectively accumulate in the apical cytoplasm of nonciliated bronchiolar (Clara) cells of the rat and mouse lung (Lund et al., 1985; Brandt et al., 1985). This selective in vivo disposition is seemingly due to the presence in rat and mouse lung cytosol of a low molecular weight, acidic protein with a high affinity and capacity for binding ([^{3}H]$MeSO_2$)$_2$TCB and certain other PCB methyl sulfones (Lund et al., 1985). The protein appears to be of a secretory nature, and following in vivo administration of ([^{3}H]$MeSO_2$)$_2$TCB, ~30% of the radioactivity present in the lung was recovered in bronchoalveolar lavage fluid associated with the binding protein (Brandt et al., 1985). A binding protein for ([^{3}H]$MeSO_2$)$_2$TCB in bronchoalveolar lavage from healthy human subjects, displaying similar physicochemical characteristics, has also been identified (Lund et al., 1986).

In view of the fact that respiratory disorders have been described for human subjects accidentally or occupationally exposed to PCBs (Shigematsu et al., 1978; Warshaw et al., 1979) and the fact that more than 60 different methylsulfonyl-PCBs have been identified in lungs from accidentally exposed subjects (Haraguchi et al., 1984; 1986), the pathway for the accumulation of methylsulfonyl-PCBs described above may be of toxicological significance. Recent work demonstrating a decreased pulmonary drug metabolism in mice treated with 4-(methylsulfonyl)-2,2′,5,5′-tetrachlorobiphenyl (Lund, B. O., et al., 1986) lends further support to this notion. The present investigation describes the partial purification and immunochemical characterization of the PCB-binding protein from rat lung cytosol as a first step toward the elucidation of the physiological role of the protein as well as of its possible role in PCB-induced pulmonary toxicity.

Experimental Procedures

Chemicals. ([^{3}H]$MeSO_2$)$_2$TCB (specific activity 4 Ci/mmol) and unlabeled ($MeSO_2$)$_2$TCB were kind gifts from Drs. Å. Bergman, E. Klasson-Wehler, and C. A. Wachtmeister, Wallenberg Laboratory, Stockholm University, Stockholm, Sweden (Klasson-Wehler et al., 1983). Sephadex G-75,

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* Author to whom correspondence should be addressed.
‡ Recipient of a research fellowship from the Swedish Cancer Society.

[1] Abbreviations: PCB, polychlorinated biphenyl; ($MeSO_2$)$_2$TCB, 4,4′-bis(methylsulfonyl)-2,2′,5,5′-tetrachlorobiphenyl; *o,p′*-DDD, 1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IFPAG, isoelectric focusing in polyacrylamide gels; Ig_{13K}, monospecific antibodies to the M_r 13 000 protein; Ig_{16K}, antibodies to the M_r 16 000 protein; Ig_N, immunoglobulin fraction from preimmune rabbit serum; p*I*, isoelectric point.

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DEAE-Sepharose, CM-Sepharose, Dextran T-70, and protein A–Sepharose were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). ^{125}I-Labeled protein A and rabbit reticulocyte lysate were purchased from New England Nuclear (Boston, MA). [^{35}S]Methionine and ^{14}C-labeled protein standards were obtained from Amersham (Buckinghamshire, England). Pansorbin was purchased from Calbiochem, La Jolla, CA. Scintillator 299 was obtained from Packard Instruments Co. (Downers Grove, IL). Uteroglobin from rabbit uterus was a kind gift from Dr. E. Milgrom, INSERM U 135, Bicêtre, France, and Dr. O. Jänne, The Population Council, New York. All other chemicals were analytical grade products from Sigma Chemical Co. (St. Louis, MO) or Merck A.G. (Darmstadt, FRG).

Animals. Female Sprague–Dawley rats (200 g) obtained from Anticimex, Stockholm, Sweden, were used.

Buffers. Buffer A contained 25 mM sodium acetate and 1 mM EDTA, pH 4.5. Buffer B contained 20 mM potassium phosphate and 1 mM EDTA, pH 7.2.

Preparation of Lung Extract. Rats (50 per purification) were decapitated to ensure thorough exsanguination. The lungs were removed and placed in ice-cold redistilled H_2O. All successive work was carried out at 0–4 °C unless otherwise indicated. The lungs were first homogenized in a blender (3 mL of redistilled H_2O/g of tissue) and then in a large, tight-fitting Teflon/glass Potter–Elvehjem homogenizer. The homogenate was centrifuged at 105000*g* for 60 min. The supernatant was removed (~180 mL from 50 rats), frozen in an ethanol/dry ice bath, and then lyophilized. The dry protein was then resuspended in 80 mL of buffer A and incubated with 5×10^6 dpm ([^{3}H]$MeSO_2)_2$TCB as a tracer for 15 min at 37 °C. The incubation mixture was then centrifuged at 1500*g* for 20 min to pellet precipitated material. The supernatant (~70 mL), hereafter termed labeled lung extract, was used for purification of the PCB-binding protein.

