Purification and photoaffinity labelling of a rat cytosolic binding protein specific for 3-methylcholanthrene

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Rat hepatic cytosolic proteins which sediment at 4–5 S on sucrose gradients exhibit high-affinity saturable binding for the carcinogen 3-methylcholanthrene. A rat liver protein of Stokes' radius 3 nm, M_r by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of 39000 and with specific 3-methylcholanthrene-binding activity sedimenting at 4.5 S, has been purified 315-fold to apparent homogeneity by using affinity chromatography on a column of 1-hydroxy-3-methylcholanthrene coupled to epoxy-activated Sepharose 6B, in conjunction with two gel-filtration steps. The protein purified by this technique was shown to be associated with the observed specific 3-methylcholanthrene-binding activity by photoaffinity labelling with 1-oxo-3-methylcholanthrene.

INTRODUCTION

Non-covalent, specific, saturable, high-affinity binding of some members of a group of carcinogens, the polycyclic aromatic hydrocarbons (PAH) and tetrachlorodibenzo-*p*-dioxin (TCDD), to proteins has been found in hepatic cytosol of rats, mice, guinea pigs, hamsters, rabbits and monkeys (Okey *et al.*, 1983).

Two major hepatic specific binding proteins (SBP) are distinguishable by their sedimentation on sucrose gradients (Collins & Marletta, 1984; Hannah *et al.*, 1981; Okey & Vella, 1982). One of these sediments between 4 and 5 S regardless of ionic strength and has an M_r by gel filtration of around 50000. Binding of 3-methylcholanthrene (3MC) and benzo[*a*]pyrene (B[*a*]P) to this 4–5 S protein has a dissociation constant of 2.5 nM and is saturable with a binding capacity in Sprague–Dawley rat cytosol of about 700 fmol·mg⁻¹ (Collins & Marletta, 1984; Heintz *et al.*, 1981; Holder *et al.*, 1981; Sarraf *et al.*, 1975; Tierney *et al.*, 1980, 1983; Zytkevich, 1982). The other specific binding protein sediments between 8 and 9 S (Carlstedt-Duke *et al.*, 1981; Okey *et al.*, 1979, 1980). Although differences have been found in M_r (Carlstedt-Duke *et al.*, 1981; Gasiewicz & Neal, 1982; Poland *et al.*, 1976), the 8–9 S specific binding protein binds TCDD with high affinity (dissociation constant of between 0.12 and 3.0 nM) and has a binding capacity of about 60 fmol·mg⁻¹ in Sprague–Dawley rat hepatic cytosol (Carlstedt-Duke *et al.*, 1980; Gasiewicz & Neal, 1982; Okey *et al.*, 1979; Poland *et al.*, 1976).

Whereas TCDD specifically binds only to the 8–9 S species (called the Ah receptor), 3MC and B[*a*]P bind specifically to both the 8–9 S and the 4–5 S species. Other PAH which are inducers of cytochrome *P*₁-450 activity also bind with a lower affinity to the 4–5 S proteins (Collins & Marletta, 1984; Heintz *et al.*, 1981; Tierney

et al., 1983) or to the 8–9 S protein (Bigelow & Nebert, 1982).

Our interest in cytosolic proteins with specific binding of 3MC derives from their potential effect on movement and metabolism of PAH as carcinogens within the cell and, in particular, their possible role in the specific induction of cytochrome *P*₁-450 by PAH. In order to examine the function of the 4–5 S specific binding protein, an affinity resin has been developed to purify it for further studies. Photoaffinity labelling, a technique used to localize and characterize steroid receptors (Gronemeyer, 1985), has been employed here to establish that the purified protein band observed by SDS/PAGE does exhibit specific binding activity.

MATERIALS AND METHODS

Materials

3MC generally labelled with tritium (34 Ci·mmol⁻¹) was obtained from Amersham. The sources of other chemicals used in this study were: 3MC, dithiothreitol and phenylmethanesulphonyl fluoride, Aldrich; acrylamide, bisacrylamide, calibration chemicals for SDS/PAGE, DMF, dimethyl sulphoxide and lead tetra-acetate, Sigma; dioxan and ethanolamine, Fisons; Optiphase 'MP' scintillant, LKB; NaBH₄, BDH; Sephacryl S-200, Sephadex G-150 and epoxy-activated Sepharose 6B, Pharmacia; Bio-Rad protein determination reagent, from Bio-Rad; calibration proteins for gel filtration, Boehringer Mannheim.

