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

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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 (NH₄)₂SO₄ 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 (NH₄)₂SO₄ 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 micosomal 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. The accompanying paper (33) describes the ligand specificities of the soluble bean ABP.

MATERIALS AND METHODS

Purification of Pea and Bean ABP. Presoaked 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 H₂O 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 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 H₂O, 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 70°C in a Waring Blender for 1 min. The homogenate was filtered through Miracloth and the filtrate centrifuged at 35,000g for 40 min. The supernatant was brought to 30% (NH₄)₂SO₄ saturation, the resulting precipitate being collected by centrifugation and discarded. The resulting supernatant was brought to 50% (NH₄)₂SO₄ 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 × 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 × 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 (NH₄)₂SO₄ (in buffer A) (Fig. 1b). The active fractions were pooled, concentrated by precipitation at 80% (NH₄)₂SO₄ 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-hydroxysulfonic acid); Bis-Tris: bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane.
ABP preparations for equilibrium dialysis experiments, 2-mercaptoethanol was omitted and for bean ABP preparations for equilibrium dialysis the DEAE-Sepharose step was also omitted to avoid consequent lability of the bean ABP. Polycaract (insoluble PVP) was also added to the homogenizing medium in some pea and bean ABP preparations for equilibrium dialysis experiments.

(NH₄)₂SO₄ Precipitation Assay. The (NH₄)₂SO₄ precipitation procedure employed to measure IAA binding was similar to that

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FIG. 4. Sequential purification of bean ABP/RuBPCase by column chromatography on (a) a Sephacryl S-200 column (2.5 cm × 70 cm) eluted with buffer C (50 mM Tris [Cl−, pH 8.0], 10 mM EDTA); (b) a DEAE-Sephacel column (4 cm × 9 cm) eluted with a linear gradient of increasing [(NH₄)₂SO₄] in buffer C; (c) a DEAE-Sephacel column (4.9 cm × 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. (○—○): A₀₂₆₀; (■—■): 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 (ΔA₂₆₀/min); (□—□): eluate pH.

described previously (32). The assay medium (1 ml total volume) contained 2 × 10⁻⁷ M IAA (specific radioactivity of [2,⁴¹⁴C]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 (NH₄)₂SO₄. The final pH of the assay was 8.0 and assays were routinely conducted at 4 °C. The order of additions was [2,⁴¹⁴C]IAA, protein and finally (NH₄)₂SO₄ solution. The protein precipitate was collected by centrifugation at 12,000g 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 H₂O, 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.

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 [(NH₄)₂SO₄]; (c) DEAE-Sephacel eluted with a gradient of decreasing pH. All other details as for Figure 4.

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 [2,⁴¹⁴C]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 [2,⁴¹⁴C]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 [2,⁴¹⁴C]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.).
Washed with equilibrated mM (\(\text{NH}_4\))_2SO_4 in 50 mM Tris (Cl\(^-\), pH 8.0). Pea ABP (purified on DEAE-Sephalc) was applied to the column in 25% (w/v) (\(\text{NH}_4\))_2SO_4 in 50 mM Tris (Cl\(^-\), pH 8.0). The column was initially washed with a linear gradient of decreasing (\(\text{NH}_4\))_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 \(2 \times 10^{-5}\) M); ( - - - - ); RuBPCase (\(\Delta A_{340/\text{min}}\)).

FIG. 6. Purification of pea leaf ABP/RuBPCase by hydrophobic chromatography. A column (3.5 cm \(\times\) 5 cm) of octyl Sepharose CL-4B was equilibrated with 25% (w/v) (\(\text{NH}_4\))_2SO_4 in 50 mM Tris (Cl\(^-\), pH 8.0). Pea ABP (purified on DEAE-Sephalc) was applied to the column in 25% (w/v) (\(\text{NH}_4\))_2SO_4 in 50 mM Tris (Cl\(^-\), pH 8.0). The column was initially washed with a linear gradient of decreasing (\(\text{NH}_4\))_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 \(2 \times 10^{-5}\) M); ( - - - - ); RuBPCase (\(\Delta A_{340/\text{min}}\)).

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 gels 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 \(\mu\)l of the enzyme and \(\Delta A_{340/\text{min}}\) 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).

