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Co-purification of Pea and Bean Leaf Soluble Auxin-binding Proteins with Ribulose-1,5-Bisphosphate Carboxylase[1]

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ABSTRACT

Soluble auxin-binding proteins (ABPs) were purified to constant specific activity from bean and pea leaves by a procedure involving $(NH_4)_2SO_4$ fractionation, anion exchange chromatography and gel filtration. Pea and bean ABPs exactly co-purify with ribulose-1,5-bisphosphate carboxylase (RuBPCase) in a variety of chromatographic separation procedures. The subunit compositions, electrophoretic purities and indole-3-acetic acid (IAA)-binding stoichiometries of the purified ABPs provide further evidence for the identity of RuBPCase and ABP. Pea ABP and bean ABP have dissociation constants for IAA of 0.8 and 1.3 micromolar, respectively, as determined by an $(NH_4)_2SO_4$ precipitation assay for IAA-binding to insolubilized ABP. IAA can bind to soluble bean and pea ABP (RuBPCase) as determined by equilibrium dialysis with affinities and stoichiometries similar to those determined for insolubilized ABP.

A current hypothesis for the mechanism of action of auxin assumes that the initial event involves the binding of the hormone to a specific cell receptor. Auxin-binding would modify the structure/activity of the receptor, resulting in a perturbation of a biochemical process that initiates a series of biochemical events resulting in the ultimate physiological response to the hormone. The stringency of the ligand specificity requirements for such a receptor suggests that the auxin receptor would be a protein. The properties of a variety of high affinity plant auxin-binding proteins have been described in recent years.

Auxin-binding sites are associated with membranes isolated from coleoptiles and primary leaves of *Zea mays* (1, 2, 5–7, 13, 19, 20, 26–28, 31), epicotyls and roots of *Pisum sativum* (8), soybean hypocotyls (36), mung bean hypocotyls (18), and zucchini hypocotyls (16). Solubilization of such auxin-binding sites from *Z. mays* has been achieved (5, 7, 31). The solubilized microsomal auxin-binding protein from *Z. mays* has a specificity for natural and synthetic auxins that parallels the ligand specificity of the membrane-associated protein and the biological activities of the auxin analogues tested (5). On current evidence this membrane-associated auxin-binding protein may well be the (an) auxin receptor. In the absence of evidence for an auxin-induced functional change in this protein the possibility exists that this protein has other than a receptor function. Apparently soluble auxin-binding proteins have been reported (15, 21, 29, 32) including soluble auxin-binding proteins that can apparently affect *in vitro* transcription (29). However, explicit functions have yet to be assigned to these proteins.

This paper describes the extensive purification of soluble auxin-binding proteins from pea and bean leaves (32) and presents evidence for the identity of these proteins with RuBPCase[2]. The accompanying paper (33) describes the ligand specificities of the soluble bean ABP.

MATERIALS AND METHODS

Purification of Pea and Bean ABP. Presterilized seeds of dwarf beans (*Phaseolus vulgaris* var. Royal Windsor) and of peas (*P. sativum* var. Greenfeast) were obtained from the Yates Seed Co., West Heidelberg, Melbourne. Seeds were briefly soaked in H_2O prior to planting in Vermiculite. Seedlings were grown at about 20 C with a 14 h/10 h light/dark cycle with light provided by two banks of 4 40-w Bio-lux fluorescent tubes at a distance of 48 cm above the seedling trays and supplemented by three 15-w Osram tungsten filament lights.

Primary bean seedling leaves were excised from seedlings 8–14 days after sowing the seed, washed with distilled H_2O, and sliced with scissors. All subsequent operations were carried out at 0–4 C. The sliced leaves were suspended in five volumes of an extraction medium containing 50 mM Tris (Cl^-, pH 8.0), 10 mM EDTA, 0.1% (v/v) 2-mercaptoethanol, 0.5 mM PMSF, and 0.2% (v/v) ethanol, and homogenized at stop 1 in a Waring Blendor for 1 min. The homogenate was filtered through Miracloth and the filtrate centrifuged at 35,000*g* for 40 min. The supernatant was brought to 30% $(NH_4)_2SO_4$ saturation, the resulting precipitate being collected by centrifugation and discarded. The resulting supernatant was brought to 50% $(NH_4)_2SO_4$ saturation and the precipitate collected by centrifugation and dissolved in a minimum volume of buffer A containing 50 mM Tris (Cl^-, pH 8.0), 10 mM EDTA, and 0.1% (v/v) 2-mercaptoethanol. This solution was applied to a column (2.5 cm^2 × 70 cm) of Ultrogel AcA-34 and eluted with buffer A (Fig. 1a). The IAA-binding fractions were pooled and applied to a column (4.0 cm^2 × 9.0 cm) of DEAE-Sephacel equilibrated with buffer A. The DEAE-Sephacel column was eluted with a linear gradient from 0 to 0.25 M $(NH_4)_2SO_4$ (in buffer A) (Fig. 1b). The active fractions were pooled, concentrated by precipitation at 80% $(NH_4)_2SO_4$ saturation, and reapplied (in buffer A) to the Ultrogel AcA-34 column and eluted in buffer A. One protein peak associated with a constant specific activity IAA-binding activity peak is observed at this stage (Fig. 1c). The purification schedule is presented in Table I. The same scheme was applied to the purification of ABP from the leaves of pea seedlings. Pea leaves were harvested 10–14 days after sowing the seed. Variants of this basic purification schedule were employed for specific purposes. For

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[2] Abbreviations: RuBPCase: ribulose-1,5-bisphosphate carboxylase; ABP: soluble bean or pea leaf auxin-binding protein; PMSF: phenylmethylsulfonyl fluoride; Pipes: piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Bis-Tris: bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane.