Purification of the PCB-Binding Protein. The labeled lung extract was first applied to a CM-Sepharose column (4 × 27 cm), equilibrated in buffer A, at a flow rate of 4.6 mL cm^{-2} h^{-1}, and 9-mL fractions were collected. The column was then washed with buffer A at a flow rate of 15 mL cm^{-2} h^{-1}. Aliquots (0.5 mL/fraction) were removed for liquid scintillation counting, and the radioactive peak fractions were pooled (~35 mL) and concentrated by ultrafiltration with an Amicon cell fitted with a YM-2 membrane (Amicon Corp., Lexington, MA). The concentrate (~6 mL) was applied to a Sephadex G-75 column (2.6 × 58 cm) equilibrated in buffer B and chromatographed at a flow rate of 7 mL cm^{-2} h^{-1}. Fractions of 2.5 mL were collected, and aliquots (0.1 mL) were removed for determination of radioactivity. The pooled peak fractions from the gel permeation chromatography (~25 mL) were applied to a DEAE-Sepharose column (1.7 × 3.2 cm), equilibrated in buffer B, at a flow rate of 20 mL cm^{-2} h^{-1}. Fractions of 2.5 mL were collected. The column was washed with buffer B, and the protein was then eluted with a linear 0–150 mM NaCl gradient. Aliquots were removed for liquid scintillation counting, and the pooled peak fractions (~15 mL) were dialyzed overnight against redistilled H_2O, lyophilized, and finally taken up in a small volume of redistilled H_2O. The final preparation, as well as aliquots that had been removed at various stages of purification, was then analyzed by an in vitro binding assay (Lund et al., 1985) to determine the maximal number of binding sites for ([^{3}H]$MeSO_2)_2$TCB.

SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE). Polyacrylamide slab gel electrophoresis was performed in the presence of SDS essentially as described by Laemmli (1970) on a Bio-Rad Protean apparatus (Richmond, CA). The gels were linear gradient gels, 8–18% and 8–25%, formed in an LKB 2001–500 gradient gel former (LKB, Bromma, Sweden). Gels were stained with Coomassie brilliant blue; stained gels were scanned in a Beckman R-112 densitometer at 600 nm with slit 5 × 0.4 mm.

Isoelectric Focusing in Polyacrylamide Gels (IFPAG). Ready-made polyacrylamide gels with pH range 3–9 (LKB, Bromma, Sweden) were used, and isoelectric focusing was performed as previously described (Lund et al., 1985).

Antigen Preparation and Immunization. Coomassie-stained protein bands were cut out of the SDS–PAGE gels, and the proteins were electroeluted as described by Strålfors and Belfrage (1983). The electroeluted protein (100 μg/0.5 mL) was emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously at multiple sites on the back of rabbits (100 μg of protein per animal). Two 1-year-old female mixed-breed rabbits were immunized with the 13-kDa and 16-kDa proteins, respectively (see Results). The rabbits had been bled prior to immunization to obtain preimmune serum. The rabbits were booster injected 3–4 times at 3-week intervals with 25–50 μg of protein in Freund's incomplete adjuvant. Blood was collected 1 week after each booster injection, and serum was prepared.

Preparation of Antibodies. Immunoglobulins (Ig) were prepared by adsorption of serum onto a column of protein A–Sepharose (1.5 × 5 cm) equilibrated in sodium phosphate buffer (50 mM, pH 7.4). After the column was washed with several column volumes of phosphate buffer, the Ig fraction was eluted with 0.1 M acetic acid. The fractions containing Ig were pooled and dialyzed overnight against sodium phosphate buffer (50 mM, pH 7.4). Approximately 7 mL of serum was used for each purification of Ig.

Western Blot Immunoassay. Proteins from SDS–PAGE were blotted onto nitrocellulose filters and detected immunologically as described previously (Haaparanta et al., 1983). Antibody–antigen complexes were visualized by reaction with ^{125}I-labeled protein A and autoradiography.

RNA Isolation, Cell-Free Translation, and Immunoprecipitation of Radiolabeled 13-kDa Protein. Total RNA was prepared from female rat lungs by homogenization in 4 M guanidinium thiocyanate and centrifugation in cesium chloride (Chirgwin et al., 1979). Poly(A+) mRNA was isolated by using oligo(dT)–cellulose (Maniatis et al., 1982). RNA concentrations were determined from the optical density at 260 nm. Aliquots of either total RNA (20 μg) or poly(A+) mRNA (1 μg) were translated in vitro with a rabbit reticulocyte lysate translation kit and [^{35}S]methionine to radiolabel newly synthesized proteins.