Chemicals. Sephacryl S-200, DEAE-Sephalc, 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 Radiocchemical 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-Sephalc (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 (\(\text{NH}_4\))_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-Sephalc eluted with a 0-0.2 M (\(\text{NH}_4\))_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 \(\times\) 10^8 dalton protein in the standard assay with an IAA concentration of 2 \(\times\) 10^{-6} M (Table I). The pea leaf ABP was purified to constant specific activity on gel filtration.
The pea ABP and pea RuBPCase exactly co-chromatograph in a chromatographic sequence involving gel filtration through Sephacryl S-200 (Fig. 5a). \((\text{NH}_4)_2\text{SO}_4\) 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\(^2\) x 5 cm) by distilled H\(_2\)O after application and washing as for the octyl Sepharose CL-4B column. In addition to precipitating in the same \((\text{NH}_4)_2\text{SO}_4\), 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 \(K_a\) for the high affinity component being 5 \(\times\) 10\(^{-7}\) mol. 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 \(K_a\) of the purified bean ABP for IAA is 0.8 (±0.3) \(\times\) 10\(^{-6}\) mol (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

**Fig. 9.** Scatchard analysis of IAA binding to ABP in the conditions of the standard \((\text{NH}_4)_2\text{SO}_4\) 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), \((\text{NH}_4)_2\text{SO}_4\) concentration gradient elution from DEAE-Sephacel (Fig. 4b) and pH gradient elution from DEAE-Sephacel (Fig. 4c).
stoichiometries may represent underestimates for reasons associ-
ated 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 $1.3 \times 10^{-8}$ M for IAA. Assuming that
ABP and RuBPCase (mol wt 550,000) are identical, the stoichi-
ometry 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. Accord-
ingly, 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. At-
ttempts to find conditions that would stabilize IAA-binding activity
were unsuccessful—inclusion of 10 mg/ml casein, 50% saturated
(NH$_4$)$_2$SO$_4$, 10% (w/v) glycerol, 10% sucrose, or 5 mM
ATP-2.5 mM MgSO$_4$ in the stored preparations fail to signifi-
cantly 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 mM (NH$_4$)$_2$SO$_4$ 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 appar-
etly 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$_4$)$_2$SO$_4$ precipitation assay. In this assay the IAA-insul-
obilized ABP complex is pelleted through a solution containing the
equilibrium concentration of free IAA, i.e. the equilibrium distri-
bution of ligand being examined is that obtaining after addition
of saturated (NH$_4$)$_2$SO$_4$ solution. The [2-$^14$C]IAA bound to the
insolubilized ABP is largely displaced by addition of excess un-
labeled IAA either before or after addition of saturated (NH$_4$)$_2$SO$_4$
solution and ABP is not inactivated by repeated (NH$_4$)$_2$SO$_4$
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$_4$)$_2$SO$_4$ precipitation assay has been applied as a
reliable procedure in other ligand-binding studies (4, 10, 22, 23).
However, given the evidence for the identity of RuBPCase and Ru-
BPCase an important question is whether IAA can bind to the
soluble as well as to the insolubilized form of the protein.

A large number of equilibrium dialysis experiments were con-
ducted in an effort to quantitate IAA-binding to soluble ABP. In
22 extensive equilibrium dialysis experiments involving 20 separ-
ate 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$_4$)$_2$SO$_4$ 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 for IAA binding to an "active" soluble bean ABP pre-
paration as determined by equilibrium dialysis. Both high and
low affinity IAA binding is apparent. The high affinity $K_d$ value
is $8 \times 10^{-9}$ 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 $5 \times 10^{-7}$ 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$_4$)$_2$SO$_4$ 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 Ru-
BPCase and ABP. We have found no soluble IAA-binding activity
in bean roots, consistent with our identification of ABP as Ru-
BPCase. We do not know what accounts for high affinity auxin-
binding is a general property of RuBPCases. We have been unable
to detect IAA-binding using the (NH$_4$)$_2$SO$_4$ 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 pheno-
menon may be peculiar to specific RuBPCases and/or to the devel-
opmental/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 "superunatant factor" from maize
coleoptiles inhibits NAA- and IAA-binding to a membrane-lo-
cated auxin-binding site (28) and is not endogenous IAA (27). The
nature of the inhibitory fractions obtained from pea and bean
leaves in the present study and their relationship to maize "super-
unatant factor" are not known. Inhibitory material resolved from
bean ABP by gel filtration precipitates at 100% (NH$_4$)$_2$SO$_4$
satu-
ration 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).

![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 S.D.](https://example.com/fig10.png)
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^{-4} mol/kg and free IAA at about 10^{-5} mol/kg (34). For the purposes of argument we can conservatively translate these levels into concentration in a one-compartment system occupying 10% of the cell volume. If the RuBPCase (concentration 10^{-3} 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 possibility 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 determined 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 glyceroldehyde-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 artificially 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, anti-auxins, and auxin transport inhibitors.

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