Preparation of cytosol and assay for the specific binding of 3MC to protein

Male Wistar rats weighing 50–100 g were killed by cervical dislocation and livers were perfused with

Abbreviations used: B[*a*]P, benzo[*a*]pyrene; DMF, dimethyl formamide; 3MC, 3-methylcholanthrene; SBP, specific 3-methylcholanthrene binding protein; SDS/PAGE, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis; TCDD, tetrachlorodibenzo-*p*-dioxin; TEDGP, 20 mM-Tris/HCl, 1 mM-Na₂EDTA, 1 mM-dithiothreitol, 10% (v/v) glycerol and 0.5 mM-phenylmethanesulphonylfluoride, pH 7.4; 1-OH-3MC, 1-hydroxy-3-methylcholanthrene; 3MC-1-one, 1-oxo-3-methylcholanthrene; PAH, polycyclic aromatic hydrocarbons.

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TEDGP at 4 °C before preparation of hepatic cytosol as described by Tierney *et al.*, (1983). Quantification of specific 3MC binding activity was by sucrose gradient assay (Tierney *et al.*, 1983).

Preparation of 1-OH-3MC and 3MC-1-one from 3MC and competition of 1-OH-3MC and 3MC-1-one for 3MC binding to the 4-5 S specific binding protein

Purified 3MC was oxidized to give 3MC-1-one by lead tetra-acetate, as previously described (Fieser & Hershberg, 1938; Cavalieri *et al.*, 1978); this was reduced to 1-OH-3MC by reaction with NaBH₄ in dry tetrahydrofuran, followed by recrystallization of both compounds from benzene (Cavalieri *et al.*, 1978). Both chemical structures were established by mass and u.v. spectra (Sims, 1966) and the presence of a ketone group in 3MC-1-one was confirmed by i.r. spectrophotometry. [³H]3MC-1-one, which co-eluted with 3MC-1-one on h.p.l.c., was prepared as above from [³H]3MC (sp. radioactivity 34 Ci · mmol⁻¹). Competition of 1-OH-3MC and 3MC-1-one for 3MC binding to the 4-5 S sedimenting protein was assessed by using the sucrose gradient assay as previously described (Tierney *et al.*, 1983).

Preparation of 1-OH-3MC-Sephacrose 6B affinity resin

Swelling, coupling, blocking and washing of epoxy-activated Sepharose 6B was done according to the manufacturer's instructions. Briefly, 1-OH-3MC was coupled to 50 ml (settled bed volume) of epoxy-activated Sepharose 6B, by shaking at 40 °C and pH 11 for 24 h at a molar ratio of substituent to epoxy groups of 2:1 in DMF/water (1:1, v/v). The coupled resin was washed with DMF and dioxan until no more 1-OH-3MC, as quantified by A₂₉₅, appeared in the washings. Ethanolamine (1 M) was used to block unreacted groups (pH 11, 24 h, 40 °C). The extent of derivatization of 1-OH-3MC-Sephacrose 6B was determined spectrally at 295 nm after suspension of the desiccated gel in 80% (v/v) aqueous glycerol.

Purification of 4-5 S specific 3MC-binding protein from cytosol

Cytosol (150 ml) was incubated for 30 min with 20 µCi of dry [³H]3MC before pumping onto a column (100 cm × 5 cm), containing Sephacryl S-200 pre-equilibrated in TEDGP. The column was eluted with TEDGP at 30 ml/h with continuous A₂₈₀ monitoring and collection of 15 ml fractions. Aliquots were counted for ³H and ³H peaks were assayed for protein (Bio-Rad protein assay) and specific binding activity.

Fractions from the Sephacryl S-200 column, which contained the 4-5 S specific 3MC-binding activity, were pooled (total volume of 150 ml) and incubated with end-over-end mixing for 16 h at 4 °C with 50 ml settled bed volume of 1-OH-3MC-Sephacrose 6B affinity resin pre-equilibrated in TEDGP. The resin was then separated from unbound proteins by pouring into a column (10 cm × 2.6 cm) and the packed resin washed with 400 ml of TEDGP over 4 h. Elution of 3MC-binding proteins from the resin was achieved with a 600 ml gradient from 5% (v/v) to 20% (v/v) DMF in TEDGP and a flow rate of 20 ml/h. The A₂₈₀ was continuously monitored and 15 ml fractions were collected. The eluted

samples were dialysed as 60 ml lots throughout the gradient against three changes of 20 vol. of TEDGP over 72 h. Samples were assayed for protein and specific 3MC-binding activity and by 12% SDS/PAGE.