Newly synthesized radiolabeled 13-kDa protein was isolated from total cell-free translation products by immunoprecipitation with Ig_{13K} and *Staphylococcus aureus* cell membranes (Pansorbin; Calbiochem). Routinely, 20 μL of translation products was incubated with 40 μg of preimmune IgG in a total volume of 0.5 mL of buffer (50 mM Tris, 0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 50 mM methionine; pH 7.5) for 30 min at 4 °C. Pansorbin (0.1 mL; 10% w/v; equilibrated by four washes with buffer) was added and the mixture incubated with rotation for 30 min at 4 °C. The Pansorbin was then pelleted, and the supernatants were transferred to new tubes. Ig_{13K} (40 μg) was then added, and the resultant mixture was incubated overnight at 4 °C with end-over-end mixing. The incubations were mixed with 0.1 mL of Pansorbin (10% w/v) for 2 h at 4 °C. The Pansorbin was then pelleted and washed four times with buffer (50 mM

Tris, 0.15 M NaCl, 2% sodium deoxycholate, 2% Triton X-100; pH 7.5). After the final wash, the pellet was resuspended in 60 μL of SDS–PAGE sample buffer and boiled for 5 min. The Pansorbin was pelleted and the entire supernatant analyzed by SDS–PAGE. Immunoisolated proteins were subjected to SDS–PAGE on 12% polyacrylamide gels. Electrophoresis was followed by treatment of the gels with Amplify, drying, and autoradiography. The M_r's of the separated proteins were calculated by the least-squares regression analysis method using commercially available ^{14}C-labeled standard proteins in the M_r range 2350–200 000.

Immunopurification of the 13-kDa Protein. An immunoaffinity matrix was prepared by cross-linking affinity-adsorbed Ig_{13K} antibodies to protein A–Sepharose through the Fc fragment according to the method of Schneider et al. (1982). Antibody attached to protein A–Sepharose was 90–95% of input. Gels containing 10–15 mg of antibody/mL were used. Preimmune rabbit antibodies (Ig_N) were also cross-linked to protein A–Sepharose under identical conditions.

The 13-kDa protein was purified from rat lung cytosol by a combination of column and batchwise chromatography using three successive matrices: (a) protein A–Sepharose, (b) Ig_N–protein A–Sepharose, and (c) Ig_{13K}–protein A–Sepharose. Cytosolic preparations (10–15 mg of protein/mL) were passed through columns containing (a) and (b), and the flow-through fractions were collected. These fractions were pooled and incubated batchwise with (c) overnight at 4 °C with end-over-end rotation. The matrix was pelleted by centrifugation and sequentially washed with buffer B, buffer B containing 0.15 M NaCl, and buffer B containing 0.5 M NaCl. Bound material was finally eluted with either 0.1 M acetic acid or 3% SDS.

In general, a yield of 13-kDa protein of ~20–25% could be estimated from the amount of 13-kDa protein present in the cytosolic preparations as judged by the in vitro ligand-binding assay and protein determination on the eluate by the fluorescamine assay (see below) followed by SDS–PAGE to check for purity.

General Procedures. Protein analysis was performed as described by Lowry et al. (1951) or, in the case of column eluates, with fluorescamine as described by Böhlen et al. (1973) with bovine serum albumin as standard. Radioactivity was measured in Scintillator 299 in an LKB-Wallac 1216 Rackbeta II scintillation spectrometer (Bromma, Sweden) with an average counting efficiency of 40%.

Results

Partial Purification of the PCB-Binding Protein from Rat Lung. The PCB-binding protein was isolated from rat lung extract by sequential chromatography as outlined in Figure 1. Due to the fact that the labeled lung extract had a pH value (pH 5.0–5.2) slightly below the p*I* of the binding protein [see Lund et al. (1985)], the protein was retarded but not retained when chromatographed on a cation-exchange column, CM-Sepharose. The PCB-binding protein thus eluted after the proteins in the flow-through fractions, and no gradient was needed for elution (Figure 1a). This increased the speed of separation and diminished the risk of contamination of the PCB-binding protein with proteins retained on the column. The pooled peak fractions from the CM-Sepharose column were concentrated by ultrafiltration, and the PCB-binding protein was further purified by gel permeation chromatography on Sephadex G-75 in buffer A, pH 7.2 (Figure 1b), followed by anion-exchange chromatography on DEAE-Sepharose (Figure 1c). The peak fractions eluted with a linear NaCl gradient (0–150 mM) from DEAE-Sepharose were pooled,