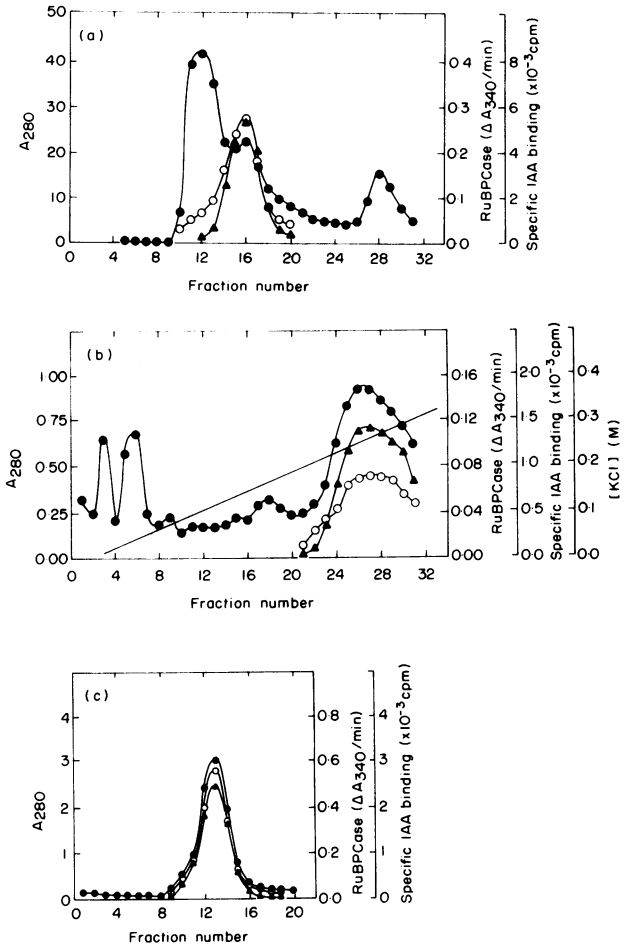

FIG. 1. Sequential purification of bean leaf ABP/RuBPCase by column chromatography on (a) an Ultrogel AcA-34 column (2.5 cm^2 × 70 cm) eluted with buffer A; (b) a DEAE-Sephacel column (4 cm^2 × 9 cm) eluted with a linear gradient of increasing [KCl] in buffer A; (c) a final Ultrogel AcA-34 column (2.5 cm^2 × 70 cm) eluted with buffer A. (●——●): A_{280}; (○——○): specific IAA-binding determined by the standard assay; (▲——▲): ΔA_{340}/min determined in the RuBPCase assay described.

Table I. *Purification of the Bean Leaf Soluble Auxin-binding Protein*

Specific IAA-binding was determined in the standard assay containing a final concentration of 2 × 10^{-6} M IAA (specific radioactivity of [2-^{14}C]IAA in the assay: 49 mCi/mmol). Primary leaves (55 g) of 2-week-old dwarf bean seedlings were extracted.

Purification Stage	Protein	Specific IAA-binding	Specific IAA-binding
	mg	*nmol*	*pmol/mg protein*
High speed supernatant	1,550	11.3	7.6
30–50% $(NH_4)_2SO_4$ fraction	501	11.0	22.0
Ultrogel AcA-34	248	86	349
DEAE-Sephacel	203	185	908

ABP preparations for equilibrium dialysis experiments, 2-mercaptoethanol was omitted and for bean ABP preparations for equilibrium dialysis the DEAE-Sephacel step was also omitted to avoid consequent lability of the bean ABP. Polyclar AT (insoluble PVP) was also added to the homogenizing medium in some pea and bean ABP preparations for equilibrium dialysis experiments.

$(NH_4)_2SO_4$ Precipitation Assay. The $(NH_4)_2SO_4$ precipitation procedure employed to measure IAA binding was similar to that

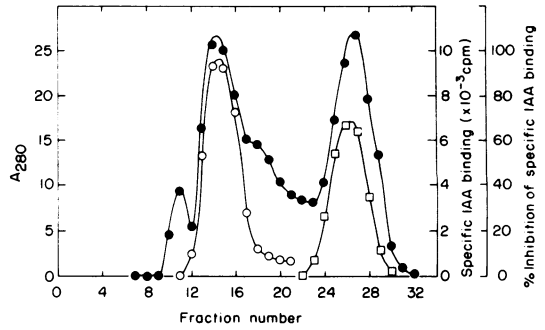

FIG. 2. Resolution of bean leaf ABP from an inhibitor of IAA binding. A 30–50% $(NH_4)_2SO_4$ saturation fraction was applied to an Ultrogel AcA-34 column (2.5 cm^2 × 70 cm) and eluted with buffer A. (●——●): A_{280}; (○——○): IAA-binding determined in the standard assay; (□——□): per cent inhibition of IAA-ABP binding of fraction 15 by addition of 50 μl of the indicated fractions in the standard assay.