The eluted 3MC-binding activity was concentrated from 600 ml to 40 ml by ultrafiltration with Amicon filter 5 YM30, before re-incubation with 10 µCi of [³H]3MC prior to pumping onto a Sephadex G-150 column (90 cm × 2.6 cm) at 20 ml/h. The column was equilibrated and eluted with TEDGP containing 0.05 M-KCl and had previously been calibrated with protein standards: ferritin (M_r 450000), bovine serum albumin (68000), ovalbumin (45000), chymotrypsin (25000) and cytochrome *c* (12500). The A₂₈₀ was continuously monitored, 5 ml fractions were collected and aliquots removed for assay of protein, ³H and for SDS/PAGE. The ³H peak was pooled for sucrose gradient assay. After elution, but before being re-used, the 1-OH-3MC-Sephacrose 6B affinity resin was washed at a flow rate of 10 ml/h with 250 ml of TEDGP at pH 2 (with 1 M-HCl) and then re-equilibrated at 10 ml/h with 500 ml of TEDGP.

Photoactivation

Samples of specific 3MC-binding protein, which had been partially purified by affinity chromatography (100 µg of protein with a specific activity of 3.289 × 10⁶ dpm · mg⁻¹), were incubated for 3 h at 4 °C in TEDGP and under different conditions: (a) 50 pmol of [³H]3MC-1-one with and without 5 nmol of 3MC-1-one; (b) 50 pmol of [³H]3MC-1-one with and without 5 nmol of 3MC; (c) 50 pmol of [³H]3MC-1-one with and without heat inactivation at 60 °C for 20 min. Two further samples were incubated under condition (a). These were 1 mg of affinity wash sample which contained no specific binding activity and TEDGP which contained no protein.

Samples were incubated with 3MC-1-one and 3MC that had been dried over N₂ and, after incubation, they were photoactivated in a water-cooled cylindrical cuvette by irradiating with a 1 kW mercury xenon lamp for 10 min at 4 °C. A borosilicate glass plate was used to exclude u.v. light below 300 nm. Protein in the samples was then precipitated with 10% trichloroacetic acid for 30 min at 4 °C followed by centrifugation, and all non-covalently bound 3MC-1-one was removed with one extraction with ethyl acetate/ethanol (4:1, v/v), followed by five further extractions with ethyl acetate. Any ethyl acetate remaining in the precipitated protein was removed by heating the sample before it was dissolved in dissociation buffer in preparation for SDS/PAGE (Laemmli, 1970). Following electrophoresis the gel was fixed and stained by silver staining (Wray *et al.*, 1981) and the R_f values of standards and sample protein bands were noted before segmenting and digestion of the gel in 30% H₂O₂ (48 h at 60 °C). After addition of scintillant, samples were left at 4 °C to allow photoluminescence to subside before counting.

Liquid-scintillation counting

Optiphase 'MP' scintillant (4 ml) was added to aqueous samples (1 ml) and the radioactivity was quantified in a Packard A300CD scintillation counter. Efficiency of ³H counting was between 30 and 35%.

RESULTS

Preparation and characterization of an affinity resin which binds 3MC SBP

Preliminary competition studies, using the sucrose gradient assay, established that 1-OH-3MC competed with 3MC for the binding site on the SBP (results not shown). An affinity resin for the SBP was, therefore, prepared by coupling 1-OH-3MC to epoxy-activated Sepharose 6B (Fig. 1), giving a level of modification of 2.1 nmol of 1-OH-3MC/mg of desiccated gel. It was found that binding of the cytosolic 3MC SBP to 1-OH-3MC-Sepharose 6B was optimal with end-over-end mixing for 16 h at 4 °C when compared with a stationary incubation or an incubation of shorter duration and that the ratio of cytosol to resin required for binding 50% of the SBP was 6:1 (v/v) (results not shown). Sepharose CL-6B was used as a negative control and did not bind the SBP, nor was there any loss of activity caused by the incubation process in the absence of the affinity resin.