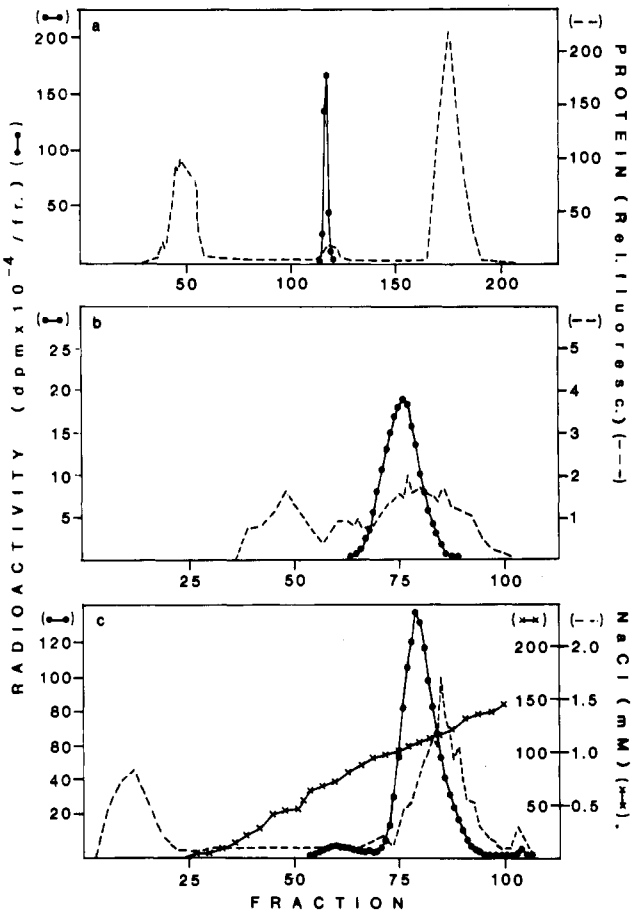

FIGURE 1: Chromatographic procedures for purifying the rat lung PCB-binding protein. Labeled lung extract from 50 female Sprague–Dawley rats (200 g) was prepared as described under Experimental Procedures, and the rat lung PCB-binding protein was purified by sequential chromatography on (a) CM-Sepharose (4 × 27 cm), (b) Sephadex G-75 (2.6 × 58 cm), and (c) DEAE-Sepharose (1.7 × 3.2 cm). For details, see Experimental Procedures.

Table I: Partial Purification of the PCB-Binding Protein from Rat Lung[a]

	protein (mg)	PBC-binding protein pmol/mg	PBC-binding protein mg	purity (%)	purifcn (x-fold)
cytosol	2524	90	2.95	0.12	1.0
labeled lung extract	1536	110	2.20	0.14	1.2
CM-Sepharose	18.68	8640	2.10	11.2	93
Sephadex G-75	4.37	19960	1.13	25.9	216
DEAE-Sepharose	0.73	45980	0.44	60.3	503

[a] The PCB-binding protein was purified from 50 female Sprague–Dawley rats (200 g) as described under Experimental Procedures. The calculation of the amount of PCB-binding protein and its purity is based on the assumptions that the protein has a molecular weight of 13 000 and that it binds one ligand per molecule. The amount of PCB-binding protein was estimated by use of the in vitro binding assay described under Experimental Procedures with ([^{3}H]$MeSO_2)_2$TCB as the radioactive ligand.

dialyzed overnight, and then lyophilized. The purified protein was redissolved in a small volume of buffer A and stored at −70 °C until further analysis.

Table I summarizes the pertinent numerical details of a typical experiment using the purification procedure illustrated in Figure 1. The starting material contained 2.95 mg of PCB-binding protein as judged by the in vitro binding assay, and after the final chromatographic step, a purity of 60% and an overall recovery of 15% had been achieved. The recovery of the PCB-binding protein in several purifications varied

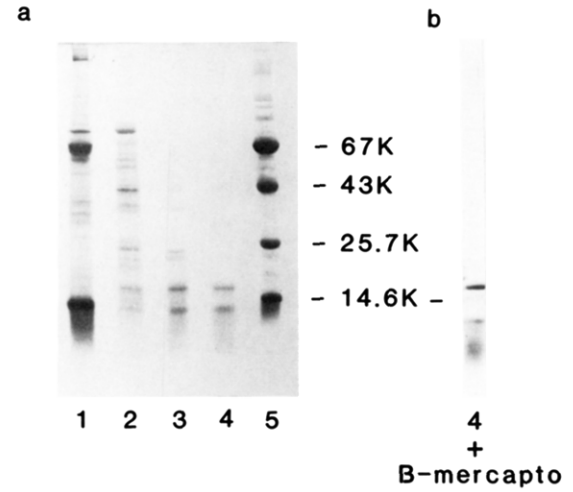

FIGURE 2: SDS–PAGE of samples from the sequential purification steps. The samples were analyzed at the protein levels indicated, and migration proceeded from top to bottom. The electrophoretic profiles of representative fractions obtained during the purification of the PCB-binding protein are shown: (a) lane 1, labeled lung extract, 30 μg; lane 2, CM-Sepharose eluate, 20 μg; lane 3, Sephadex G-75 eluate, 15 μg; lane 4, DEAE-Sepharose eluate, 10 μg; reference proteins with their respective M_r values are indicated; (b) the same sample as in lane 4 but treated with β-mercaptoethanol. Electrophoresis was performed on linear 8–18% SDS–polyacrylamide gradient gels as described under Experimental Procedures, and the gels were stained with Coomassie blue.

between 7 and 20%, and the purity of the protein ranged from 30 to 80% (mean 50%), as judged by the in vitro binding assay.

SDS–PAGE analysis of aliquots from the various chromatographic eluates is shown in Figure 2a. The final purified preparation revealed two protein bands of M_r 16 400 ± 600[2] and M_r 12 800 ± 300[2], respectively, as shown in lane 4. Due to the denaturing conditions, we could not at this point determine whether only one or both of the proteins bind PCBs.

