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Properties of a High-affinity Cytokinin-binding Protein from Wheat Germ

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Abstract. A soluble protein that interacts with a range of cytokinins was extensively purified from wheat (*Triticum aestivum* L.) germ. This protein has a K_d for kinetin of 2×10^{-7} M. The binding of kinetin to the protein is inhibited by low concentrations of synthetic and naturally-occurring cytokinins including N^6-benzyladenine, N^6-benzyladenosine, kinetin riboside, N^6-dimethylallyladenine, N^6-dimethylallyladenosine, zeatin, zeatin riboside, N^6-dimethyladenine and N^6-dimethyladenosine. Adenine, adenosine and several non-N^6-substituted adenine derivatives were ineffective as inhibitors of kinetin binding. While N^6-butyryl-3′,5′-cyclic AMP, N^6,2-O′-dibutyryl-3′,5′-cyclic AMP and 2′,3′-cyclic AMP inhibited binding of kinetin to the protein, 3′,5′-cyclic AMP was ineffective. The kinetin-binding protein is heat-labile and pronase-sensitive. Kinetin-binding activity exactly cochromatographs with a single peak of carbohydrate and protein on gel-filtration and is displaced from concanavalin A-Sepharose 4B by α-methylglucoside. On gel filtration, the kinetin-binding protein behaves as a soluble protein with an apparent molecular weight of 180,000 daltons.

Key words: Cytokinin binding – Glycoprotein – *Triticum*.

Introduction

One approach to establishing the molecular basis of hormone effects in higher plants is to isolate high-affinity hormone-binding macromolecules and to attempt to establish the functional consequences of hormone binding (for review see Kende and Gardner, 1976). High-affinity auxin-binding to plant membranes is well established (Ray et al., 1977; Ray, 1977; Venis, 1977) and some success has been achieved recently in the resolution of soluble (or solubilized) high-affinity auxin-binding proteins from higher plants (Venis, 1977; Wardrop and Polya, 1977; Roy and Biswas, 1977). However no function can yet be unambiguously assigned to these proteins although in-vitro reconstitution experiments with an auxin-binding protein have indicated transcription modification as a possible molecular mechanism of auxin action (Roy and Biswas, 1977). While hypotheses have been advanced for the action of cytokinins at the level of gene expression (see review and discussion by Burrows, 1975), the examination of such hypotheses in vitro will require detection and isolation of the cytokinin receptor(s) of higher plants.

High-affinity cytokinin-binding to wheat-germ ribosomes has been demonstrated (Fox and Erion, 1975). Cytokinin-binding to ribosomes from Chinese cabbage leaves (Berridge et al., 1970) and tobacco callus (Fox and Erion, 1975) has also been demonstrated but in these cases the affinity constants were not determined. A 5000-dalton cytokinin-binding polypeptide has been purified from tobacco leaves (Takegami and Yoshida, 1975; Yoshida and Takegami, 1977) and shown to bind to 40S ribosomal subunits (Takegami and Yoshida, 1977). The tobacco-leaf cytokinin-binding protein is similar in molecular size to a Ca^{2+}- and cytokinin-binding glycoprotein purified from aquatic fungi (Le John and Cameron, 1973). However the tobacco-leaf protein has a very high K_d for N^6-benzyladenine (40 μM) (Yoshida and Takegami, 1977). In comparison, high-affinity cytokinin-binding proteins isolated from the ribosomal and postribosomal supernatant fractions from wheat germ have K_d's for N^6-benzyladenine that are 100 times lower and may thus be visualized as having cytokinin-receptor functions (Fox and Erion, 1977). In the present paper we describe the partial purification and characterization of a soluble, high-affinity, cytokinin-binding protein that is present in large amounts in wheat germ.