The incubation of cytosol with 1-OH-3MC-Sepharose 6B in buffers of differing ionic strengths led to a number of observations. (i) Increasing ionic strength, from 0.1 M-KCl to 1.0 M-KCl, caused an increase in the amount of SBP bound to the resin, although in the absence of KCl binding was greater than at 0.1 M- and 0.3 M-KCl (almost 70%). (ii) The two buffers, 10% (v/v) DMF in TEDGP and 2 M-NaSCN in TEDGP, were established as reversible inhibitors of 3MC specific binding to the SBP, dialysis being used to recover specific activity completely. Increasing ionic strength caused a decrease in the amount of SBP eluted using either 10% DMF or 2 M-NaSCN as eluents. However, both buffers could be successfully used as eluents; 10% DMF in

Fig. 1. Partial structure of 1-OH-3MC-Sepharose 6B

Fig. 2. Sephacryl S-200 gel filtration profile of Wistar rat cytosol preincubated with [³H]3MC

[³H]3MC (20 μCi) was incubated for 1 h at 4 °C with 150 ml of cytosol before pumping onto a Sephacryl S-200 column (100 cm × 5 cm) equilibrated in TEDGP, pH 7.4. Elution with TEDGP, pH 7.4, was at 30 ml/h with collection of 15 ml fractions. The A₂₈₀ (—) was monitored and 100 μl aliquots were counted for ³H (·····). Proteins associated with radioactive peaks were analysed for specific 3MC binding by the sucrose gradient assay.

Fig. 3. Affinity chromatography of the specific 3MC binding activity (peak B) obtained from Sephacryl S-200 chromatography

After incubation (16 h at 4 °C with end-over-end mixing) of the Sephacryl S-200 peak B with 1-OH-3MC-Sepharose 6B affinity resin (3:1 v/v ratio respectively), a column (10 cm × 2.6 cm) was poured and washed (400 ml of TEDGP over 4 h) before elution at 20 ml · h⁻¹ with a 5–20% (v/v) gradient of DMF in TEDGP (total volume 600 ml); 60 ml fractions were pooled for dialysis followed by quantification of specific binding activity by sucrose gradient assays (histograms). The A₂₈₀ was monitored (—).

TEDGP gave a greater yield and -fold purification than did 2 M-NaSCN in TEDGP. (iii) Although optimum -fold purification was achieved at a salt concentration of 0.1 M-KCl (164-fold with 28% yield), maximum total yield was achieved in the absence of salt (75% at 65-fold). In the interest of maximizing total yield, therefore, it was decided to use affinity chromatography on 1-OH-3MC-Sepharose 6B in the absence of salt followed by elution with 10% DMF in TEDGP and to incorporate this in a sequence of procedures designed to purify the 4–5 S SBP.

Fig. 4. Sephadex G-150 gel filtration profile of affinity-purified specific 3MC binding protein

The fraction with specific binding activity obtained by affinity chromatography was pooled and concentrated by ultrafiltration, incubated for 1 h at 4 °C with 10 μ Ci of [3 H]3MC, then subjected to Sephadex G-150 gel filtration on a column (90 cm $\times$ 2.6 cm) eluted with TEDGP/0.05 M-KCl at a flow rate of 20 ml/h. Fractions (5 ml) were collected and aliquots were assayed for amount of protein (—), radioactivity (---) and specific 3MC binding activity. The Sephadex G-150 column was calibrated with ferritin (M_r 450 000), bovine serum albumin (68 000), ovalbumin (45 000) and chymotrypsin (25 000).

Purification of 3MC SBP

Sephacryl S-200 gel permeation chromatography of Wistar rat cytosol (specific binding activity of 16.1×10^3 d.p.m. $\cdot$ mg $^{-1}$) which had been preincubated with [3 H]3MC showed three peaks of radioactivity (Fig. 2); only peak B contained specific 3-MC binding activity (180.3×10^3 d.p.m. $\cdot$ mg $^{-1}$). This peak was pooled to give an 11.2-fold purification of the 4–5 S SBP with a yield of 134%; elevated presumably due to our definition of cytosolic SBP as 100%. This phenomenon of an increase in yield on gel filtration has been observed previously (Collins *et al.*, 1985; Tierney *et al.*, 1983).