Characterization of the 16-kDa and 13-kDa Proteins. The purified protein preparation, containing both the 16-kDa and the 13-kDa proteins, was further characterized by SDS–PAGE and IFPAG in the presence or absence of reducing agents, i.e., β-mercaptoethanol and dithioerythreitol. As shown in Figure 2b, the presence of β-mercaptoethanol in the solubilizing buffer during SDS–PAGE splits the 13-kDa protein into subunits, whereas the 16-kDa protein is unaffected by the presence of reducing agents. With the use of 8–25% gradient gels we were able to achieve a sufficient separation in the low molecular weight range to determine an approximate molecular weight of the subunit protein band, 7800 ± 500.[3] The existence of subunit polypeptides of different M_r within the rather broad protein band cannot be excluded. When the purified preparation was analyzed by IFPAG, only one sharp band corresponding to a p*I* of 5.3 was observed when the gel was stained with Coomassie blue (Figure 3, lane 2). However, when the preparation was exposed to 10 mM DTT and then subjected to IFPAG, one additional band was observed with a slightly more basic p*I* (Figure 3, lane 3). These data indicate that the 13-kDa protein is composed of subunits held together by disulfide bridges and that the subunits have a different p*I* as compared to that of the intact 13-kDa protein.

Immunochemical Characterization. The Ig fractions from antisera raised against the 13-kDa and the 16-kDa proteins were isolated by use of protein A–Sepharose. The reactivity and specificity of the antibodies were tested by immunoblotting. SDS–polyacrylamide gels were run with aliquots of rat lung cytosol, CM–Sepharose eluate, and DEAE-Sepharose eluate. The proteins were transferred to nitrocellulose filters and incubated with purified anti 13-kDa protein antibodies (Ig_{13K}) or anti 16-kDa protein antibodies (Ig_{16K}). When the acrylamide gels were stained after electrophoretic transfer, no residual protein bands could be detected. Figure 4 shows autoradiograms of nitrocellulose sheets incubated with Ig_{13K} (Figure 4a) and Ig_{16K} (Figure 4b), respectively, and autoradiographed following incubation with ^{125}I-labeled protein A. The Ig_{13K} only detected one band in all the fractions tested, corresponding to an M_r of 13 000. No cross-reactivity with the 16-kDa protein was observed. The Ig_{16K} detected mainly one protein corresponding to an M_r of 16 000, but additional protein bands of higher M_r values were also detected. No cross-reactivity of the Ig_{16K} with the 13-kDa protein was observed.

Aliquots of a partially purified protein preparation (30% pure as judged by the ligand-binding assay) labeled with ([^{3}H]$MeSO_2$)$_2$TCB were incubated for 18 h at 0–4 °C with either preimmune Ig (Ig_N), Ig_{13K}, or Ig_{16K}. After removal of

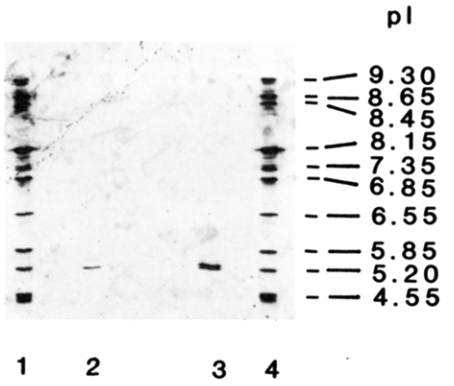

FIGURE 3: IFPAG of the DEAE-Sepharose eluate containing both the 13-kDa and 16-kDa proteins, in the presence or absence of DTT. Aliquots of the DEAE-Sepharose eluate containing both the 13-kDa and 16-kDa proteins were analyzed by IFPAG as described under Experimental Procedures, and the gels were stained with Coomassie blue. Lane 2, DEAE-Sepharose eluate, 10 μg; lane 3, DEAE-Sepharose eluate treated wtih 10 mM dithioerythreitol, 10 μg. Lanes 1 and 4 contain reference proteins (isoelectric focusing calibration kit, Pharmacia Fine Chemicals, Uppsala, Sweden), and the respective p*I* values are listed.

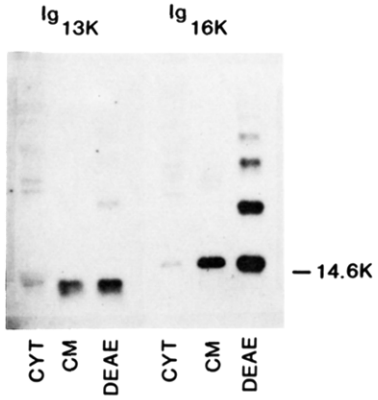

FIGURE 4: Immunoblots of samples from the sequential purification steps with (a) Ig_{13K} and (b) Ig_{16K}. Aliquots of cytosol (200 μg, CYT), CM-Sepharose eluate (20 μg, CM), and DEAE-Sepharose eluate (7 μg, DEAE) were separated on SDS–PAGE, transferred to nitrocellulose sheets, and incubated with (a) Ig_{13K} or (b) Ig_{16K}. Incubation with ^{125}I-labeled protein A in combination with autoradiography was used to visualize the respective immune complexes. ^{125}I-Labeled lysozyme (14.6 kDa) was used as reference. For details, see Experimental Procedures.

[2] Values represent mean ± SD of six determinations.

[3] The value represents mean ● SD of four determinations.