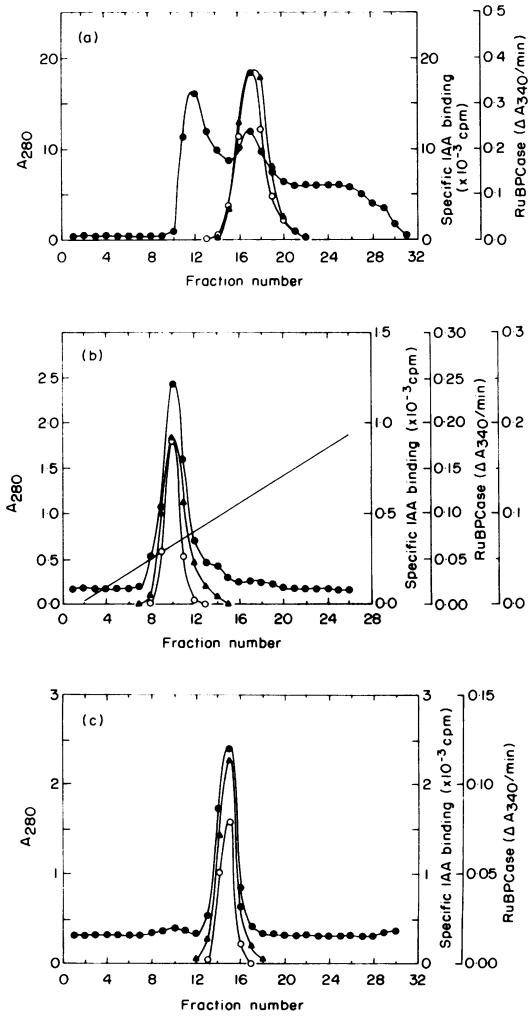

FIG. 3. Sequential purification of pea leaf ABP/RuBPCase by column chromatography on (a) an Ultrogel AcA-34 column (2.5 cm^2 × 70 cm) eluted with buffer B (50 mM Tris [Cl^-, pH 8.0], 10 mM 2-mercaptoethanol); (b) a DEAE-Sephacel column (4 cm^2 × 9 cm) eluted with a linear gradient of increasing [$(NH_4)_2SO_4$] in buffer B; (c) a final Ultrogel AcA-34 column (2.5 cm^2 × 70 cm) eluted with buffer B. (●——●): A_{280}; (○——○): specific IAA-binding; (▲——▲): RuBPCase (ΔA_{340}/min). For b and c IAA-binding was determined in the standard assay (IAA concentration, 2 × 10^{-7} M). For a, the IAA concentration in the assays was 2 × 10^{-6} M.

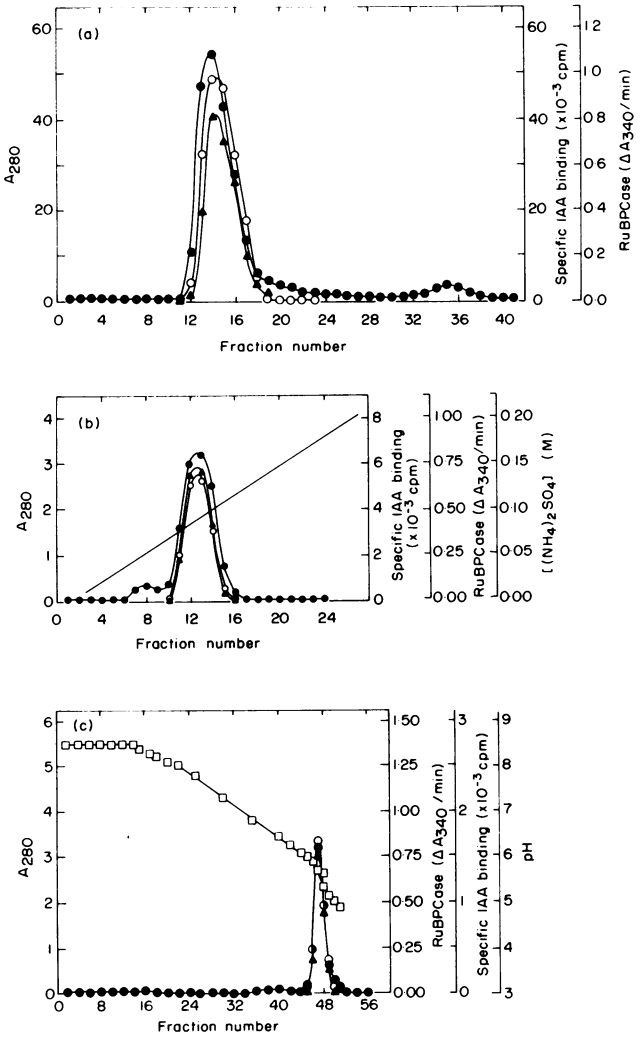

FIG. 4. Sequential purification of bean ABP/RuBPCase by column chromatography on (a) a Sephacryl S-200 column (2.5 cm^2 × 70 cm) eluted with buffer C (50 mM Tris [Cl^-, pH 8.0], 10 mM EDTA)); (b) a DEAE-Sephacel column (4 cm^2 × 9 cm) eluted with a linear gradient of increasing [$(NH_4)_2SO_4$] in buffer C; (c) a DEAE-Sephacel column (4.9 cm^2 × 4 cm) equilibrated with buffer C and eluted with a pH gradient determined by two reservoirs containing 20 mM Tris-20 mM Bis-Tris (pH 8.0) and 50 mM acetic acid. (●——●): A_{280}; (○——○): IAA-binding determined in the standard assay with 2×10^{-7} M IAA (for b and c) or 1×10^{-6} M IAA (for a); (▲——▲): RuBPCase (ΔA_{340}/min); (□——□): eluate pH.

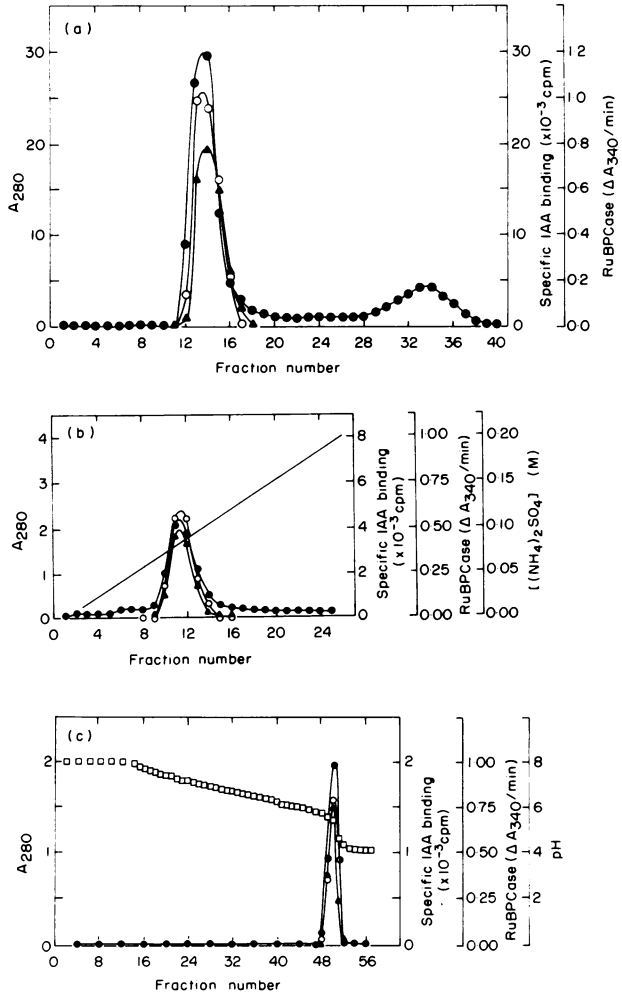

FIG. 5. Sequential purification of pea leaf ABP/RuBPCase by column chromatography on (a) Sephacryl S-200; (b) DEAE-Sephacel eluted with a linear gradient of increasing [$(NH_4)_2SO_4$]; (c) DEAE-Sephacel eluted with a gradient of decreasing pH. All other details as for Figure 4.