The peak B from Sephacryl S-200 gel filtration was incubated with the 1-OH-3MC–Sephacrose 6B affinity chromatography resin as described in the Materials and methods section (Fig. 3). Dialysis of the affinity eluate was essential for reactivation of specific binding activity and the large volume of eluate necessitated concentration of the purified sample by ultrafiltration. This concentrated sample was reincubated with [3 H]3MC prior to Sephadex

G-150 gel filtration (Fig. 4). Only one protein-associated peak of 3 H at a Stokes' radius of 3 nm was eluted {the Stokes' radius of the SBP was determined from a graph of the square root of the negative logarithm of the partition coefficient for the standard proteins [$(-\log K_{av})^{1/2}$] against their respective Stokes' radii [Siegel & Monty (1966)]}. This peak had an average specific 3MC-binding activity of 5.088×10^6 d.p.m. $\cdot$ mg $^{-1}$ by sucrose gradient assay and represents a 316-fold purification of the SBP from cytosol (Table 1).

SDS/PAGE of each purification step from cytosol to Sephadex G-150 gel filtration was associated with an increase in the amount of a protein with an M_r of 39 000, relative to other proteins in the sample (Fig. 5). Only two other proteins of M_r of 21 500 and 85 000 occurred in the pooled Sephadex G-150 fraction; all three proteins presumably have similar molecular size on gel filtration as other proteins with M_r values close to 39 000 are resolved by this step (Fig. 5). To determine which protein was responsible for specific binding of 3MC, photo-

Table 1. Purification procedure used to isolate specific 3-methylcholanthrene binding protein from Wistar rat liver cytosol

Purification step	Total protein (mg)	$10^{-3} \times$ Specific activity (d.p.m. $\cdot$ mg $^{-1}$)	Purification (-fold)	$10^{-3} \times$ Total activity (d.p.m.)	Recovery (%)
Cytosol	1781.00	16.1	1.0	28 674.1	100*
Sephacryl S-200	214.00	180.3	11.2	38 584.2	134
1-OH-3MC–Sephacrose 6B affinity resin	7.68	3289.3	204.3	25 261.8	88
Sephadex G-150	1.40	5087.6	315.9	7122.6	25

* Cytosolic specific binding activity was taken as 100%.

Fig. 5. SDS/polyacrylamide-gel electrophoresis of samples at each stage of the purification of proteins containing specific 3MC-binding activity

Electrophoresis was on 12% polyacrylamide gel by using the procedure of Laemmli (1970) and proteins were visualized by silver staining (Wray *et al.*, 1981). Each lane contained the following protein fractions; the amount and specific activity are given in parentheses: lane 1, cytosol ($5 \mu\text{g}$ of 16.1×10^3 d.p.m. $\cdot$ mg $^{-1}$); lane 2, Sephadex S-200 Peak A ($10 \mu\text{g}$, no activity); lane 3, Sephadex S-200 Peak B ($5 \mu\text{g}$ of 180×10^3 d.p.m. $\cdot$ mg $^{-1}$); lane 4, affinity wash ($2 \mu\text{g}$ of 79×10^3 d.p.m. $\cdot$ mg $^{-1}$); lane 5, affinity-purified sample ($6 \mu\text{g}$ of 3289×10^3 d.p.m. $\cdot$ mg $^{-1}$); lane 6, 1/10 dilution of lane 5; lane 7, affinity-purified peak activity ($4 \mu\text{g}$ of 4759×10^3 d.p.m. $\cdot$ mg $^{-1}$); lane 8, 1/10 dilution of lane 7; lane 9, Sephadex G-150 elution volume (see Fig. 4) 201–220 ml (no protein, no activity); lane 10, Sephadex G-150 elution volume (see Fig. 4) 221–260 ml ($1\text{--}2 \mu\text{g}$, no activity); lane 11, Sephadex G-150 elution volume (see Fig. 4) 261–300 ml ($1\text{--}2 \mu\text{g}$, no activity); lane 12, Sephadex G-150 elution volume (see Fig. 4) 301–340 ml ($1\text{--}2 \mu\text{g}$, high specific activity); lane 13, Sephadex G-150 elution volume (see Fig. 4) 341–380 ml ($1\text{--}2 \mu\text{g}$, high specific activity). The specific activity of pooled elution volume 301–380 ml from Sephadex G-150 was 5088×10^3 d.p.m. $\cdot$ mg $^{-1}$.

affinity labelling of the SBP was attempted using 3MC-1-one. Preliminary experiments indicated that this compound could compete with 3MC for the binding site on the SBP, although SBP had a comparative lower affinity for 3MC-1-one when compared with 3MC (results not shown).