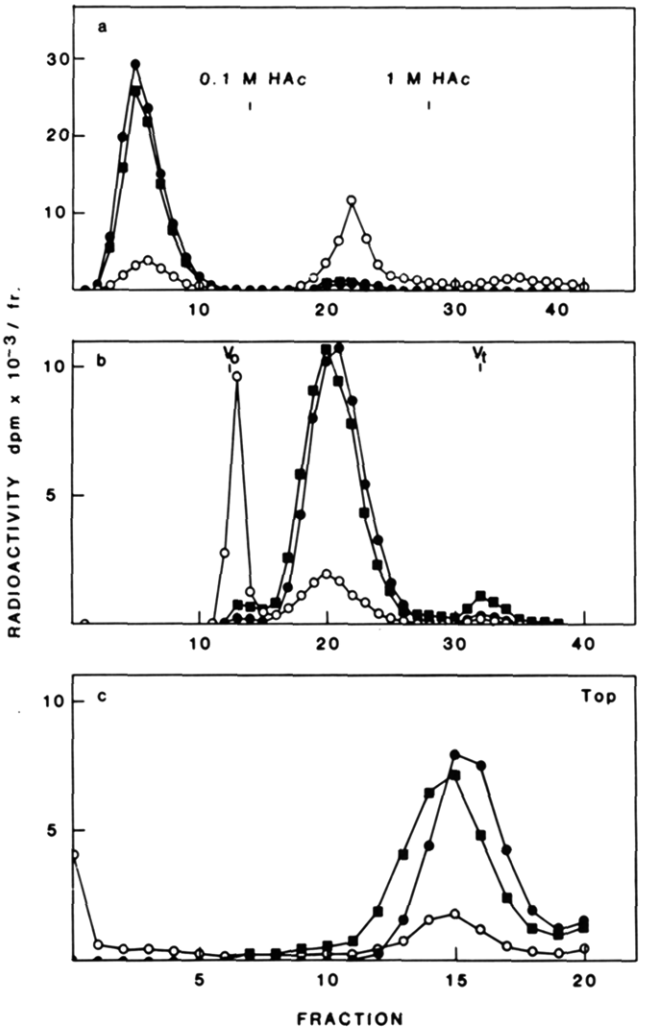

FIGURE 5: Protein A–Sepharose chromatography, gel permeation chromatography, and density gradient centrifugation of ([^{3}H]-$MeSO_2)_2$TCB-labeled partially purified binding protein treated with Ig_N, Ig_{13K}, or Ig_{16K}. (a) A 10-μL aliquot of partially purified binding protein (1.4 μg, 25% purity) was incubated with 250 μL (1.6 mg/mL) of Ig_N (■), Ig_{13K} (○), or Ig_{16K} (●) for 18 h at 0–4 °C; 250 μL of 0.5% (w/v) dextran-coated charcoal was added to each incubation, which was left on ice for 10 min. The charcoal was pelleted by centrifugation at 1500g for 15 min. A 400-μL aliquot of each supernatant was then chromatographed on protein A–Sepharose columns (2.5 mL). After the antibodies were allowed to bind to protein A for 10 min, the columns were washed with buffer B and then sequentially eluted with 0.1 and with 1 M acetic acid (HOAc); 0.5-mL fractions were collected and the fractions assayed for radioactivity. The amount of radioactivity applied was 110 000 dpm (Ig_N), 61 000 dpm (Ig_{13K}), and 120 000 dpm (Ig_{16K}). The recovery of radioactivity was ~90%. Aliquots from incubations identical with those in (a) were also applied to (b) a Sephadex G-75 column (1 × 49 cm, 200 μL applied) and to (c) linear 5–20% sucrose density gradients (100 μL applied).

any unbound ligand by dextran-coated charcoal treatment (1:1 v/v; 0.5% charcoal, 0.05% Dextran T-70, w/v), aliquots were applied to protein A–Sepharose columns (1 × 3 cm) equilibrated in buffer B. The columns were washed with the starting buffer, and bound material was then sequentially eluted with 0.1 M acetic acid and 1 M acetic acid, respectively. When the samples that had been incubated with either Ig_N or Ig_{16K} were applied to protein A–Sepharose, 98 and 96%, respectively, of the radioactivity eluted in the flow-through of the columns (Figure 5a). However, in the presence of Ig_{13K}, 73% of the radioactivity was retained on the column and could be eluted with the acetic acid washes.

Aliquots of the partially purified protein preparation incubated with the different Ig fractions (cf. above) were also

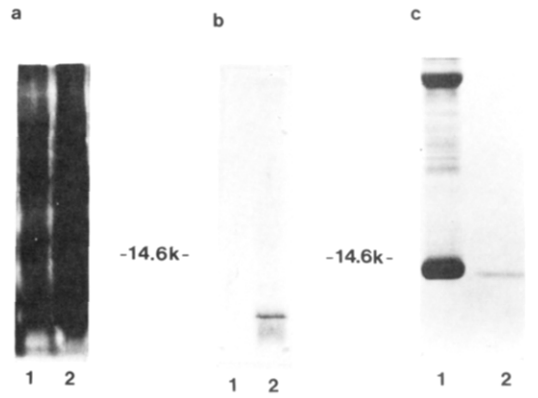

FIGURE 6: Cell-free translation of rat lung RNA and immunoprecipitation using Ig_{13K}. Poly(A+) mRNA (1 μg/incubation) from rat liver (a and b, lanes 1) or rat lung (a and b, lanes 2) were translated in vitro by using a rabbit reticulocyte lysate system. SDS–PAGE analysis was performed on 12% polyacrylamide gels. Gels were treated with Amplify, dried, and subjected to autoradiography. (a) Total in vitro translation products and (b) immunoprecipitated products using Ig_{13K}. (c) For comparison, an SDS–PAGE analysis of rat lung cytosol (lane 1, 50 μg of cytosolic protein) and of the eluate from an Ig_{13K}–protein A–Sepharose immunoaffinity matrix (lane 2, 5 μg of protein) is shown. For details, see Experimental Procedures.