described previously (32). The assay medium (1 ml total volume) contained 2×10^{-7} M IAA (specific radioactivity of [2-^{14}C]IAA : 49 mCi/mmol), 50 mM Tris (Cl^-, pH 8.0), the protein sample being assayed (routinely added in 50 μl of solution) and 85% saturated $(NH_4)_2SO_4$. The final pH of the assay was 8.0 and assays were routinely conducted at 4 C. The order of additions was [2-^{14}C]IAA, protein and finally $(NH_4)_2SO_4$ solution. The protein precipitate was collected by centrifugation at 12,000*g* for 10 min in the SS-34 rotor of a Sorvall RC-2B centrifuge. The supernatant was removed carefully by aspiration. The pellet was solubilized in 1 ml of 1% (w/v) SDS in H_2O, added to 10 ml of scintillation fluid A (24), and counted. Assay counts were routinely corrected by subtraction of counts obtained in the same assay conducted in the presence of 0.2 μmol unlabeled IAA to obtain a measure of specific IAA-binding. Specific IAA-binding was routinely 80% of total IAA binding and SDS in the binding assays were less than 9% of the means, in assays in which about 30% of label was bound SDS were routinely about 2.5% of the means. In some assays the concentration of IAA was 2×10^{-6} M (199,700 cpm in the assay) (Table I, Fig. 3a) or 1×10^{-6} M (101, 320 cpm in the assay) (Figs. 4a and 5a); in these assays the specific radioactivity of [2-^{14}C]IAA was the same (49 mCi/mmol) as in the standard assay (IAA concentrations, 2×10^{-7} M; total cpm 20,677). The proportion of label specifically bound to ABP in the binding assays depends on the concentration of IAA and protein and ranged up to about 50% in the standard assay (*e.g.* Fig. 2) and in the assay containing 1×10^{-6} M IAA (*e.g.* Fig. 4a). We have previously shown that the labeled compound binding to bean ABP co-purifies chromatographically with [2-^{14}C]IAA (32).

Equilibrium Dialysis. Size 8/32 dialysis tubing (Visking Co., Chicago, Ill.) was cleaned before use by the procedure of Brewer (3). Uniform (18 cm) lengths of cleaned dialysis tubing were used. Dialysis tubing sacs containing 0.5 ml of ABP were double-knotted at each end and suspended in 10 ml of a solution containing [2-^{14}C]IAA, 100 mM Pipes (Cl^-, pH 7.0), and 100 mM KCl. The equilibrium dialysis experiments were conducted within capped 20 ml scintillation vials which were shaken at 120 oscillations/min at 30 C for 42 h in the dark in a thermostatted shaking water bath (model RW 1812, Paton Industries, South Australia). Samples (0.4 ml) of the inside and outside solutions were added to 4 ml of scintillation fluid A and counted using a Packard 3003 Series Tri-Carb liquid scintillation spectrometer (Packard Instrument Co.).

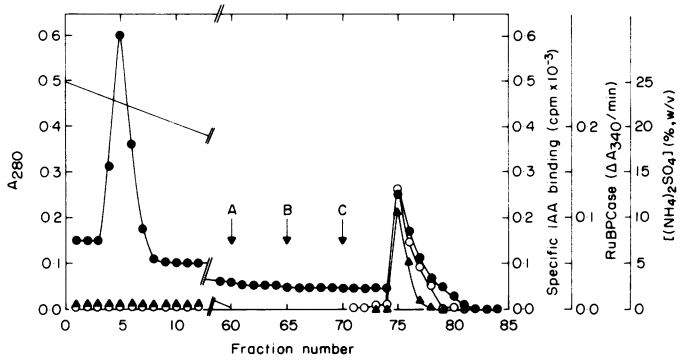

FIG. 6. Purification of pea leaf ABP/RuBPCase by hydrophobic chromatography. A column (3.5 cm^2 × 5 cm) of octyl Sepharose CL-4B was equilibrated with 25% (w/v) $(NH_4)_2SO_4$ in 50 mM Tris (Cl^-, pH 8.0). Pea ABP (purified on DEAE-Sephacel) was applied to the column in 25% (w/v) $(NH_4)_2SO_4$ in 50 mM Tris (Cl^-, pH 8.0). The column was initially washed with a linear gradient of decreasing $(NH_4)_2SO_4$ concentration in 50 mM Tris (Cl^-, pH 8.0). The column was then washed successively with 25 ml 50 mM Tris (Cl^-, pH 8.0) (arrow A), 25 ml 10 mM Tris (Cl^-, pH 8.0) (arrow B) and then distilled H_2O (arrow C) as indicated; 5-ml fractions were collected. (●——●): A_{280}; (○——○): IAA-binding determined in the standard assay (IAA concentration 2 × 10^{-7} M); (△——△): RuBPCase (ΔA_{340}/min).

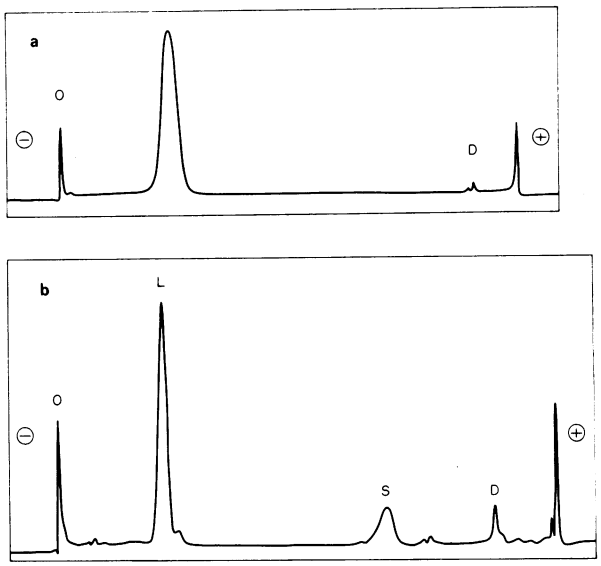

FIG. 7. a: Nondissociating 5% polyacrylamide gel electrophoresis of purified bean leaf ABP (RuBPCase) in 0.1 M Tris-glycine (pH 9.0). b: Dissociating 0.1% SDS-10% polyacrylamide gel electrophoresis of purified bean leaf ABP (RuBPCase) in 0.1% SDS-0.1 M Na-phosphate (pH 7.1). Gels were stained and gel scans obtained as described. The sharp peaks at the ends of the traces correspond with the ends of the gels: O, origin (cathodic end); S, small RuBPCase subunit; L, large RuBPCase subunit; D, position of the bromphenol blue tracker dye.