Photoaffinity labelling of 3MC SBP with 3MC-1-one

The SBP sample used for photoactivation was the eluent from the affinity chromatography step prior to Sephadex G-150 gel filtration and, therefore, contained a number of different proteins. The addition of [^3H]3MC-1-one, followed by irradiation with a 1 kW mercury xenon lamp, resulted in covalent [^3H]3MC-1-one-protein complexes. These were separated by SDS/PAGE and the resulting gel was stained to visualize the proteins and then segmented and the radioactivity determined. The covalently bound radioactivity present, after SDS/PAGE, represented 1% of the total [^3H]3MC-1-one which was available for binding and a peak of radioactivity was found to be associated with the protein band of M_r 39000 (Fig. 6). Two other

Fig. 6. Photoaffinity-labelling of specific 3MC-binding protein

Samples of specific 3MC-binding protein which had been partially purified by affinity chromatography, but had not been further purified on Sephadex G-150 (specific activity 3289×10^3 d.p.m. $\cdot$ mg $^{-1}$), were incubated for 3 h at 4°C under different regimes: (a) 50 pmol of [^3H]3MC-1-one with (■) and without (□) 5 nmol of 3MC-1-one; (b) 50 pmol of [^3H]3MC-1-one with (■) and without (□) 5 nmol of 3MC; (c) 50 pmol of [^3H]3MC-1-one with (■) and without (□) heat inactivation at 60°C for 20 min. After incubation, samples were irradiated for 10 min with a 1 kW mercury xenon lamp prior to preparation for and analysis by SDS/PAGE (see the Materials and methods section). After silver staining, lanes (three lanes per sample) were segmented, dissolved and counted. M_r values of radioactive peaks were determined by migration relative to stained markers. Bromophenol Blue (BPB) was used as the low- M_r marker.

peaks of radioactivity were also observed; one migrated with the tracking dye and the other was associated with high- M_r or non-polar material. The photoactivated binding of [^3H]3MC-1-one to the protein of M_r 39000 could be decreased by the inclusion (in the incubation mixture) of a 100-fold excess of 3MC-1-one (Fig. 6a) or

3MC (Fig. 6b), or by heating the incubation mixture at 60 °C for 20 min (Fig. 6c), indicating that this protein does have specific binding activity. Other proteins which were present in the incubation mixture (Fig. 5, lane 5) did not exhibit specific binding, and as an additional control for specificity those proteins which were not retained by the affinity column (affinity wash; Fig. 5, lane 4) and which did not have specific binding activity by the sucrose gradient assay also showed no specific binding peak following photoaffinity labelling experiments. In addition, TEDGP containing no protein but photoactivated in the presence of [³H]3MC-1-one gave no radioactive segments (results not shown).

DISCUSSION

This paper reports the preparation of an affinity resin which can be used in conjunction with gel filtration methods to purify milligram amounts of 4–5 S specific 3MC binding protein to apparent homogeneity. The purification can be done in 7 days (including 3 days for dialysis). Purification of the specific binding activity is associated with increased amounts, on SDS/PAGE, of a protein of M_r 39000 relative to other proteins in the sample and this protein demonstrates specific binding activity for [³H]3MC-1-one during photoaffinity-labelling experiments. Given an M_r of 39000, and assuming that each SBP molecule will bind one 3MC molecule, it is possible to calculate an expected specific binding activity of 1.95×10^9 d.p.m. $\cdot$ mg⁻¹. This value is about 380-fold higher than the actual specific activity reported here. It could be argued that the SBP requires further purification; however, inactivated SBP may be co-purified with SBP, lowering the resulting -fold purification. The latter possibility is supported by the observed homogeneity of this protein on silver-stained SDS/PAGE. In addition, under the assay conditions used here, one SBP molecule may not bind one 3MC molecule, since an increase in dithiothreitol concentration causes a substantial increase in the observed specific binding activity (P. S. Arnold, unpublished work). This is thought to be due to activation, by thiol anions, of disulphide groups associated with the specific binding site on the SBP.