subjected to gel permeation chromatography and density gradient centrifugation. In the presence of Ig_N or Ig_{16K}, radioactive peaks corresponding to a Stokes radius of 22 Å and an s value of 1.7 S were observed with Sephadex G-75 and 5–20% (w/v) sucrose gradients, respectively (Figure 5b,c). The presence of Ig_{13K} shifted the radioactivity to the void volume of the Sephadex G-75 column and caused the radioactivity to pellet during the density gradient centrifugation. It appeared as if the presence of Ig_{13K} caused precipitation of some ligand-bound material during the incubation and that this material was pelleted together with the dextran-coated charcoal, since only ~60% of the amount of radioactivity recovered in the supernatant from the Ig_N and Ig_{16K} incubation mixtures could be recovered in the Ig_{13K} incubations. Thus, the amount of ([^{3}H]$MeSO_2)_2$TCB-labeled material recognized by the Ig_{13K} probably represents an underestimation of the true value, since it is reasonable to assume that the pelleted immune complexes would also have been retained on protein A–Sepharose, eluted in the void volume of the Sephadex G-75 column, and pelleted during density gradient centrifugation. These data further support the contention that the 13-kDa protein constitutes the major PCB-binding protein in lung cytosol, since Ig_{13K} but not Ig_{16K} selectively recognizes the protein-bound radioactivity in a labeled mixture of 13-kDa and 16-kDa proteins, as analyzed by three independent methods under nondenaturing conditions.

Cell-Free Translation of Rat Lung RNA and Immunoprecipitation of Translation Products Using Ig_{13K}. Poly(A+) mRNA was isolated from female rat lungs and translated in a cell-free system by using rabbit reticulocyte lysate and [^{35}S]methionine. Newly synthesized proteins were immunoprecipitated from aliquots of the translation mixture by using Ig_{13K}. The results demonstrate that Ig_{13K} will precipitate a single ^{35}S-labeled protein with an M_r of ~8000 when subjected to SDS–PAGE (Figure 6b, lane 2). Cell-free translation of control RNA did not give rise to any immunoprecipitable proteins (Figure 6b, lane 1), nor did preimmune Ig precipitate any ^{35}S-labeled proteins from translation mixtures obtained from cell-free translation of rat lung RNA (data not shown). Similar results were obtained when total (unfractionated) RNA was used for cell-free translation (data not shown). An M_r of ~8000 corresponds well with the size of the subunits

of the 13-kDa protein as observed on SDS–PAGE in the presence of reducing agents. For comparison, Figure 6c shows an SDS–PAGE analysis of rat lung cytosol (lane 1) and an eluate from an Ig_{13K}–protein A–Sepharose immunoaffinity matrix (lane 2). Chromatography on the immunoaffinity matrix leads to the selective purification of a single 13-kDa protein. A reasonable interpretation of the results from cell-free translation of lung RNA is thus that the 13-kDa protein is synthesized as M_r ~8000 subunits and that two subunits joined by disulfide bonds constitute the mature protein.

Cross-Reactivity of Ig_{13K} with Rabbit Uteroglobin. Due to the striking similarities in physicochemical characteristics and ligand binding properties between the PCB-binding protein and uteroglobin (Lund et al., 1985), a protein purified from rabbit lung and uterus, the Ig_{13K} was tested for cross-reactivity to this protein. The two proteins appear to have identical M_r values when analyzed by SDS–PAGE and also split up into subunits in a similar fashion in the presence of 10 mM DTT (data not shown). However, the Ig_{13K} does not recognize purified uteroglobin as judged by Western immunoblots, even when the amount of applied uteroglobin was in excess of the 13-kDa protein by a factor of ~6 (data not shown). Furthermore, Ig_{13K} recognized the subunits of the 13-kDa protein but not those of uteroglobin. The purified uteroglobin preparation used displayed the characteristics expected for the native protein as judged by its behavior on Sephadex G-75 and sucrose density gradients as well as by its ligand-binding properties. Also under nondenaturing conditions the Ig_{13K} failed to recognize ($[^3H]MeSO_2)_2TCB$-labeled uteroglobin as analyzed by protein A–Sepharose chromatography, gel permeation chromatography, and density gradient centrifugation. The two proteins thus appear similar but not identical.

Discussion

In the present report, we describe the partial purification of a binding protein for certain methylsulfonyl-PCBs from rat lung cytosol by sequential chromatography on CM-Sepharose, Sephadex G-75, and DEAE-Sepharose. SDS–PAGE analysis of the final DEAE eluate revealed two protein bands of M_r 13 000 and 16 000. Antibodies were raised against the 13-kDa and the 16-kDa proteins and were used to demonstrate that the 13-kDa protein appears to be responsible for the binding of methylsulfonyl-PCBs.