RuBPCase and Protein Assays. RuBPCase was assayed at 30 C in a final assay volume of 0.55 ml by the method of Racker (25). The reaction was initiated by addition of 50 μl of the enzyme and Δ_{340}/min determined using an Hitachi Model 181 spectrophotometer coupled to a recorder. Protein was determined by the modified biuret-Folin procedure of Dorsey *et al.* (9) using crystalline BSA as a standard.

Electrophoresis. Nondissociating 5% polyacrylamide gel electrophoresis (in 100 mM Tris-glycine, pH 9.0) and 0.1% SDS-10% polyacrylamide gel electrophoresis were conducted as described previously (22). Densitometer tracings of disk gels at 600 nm were made using a Gilford model 1520 gel scanner attached to a Gilford model 240 spectrophotometer (Gilford Instrument Labs., Oberlin, Ohio).

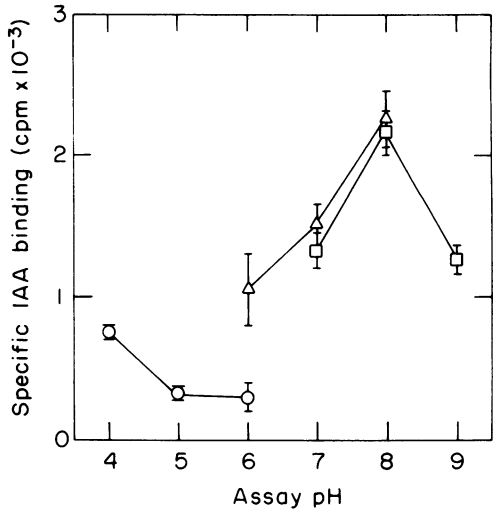

FIG. 8. Dependence of IAA binding to bean ABP on assay pH. IAA-binding was determined in triplicate in the standard assay but with the inclusion of the following final concentrations of various buffers to achieve the indicated assay pH values: (○——○), 0.1 M acetate; (△——△), 0.1 M Bis-Tris; (□——□), Tris. The error bars indicate SDS.

Chemicals. Sephacryl S-200, DEAE-Sephacel, octyl Sepharose CL-4B, and phenyl Sepharose 4B were obtained from Pharmacia Fine Chemicals, AB, Uppsala, Sweden; Ultrogel AcA-34 from LKB-Produkter AB, Bromma, Sweden; [2-^{14}C]IAA from the Radiochemical Centre, Amersham, U.K.; BSA, PMSF, Tris, Bis-Tris, and Pipes from the Sigma. Crystalline rabbit muscle glyceraldehyde-3-P dehydrogenase and 3-phosphoglycerate kinase were kindly provided by Dr. R. K. Scopes, Biochemistry Department, La Trobe University. All other chemicals were of analytical reagent grade.

RESULTS

Purification of the Pea and Bean ABPs. The bean leaf ABP was purified to constant specific activity on gel filtration (Fig. 1c) by the procedure described in Materials and Methods; the purification schedule is given in Table I. Large increases in IAA-binding are observed after gel filtration and also after ion exchange chromatography on DEAE-Sephacel (Table I). The increase in IAA-binding activity after gel filtration is attributable to the resolution of the bean ABP from low mol wt inhibitor(s) of IAA-binding that elute from the Ultrogel AcA-34 column in buffer A slightly before the salt peak (Fig. 2). The nature of the inhibitory material that precipitates with bean ABP in the 30–50% saturation $(NH_4)_2SO_4$ fraction is not known. The inhibitory fractions resolved on gel filtration (Fig. 2) are yellow-colored, but although inclusion of 2 g Polyclar AT/g leaves in the homogenizing medium resulted in removal of yellow-colored material, enhancement of auxin-binding by bean ABP was still observed after the subsequent gel filtration step. Further removal of inhibitory material was effected by ion exchange chromatography on DEAE-Sephacel eluted with a 0–0.2 M $(NH_4)_2SO_4$ gradient (Table I). The over-all yield of auxin-binding activity is difficult to assess because of the removal of inhibitors during the procedure. On a protein basis the purification was at most only 7.6-fold (Table I) but the stoichiometry of IAA-binding in the purified fraction is very high, corresponding to 0.45 mol IAA bound/mol 5 × 10^5 dalton protein in the standard assay with an IAA concentration of 2 × 10^{-6} M (Table I). The pea leaf ABP was purified to constant specific activity on gel filtration