Two methods describing the partial purification of the SBP from mice (Collins *et al.*, 1985) and rats (Tierney *et al.*, 1983) have previously been reported. The major protein purified by Collins *et al.*, (1985) had a dissociated M_r of 32000 and a similar molecular size protein was purified by Tierney *et al.*, (1983). In this latter study a fraction with higher specific binding activity was also purified which contained an M_r 39000 protein, amongst others. The protein purified here to homogeneity with 3MC specific binding properties has an M_r of 39000. The major purification step employed by Tierney *et al.*, (1983) used 10% (v/v) DMF as an eluent in hydrophobic interaction chromatography. Collins *et al.*, (1985) used 2 M-NaSCN as an eluent with 1-aminopyrene–Sephacrose 6B column affinity chromatography. We found both these eluents were inhibitors of the interaction of 3MC with SBP at 4 °C but this inhibition was reversible by dialysis. The lack of a dialysis step, together with carrying out the binding incubation at 20–22 °C, may explain the low yield of active SBP obtained from 1-aminopyrene–Sephacrose 6B affinity chromatography (Collins *et al.*, 1985). Stability studies on partially purified SBP in TEDGP have suggested that the SBP is heat-labile at 20 °C, so all

stages of purification reported here have been done at 4 °C.

Various Stokes' radii from 2.7 nm (Tierney *et al.*, 1983) to 3.1 nm (Zytkovicz, 1982) have been reported for the SBP sedimenting at 4–5 S. We found this protein to have a Stokes' radius of 3.0 nm by Sephadex G-150 gel filtration. The selection of proteins with M_r values around 50000 in gel filtration steps used here would exclude purification of the TCDD SBP sedimenting at 8–9 S, due to its higher molecular size; however, potentially this protein should be purified on 1-OH-3MC–Sephacrose 6B affinity chromatography, due to its reported specific binding of 3MC. We have not observed a specific 3MC-binding peak sedimenting at 8–9 S on sucrose gradients at any stage in the purification used here.

The functional role of this 3MC SBP is not known at present. We did not find it had any glutathione *S*-transferase activity, nor does it appear to be rat serum albumin on the basis of its molecular size. The specific binding characteristics used here to purify the protein reflect the properties one would expect of a receptor protein involved in the induction of cytochrome *P*₁-450 (Poland *et al.*, 1976). Studies which have been carried out on crude cytosolic preparations of the SBP and a TCDD-binding protein sedimenting at 8–9 S on sucrose gradients have shown that such proteins have high-affinity saturable binding (Collins & Marletta, 1984; Heintz *et al.*, 1981; Slaga *et al.*, 1973a,b; Tierney *et al.*, 1980; Tierney & Bresnick, 1981) and are capable, as complexes with ligands, of temperature-dependent nuclear translocation (Holder *et al.*, 1981; Tierney *et al.*, 1980) and interaction with DNA (Weaver *et al.*, 1980). In addition, the TCDD-binding protein has been shown to be involved in the dose-dependent induction of specific mRNA coding for cytochrome *P*₁-450 (Tukey *et al.*, 1982). These latter observations on the TCDD-binding species, together with the reported lack of specific binding of TCDD by the protein sedimenting at 4–5 S on sucrose gradients (Hannah *et al.*, 1981; Okey & Vella, 1982), have led several groups to propose exclusively that the 8–9 S TCDD-binding species is the receptor involved in the induction of cytochrome *P*₁-450, termed the Ah receptor (Nebert *et al.*, 1981). Further studies are necessary on these proteins in their purified form in order to establish precisely the role they might have in cytochrome *P*₁-450 induction.

Clearly, those reports in which no specific binding of 3MC was observed to protein sedimenting in the 4–5 S region (Hannah *et al.*, 1981; Okey & Vella, 1982), or which accounted for such specific binding as being due to contaminating serum proteins (Poellinger *et al.*, 1983), are in error. The protein fraction purified here is similar to that used to demonstrate transport of polycyclic aromatic hydrocarbons to the membrane-bound cytochrome *P*₁-450 in the endoplasmic reticulum and nucleus of the liver cell (Dixit *et al.*, 1982; Hanson-Painton *et al.*, 1981; Holder *et al.*, 1981) and has been indicated as binding to the structural gene for cytochrome *P*₁-450 (Houser *et al.*, 1985). Further studies on the purified 4–5 S protein are needed to examine its function in such a transport process.

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