SDS–PAGE and IFPAG analysis of the 13-kDa protein demonstrated its sensitivity to reducing agents such as dithioerythreitol or β-mercaptoethanol. Reduction of the protein led to the disappearance of the 13-kDa protein on SDS–PAGE with the concomitant appearance of a broad protein band with higher electrophoretic mobility. This would suggest that the 13-kDa protein consists of subunits held together by disulfide bridges. Cell-free translation of RNA from rat lung followed by immunoprecipitation using Ig_{13K} yielded a single ^{35}S-labeled protein with an M_r of ~8000, whereas chromatography on an immunoaffinity matrix selectively enriches a protein of M_r ~13 000 from rat lung cytosol, lending further support to the notion that the 13-kDa protein is synthesized as an M_r ~8000 subunit and that the mature protein is made up of two subunits joined by disulfide bridges.

The physicochemical characteristics of the 13-kDa protein are similar to those of a protein purified from rabbit uterus and lung, i.e., uteroglobin [for a review, see Savouret and Milgrom (1983)]. Uteroglobin has been shown to consist of two identical subunits of 70 amino acids held together by two disulfide bonds and to migrate on SDS–PAGE corresponding to an M_r of 12 000–13 000 in the absence of reducing agents (Nieto et al., 1977; Torkkeli et al., 1978). The PCB-binding protein and uteroglobin have previously been shown to have similar ligand-binding characteristics (Lund et al., 1985). The 13-kDa protein and uteroglobin were compared in the present study and were found to have an identical M_r. However, the monospecific antibodies raised against the 13-kDa protein failed to recognize uteroglobin, as analyzed by Western immunoblots. Thus, the proteins appear similar but not identical.

Both the physiological role of the 13-kDa protein and the toxicological implications of its interaction with methylsulfonyl-PCBs remain to be determined. The strikingly selective in vivo accumulation of methylsulfonyl-PCBs in the Clara cells of rat and mouse bronchiolar epithelium (Brandt et al., 1985) suggests that the protein is localized to these cells. This hypothesis is testable with the use of the antibodies produced in this study and, if correct, would focus the interest on the Clara cells. The function of the Clara cells appears to be less well understood. The cells are clearly of a secretory nature, although the exact mechanism of secretion is debated and the nature of the secretion has not been well characterized. Again, the immunochemical probes now available would allow studies of what appears to be a secretory product of the Clara cells. Immunochemical studies and enzymatic studies with freshly isolated lung cells have demonstrated that the Clara cell is also a major site of cytochrome P-450 dependent activities [for a review, see Bend et al. (1985)]. A recent paper (Lund, B. O., et al., 1986) has demonstrated that the in vivo treatment of mice with 4-(methylsulfonyl)-2,2′,5,5′-tetrachlorobiphenyl, a methylsulfonyl-PCB shown to possess high affinity for the rat PCB-binding protein (Lund et al., 1985), results in a significant decrease in the in vitro pulmonary N-demethylation of aminopyrine and a decrease in the in vivo covalent binding of *o,p′*-DDD. Since both the N-demethylation of aminopyrine and the activation of *o,p′*-DDD to a reactive metabolite appear to be cytochrome P-450 dependent events, these data would suggest that certain methylsulfonyl-PCBs can influence the levels of some cytochrome P-450 dependent activities. The presence of high levels of cytochrome P-450 in Clara cells and high levels of a high-affinity binding protein concentrating such methylsulfonyl-PCBs in the Clara cells thus offers an intriguing hypothesis for a causal relationship between the pathway for accumulation of certain methylsulfonyl-PCBs and the observed decrease in pulmonary drug metabolism. No information is available regarding the effect of methylsulfonyl-PCBs on the levels of individual cytochrome P-450 isoenzymes, but earlier studies have shown certain P-450 isozymes to be repressed by PCBs (Serabjit-Singh et al., 1983). The individual PCB isomer(s)/metabolite(s) responsible for this repression was (were) not identified. Clearly, further studies regarding the effects of methylsulfonyl-PCBs on the levels of individual pulmonary isozymes of cytochrome P-450 would be of great interest, particularly studies trying to address the question of the mechanism(s) involved.

Other xenobiotic-binding proteins have been described that bind unmetabolized compounds such as benzo[*a*]pyrene and 3-methylcholanthrene noncovalently and with high affinity (Zytkovicz, 1982; Tierney et al., 1983; Collins & Marletta, 1984). All of these proteins have higher M_r values than the PCB-binding protein described in this study and show a different tissue distribution. A recent paper has demonstrated that benzo[*a*]pyrene, 3-methylcholanthrene, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin bind to the rat prostatic steroid-binding protein (Söderkvist et al., 1986). This is of particular interest since the rat prostatic steroid-binding protein exhibits

amino acid homologies with rabbit uteroglobin (Baker, 1983). The toxicological significance of these xenobiotic–protein interactions remains to be elucidated.

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