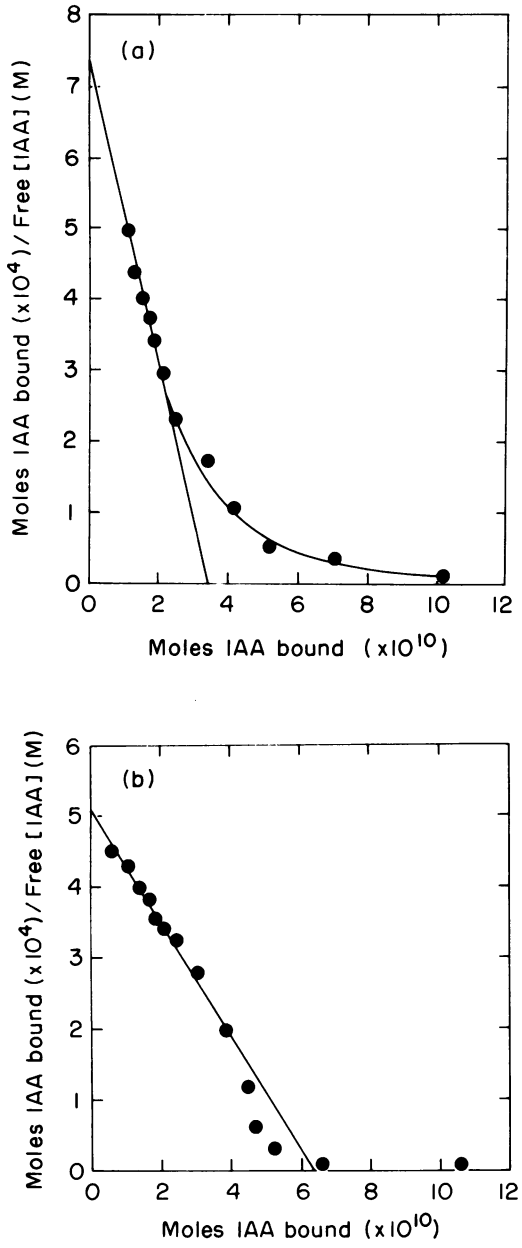

FIG. 9. Scatchard analysis of IAA binding to ABP in the conditions of the standard $(NH_4)_2SO_4$ precipitation assay. a: IAA-binding to bean ABP. b: IAA-binding to pea ABP.

(Fig. 3) by application of the same sequence of procedures used to purify bean leaf ABP.

Co-purification of ABP with RuBPCase. The large amounts of ABP present in leaves (Table I) and the elution of both pea and bean ABP close to the void volume on gel filtration through Ultrogel AcA-34 (Figs. 1 and 3) indicated that the ABPs were high incidence and high mol wt proteins. The possibility that the auxin-binding sites were associated with RuBPCase was therefore investigated. Figure 1, a to c, shows that in a bean ABP purification sequence involving successive gel filtration on Ultrogel AcA-34 (Fig. 1a), chromatography on DEAE-cellulose (Fig. 1b) and a final gel filtration on Ultrogel AcA-34 (Fig. 1c), auxin-binding and RuBPCase exactly co-chromatographed. Similarly bean ABP and RuBPCase exactly co-chromatograph in a chromatographic sequence of gel filtration through Sephacryl S-200 (Fig. 4a), $(NH_4)_2SO_4$ concentration gradient elution from DEAE-Sephacel (Fig. 4b) and pH gradient elution from DEAE-Sephacel (Fig. 4c). The pea ABP and pea RuBPCase exactly co-chromatograph in a chromatographic sequence involving gel filtration through Sephacryl S-200 (Fig. 5a), $(NH_4)_2SO_4$ gradient elution from DEAE-Sephacel (Fig. 5b) and pH gradient elution from DEAE-Sephacel (Fig. 5c). Pea ABP and RuBPCase also co-chromatograph on hydrophobic chromatography on octyl Sepharose CL-4B (Fig. 6). Neither pea ABP nor RuBPCase could be eluted from a phenyl-Sepharose column (3.5 cm^2 × 5 cm) by distilled H_2O after application and washing as for the octyl Sepharose CL-4B column. In addition to precipitating in the same $(NH_4)_2SO_4$ precipitation cut as RuBPCase the pea and bean ABPs exactly co-purify with pea and bean RuBPCases, respectively, in a variety of systems that separate proteins on the basis of Stokes radii, net charges at pH 8.0, or isoelectric points (Figs. 1 and 3–6).

Electrophoretic Analysis of Purified ABP. The purified bean ABP preparations yielded only one band on nondissociating polyacrylamide gel electrophoresis at pH 9.0 (Fig. 7a). Polyacrylamide gel electrophoresis of bean ABP in subunit dissociating conditions (in the presence of 0.1% SDS) revealed only 2 major polypeptide subunits with mol wt of 55,000–57,000 and 14,000–16,000 (Fig. 7b), the ratio of large subunits to small subunits being 0.96 mol/mol. A minor component (mol wt 49,000–53,000) was present in variable amounts in pea and bean ABP preparations (*cf.* Fig. 7b). 0.1% SDS-polyacrylamide gel electrophoresis of purified pea ABP preparations resolved only two major polypeptide bands with mol wt (determined by the use of mol wt standards as described under "Materials and Methods") of 54,000 –56,000 and 13,000–14,000. The two major polypeptides present in the pea and bean ABP preparations are clearly the large and small RuBPCase subunits. These electrophoretic results provide further evidence for the identity of ABP and RuBPCase. If ABP is a contaminant co-purifying with RuBPCase (albeit in a variety of chromatographic procedures) then it must have the same electrophoretic mobility as RuBPCase in nondissociating gel electrophoresis (Fig. 7a) and subunits with the same mol wt as one or both of the RuBPCase subunits (Fig. 7b). The possibility that the minor 50,000 dalton polypeptide detected in our preparations by dissociating gel electrophoresis (Fig. 7b) is the IAA-binding polypeptide can be excluded for reasons of IAA-binding stoichiometry as described below.

IAA-binding to Purified ABP. The pH optimum for IAA-binding to bean ABP in the standard assay is pH 8.0 (Fig. 8). The concentration dependence of IAA-binding to purified bean ABP at pH 8.0 was determined and the data analyzed by constructing Scatchard plots. A representative Scatchard plot is shown in Fig. 9a, high and low affinity IAA-binding components are apparent, the K_d for the high affinity component being 5×10^{-7} M. The stoichiometry for high affinity binding of IAA at saturation for the analysis shown in Fig. 9a is 1.1 mol IAA bound/mol of ABP, assuming a mol wt for ABP of 550,000 *i.e.* the mol wt of RuBPCase (11, 17, 37). The subunit composition of the bean ABP preparation used for the Scatchard analysis of Figure 9a is shown in Figure 7b. The minor 50,000 dalton polypeptide represents only 1.3% of the total protein. If the 50,000 dalton polypeptide (Fig. 7b) is responsible for IAA-binding then the IAA-binding stoichiometry at saturation would be 7.5 mol IAA bound/mol of this polypeptide, *i.e.* there would need to be at least eight IAA-binding sites per polypeptide chain. This would appear to be an extremely implausible stoichiometry and accordingly further supports our evidence for the identity of ABP and RuBPCase. Further, previous workers have described a minor large subunit-derived polypeptide of this size as a product from proteolytic action (11) or from sample preparation for dissociating gel electrophoresis (14). The mean estimate for the K_d of the purified bean ABP for IAA is 0.8 (±0.3) × 10^{-6} M (determinations on five separate preparations). The mean binding stoichiometry at saturation is 1.2(±0.5) mol IAA/mol RuBPCase/ABP (assuming a mol wt of 550,000). These

stoichiometries may represent underestimates for reasons associated with the lability of the bean ABP after purification on DEAE-Sephacel. A Scatchard plot for IAA binding to the purified pea ABP is shown in Figure 9b. One high-affinity binding component is apparent with a K_d of 1.3×10^{-6} M for IAA. Assuming that ABP and RuBPCase (mol wt 550,000) are identical, the stoichiometry of IAA binding at saturation is 1.0 mol/mol RuBPCase.

Stability of the Purified ABP. The bean ABP preparations become very unstable after chromatography on DEAE-Sephacel, 70% of auxin-binding activity being lost after 24 h at 4 C. Accordingly, for Scatchard analyses of IAA binding to DEAE-Sephacel-purified bean ABP IAA-binding was determined within 1 h of elution of the protein from DEAE-Sephacel. Highly purified but more stable preparations of bean ABP could be prepared by repeated gel filtration through an Ultrogel AcA-34 column in the absence of 2-mercaptoethanol; such bean ABP preparations lose about 60% IAA-binding activity after 15 days at 4 or 20 C. Attempts to find conditions that would stabilize IAA-binding activity were unsuccessful—inclusion of 10 mg/ml casein, 50% saturated $(NH_4)_2SO_4$, 10% (w/v) glycerol, 10% sucrose, or 5 mM ATP-2.5 mM $MgSO_4$ in the stored preparations fail to significantly prevent loss of IAA-binding at 4 or 20 C. Inclusion of 5 mM 2-mercaptoethanol results in enhanced loss of IAA binding activity during storage—about 80% of activity being lost at 4 or 20 C after 15 days. Pea ABP preparations are more stable than bean ABP preparations. Pea ABP, purified by repeated gel filtration in the absence of 2-mercaptoethanol, loses only 7% of IAA-binding activity after 14 days at 4 C; pea ABP, purified further by DEAE-Sephacel chromatography, loses 12% activity after 7 days at 4 C.

Although a bean ABP preparation purified on DEAE-Sephacel lost 60% IAA-binding activity in 12 h at 4 C, the RuBPCase activity of this preparation did not change. Similarly, gel filtration of crude pea ABP on Ultrogel AcA-34 in 50 mM Tris (Cl^-, pH 8.0)-10 mM 2-mercaptoethanol-0.5 M $(NH_4)_2SO_4$ yielded an active RuBPCase peak with no IAA-binding activity; rechromatography of this RuBPCase peak on the same column eluted with 50 mM Tris (Cl^-, pH 8.0)-10 mM 2-mercaptoethanol yielded coincident peaks of A_{280}, RuBPCase and IAA-binding activity. Thus apparently irreversible as well as reversible loss of IAA-binding activity co-purifying with RuBPCase can be demonstrated. While in such instances one can generate RuBPCase experimentally with no apparent associated IAA-binding, all our active IAA-binding preparations contain co-purifying RuBPCase.

IAA-binding to Soluble ABP. The foregoing results demonstrate that IAA binds tightly to pea and bean ABP in the conditions of the $(NH_4)_2SO_4$ precipitation assay. In this assay the IAA-insolubilized ABP complex is pelleted through a solution containing the equilibrium concentration of free IAA, *i.e.* the equilibrium distribution of ligand being examined is that obtaining after addition of saturated $(NH_4)_2SO_4$ solution. The [2-^{14}C]IAA bound to the insolubilized ABP is largely displaced by addition of excess unlabeled IAA either before or after addition of saturated $(NH_4)_2SO_4$ solution and ABP is not inactivated by repeated $(NH_4)_2SO_4$ precipitations (Table I). The binding of IAA to ABP is rapid, maximal binding occurring in the standard assay within the time required for centrifugal separation of bound and free hormone. Thus, the binding of IAA to ABP in these conditions is rapid and reversible. This type of $(NH_4)_2SO_4$ precipitation assay has been applied as a reliable procedure in other ligand-binding studies (4, 10, 23, 24). However given the evidence for the identity of ABP and RuBPCase an important question is whether IAA can bind to the soluble as well as to the insolubilized form of the protein.

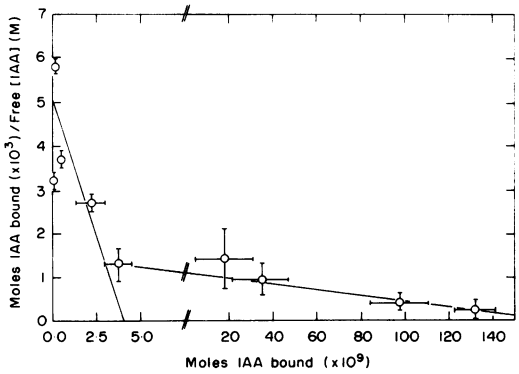

FIG. 10. Scatchard analysis of IAA-binding to soluble bean ABP in conditions of equilibrium dialysis. Bound and free IAA were determined in triplicate as described and error bars indicate SDS.

A large number of equilibrium dialysis experiments were conducted in an effort to quantitate IAA-binding to soluble ABP. In 22 extensive equilibrium dialysis experiments involving 20 separate purified pea or bean ABP preparations, IAA-binding to ABP was demonstrated in only nine experiments. All preparations used in these experiments were active in binding IAA in the conditions of the standard $(NH_4)_2SO_4$ precipitation assay. The basis for this variability in the IAA binding of the ABP preparations in our equilibrium dialysis conditions is not known. Figure 10 shows a Scatchard plot of IAA binding to an "active" soluble bean ABP preparation as determined by equilibrium dialysis. Both high and low affinity IAA binding is apparent. The high affinity K_d value is 8×10^{-7} M and the stoichiometry for high affinity IAA-binding at saturation is 0.7 mol/mol ABP. The K_d and high affinity stoichiometry values for pea ABP (as determined by equilibrium dialysis) are 5×10^{-7} M and 0.4 mol/mol ABP, respectively. While the basis for the lack of IAA-soluble ABP binding in some preparations is not known, the high affinity binding of IAA to "active" soluble pea and bean ABP preparations in conditions of equilibrium dialysis is quantitatively similar (in terms of K_d values and stoichiometries) to that obtaining in the conditions of the $(NH_4)_2SO_4$ precipitation assay.

DISCUSSION

Pea and bean ABPs exactly copurify with RuBPCase in a variety of chromatographic procedures and the purified ABP subunit compositions, electrophoretic purities and IAA-binding stoichiometries provide further evidence for the identity of RuBPCase and ABP. We have found no soluble IAA-binding activity in bean roots, consistent with our identification of ABP as RuBPCase. We do not know at present whether high affinity auxin-binding is a general property of RuBPCases. We have been unable to detect IAA-binding [using the $(NH_4)_2SO_4$ precipitation assay] to homogeneous spinach and silver beet RuBPCase, purified by the procedure of Wishnick and Lane (37). However this apparent lack of IAA binding may be due to modification of these proteins during isolation since it is possible, reversibly and also apparently irreversibly, to abolish IAA binding to pea and bean ABPs without abolition of RuBPCase. Alternatively this auxin-binding phenomenon may be peculiar to specific RuBPCases and/or to the developmental/physiological state of the plant tissue from which the RuBPCase is isolated. Analysis of plant tissues for soluble auxin-binding proteins, such as pea and bean ABPs, may be complicated by the presence of low mol wt inhibitors of IAA binding of the kind found in this study. A "supernatant factor" from maize coleoptiles inhibits NAA- and IAA-binding to a membrane-located auxin-binding site (28) and is not endogenous IAA (27). The nature of the inhibiting fractions obtained from pea and bean leaves in the present study and their relationship to maize "supernatant factor" are not known. Inhibitory material resolved from bean ABP by gel filtration precipitates at 100% $(NH_4)_2SO_4$ saturation at pH 8.0 and is therefore unlikely to be IAA. We have extracted RuBPCase from 17-day-old maize leaves but have found no associated soluble IAA-binding activity before or after gel filtration (performed as for pea and bean ABP preparations).

Given the high incidence of RuBPCase, if this enzyme has a high affinity for IAA *in vivo* then the theoretical consequences for auxin sequestration and translocation are considerable. In 12-day-old primary leaves of dwarf beans RuBPCase is present at about 10^{-5} mol/kg and free IAA at about 10^{-7} mol/kg (34). For the purposes of argument we can conservatively translate these levels into concentrations in a one-compartment system occupying 10% of the cell volume. If the RuBPCase (concentration 10^{-4} M) has a K_d of 10^{-6} M for IAA (total concentration 10^{-6} M) then the ratio of nonbound IAA to RuBPCase-bound IAA will be about 0.01. Apart from compartmentation considerations the critical problem is whether the high affinity of RuBPCase for IAA that is demonstrable *in vitro* also obtains *in vivo*.

The affinity of pea and bean RuBPCase for IAA has been determined by two equilibrium procedures, namely the $(NH_4)_2SO_4$ precipitation procedure and by equilibrium dialysis. The K_d values for IAA, determined by either procedure, are about 10^{-6} M and the high affinity IAA/RuBPCase stoichiometries at saturation are of the order of 1 mol/mol as determined by either procedure for both bean and pea RuBPCase. Nevertheless the possiblity that IAA binding by RuBPCase is conferred by the procedures used must be seriously considered. The $(NH_4)_2SO_4$ precipitation assay conditions could favor hydrophobic IAA-protein interactions (19). However specific IAA-ABP binding in the standard assay at 30 C is only 22% of that at 0 C suggesting that hydrophobic forces are not predominant in this interaction. It is possible that an insolubilized form of ABP (*e.g.* membrane-bound ABP) could bind IAA *in vivo*. RuBPCase (ABP) can be a major peripheral thylakoid protein (12) but we have not detected high affinity IAA-binding to thylakoids. The $(NH_4)_2SO_4$ precipitation assay has been applied as a reliable procedure in other ligand-binding studies (4, 10, 23, 24). We have found that the $(NH_4)_2SO_4$ precipitation assay and equilibrium dialysis yield qualitatively similar results when applied to the binding of cyclic AMP to a wheat embryo cyclic AMP-binding protein (Polya, in preparation), to binding of cyclic AMP to *Saccharomyces* glyceraldehyde-3-P dehydrogenase isozymes (4), and to cytokinin binding to a soluble wheat germ cytokinin-binding protein (23, 24). It has been pointed out that while the $(NH_4)_2SO_4$ precipitation assay may enhance the level of a preexisting protein-IAA interaction it is unlikely to artifactually produce one where none intrinsically exists (19). The binding of IAA to ABP in equilibrium dialysis conditions supports this view. However the absolute lack of IAA-binding (as determined by equilibrium dialysis) in experiments with many preparations that otherwise bound IAA in the conditions of the $(NH_4)_2SO_4$ precipitation assay suggests that there are conformers of soluble (as opposed to insolubilized) RuBPCase that are active or inactive with respect to IAA-binding. Temperature-dependent interconversions of catalytically and conformationally distinct forms of RuBPCase have been demonstrated (35) and the existence of kinetically different forms of RuBPCase is well established (17, 30). However, because we do not know the basis of the variability between our preparations we cannot yet conclude that those preparations that bind IAA in equilibrium dialysis experiments contain RuBPCase conformers likely to be present *in vivo*. In addition to a high affinity for IAA, a further criterion for a physiological function of IAA binding to ABP would be an appropriate ligand specificity. The accompanying paper (33) describes the specificity of ABP for auxins, antiauxins, and auxin transport inhibitors.

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