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Materials and Methods

Plant Material and Chemicals

Raw (non-roasted) wheat germ (percent hydration, 13%) was purchased from Heidelberg Health Foods, Melbourne, Australia. *Trans*-6-(4-hydroxy-3-methylbut-2-enyl)-aminopurine (*trans*-zeatin) was obtained from Calbiochem, San Diego, Cal., USA. All other adenine derivatives, including cytokinins and adenine-antimetabolites, and α-methylglucoside were obtained from Sigma Chemical Co., St. Louis, Mo., USA. Bovine thyroglobulin, rabbit-muscle pyruvate kinase, bovine serum albumin, egg-white Lysozyme (25,000 units/mg) and yeast alcohol dehydrogenase were also obtained from Sigma Chemical Co., pancreatic deoxyribonuclease (42,750 units/mg) and pronase (45 units/mg) from Calbiochem and ribonuclease T1 (5000 units/mg) from Sankyo Co., Tokyo, Japan. Crystalline rabbit-muscle phosphoglycerokinase was provided by Dr. R.K. Scopes, Department of Biochemistry, La Trobe University. [8-^{14}C]Kinetin (15 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, Bucks., UK; Ultrogel AcA 34 from LKB-Produckter A.B., Bromma, Sweden; Agarose A 0.5 m (50–100 mesh) from Bio-Rad Labs., Richmond, Cal., USA; Sephacryl S-200 (Superfine) from Pharmacia Fine Chemicals, Uppsala, Sweden; DEAE-cellulose (Cellex-D) and phosphocellulose (Cellex-P) from Sigma Chemical Co; and Avicel PH-101 cellulose from J. Beith and Co., Melbourne, Australia. Concanavalin A-Sepharose 4B was kindly provided by Professor R.B. Knox, Botany Department, Melbourne University. All other chemicals were of analytical reagent grade.

Protein, Carbohydrate and Enzyme Assays

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Carbohydrate was determined by the phenol-sulphuric acid procedure of Dubois et al. (1956) as modified by Immers (1964); D-glucose was used as a standard. Pyruvate kinase (EC 2.7.1.40) and phosphoglycerate kinase (EC 2.7.2.3) were measured as described by Scopes (1977). Alcohol dehydrogenase was assayed at 30° C by monitoring initial rates of increase of A_{340} in a medium containing 0.5 mM NAD^+, 0.1 M $(NH_4)_2SO_4$, 0.01 M Tris-glycine (pH 9) and 0.1 M ethanol. 2′,3′-Cyclic AMP phosphodiesterase was assayed at 30° C in a medium (100 µl final assay volume) containing 1 mM 2′,3′-cyclic AMP, 0.2 M $(NH_4)_2SO_4$ and either 0.1 M acetate (Na^+, pH 4.0) or 0.1 M Tris (Cl^-, pH 8.0). Aliquots were taken at various times for analysis by poly (ethyleneimine)-cellulose thin-layer chromatography as described in Ashton and Polya (1975).

Scintillation Counting

Scintillation fluid A, used for counting the radioactivity of aqueous samples, contained 1 volume of Triton X-114, 3 volumes of xylene, 0.3% (w/v) PPO (2,5-diphenyloxazole) and 0.02% (w/v) dimethyl-POPOP (1,4-bis-(4-methyl-5-phenyloxazol-2-yl)-benzene). Aqueous samples from kinetin-binding assays were counted in this scintillation fluid; the efficiency was 68%. Scintillation fluid B, used for counting thin-layer segments (efficiency 39%), contained 0.5% (w/v) PPO and 0.03% (w/v) dimethyl-POPOP in toluene. The samples were counted using a Packard 3003 Series Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Ill., USA).

Thin-layer Chromatography of [8-^{14}C]kinetin

Cellulose thin-layer sheets were obtained from E. Merck, Darmstadt, West Germany. Poly(ethyleneimine)-cellulose thin layers were prepared as described in Ashton and Polya (1975). After the aliquots (20 or 10 µl) had been applied to the thin layers these were dried and developed by ascending chromatography, and segments (1 cm) cut from the developed and dried thin layers were counted in 10 ml scintillation fluid B. The solvents used for thin-layer chromatography were: 5% (w/v) boric acid – 0.5 M ammonium acetate, pH 7.5 (solvent A); 0.5 M ammonium acetate, pH 7.4 (solvent B); isobutyric acid – conc. $NH_4OH - H_2O$, 66:1:33 (v/v) (solvent C).

Kinetin-binding Assay

The procedure employed to measure kinetin binding was similar to that described previously for binding of 3′,5′-cyclic AMP (Polya and Sia, 1976) and IAA-binding (Wardrop and Polya, 1977) assays. Kinetin binding was routinely assayed in triplicate in a medium (final volume 100 µl, after addition of the protein solution to be assayed) containing 12.5 mM Tris (Cl^-, pH 8.0), 125 mM $(NH_4)_2SO_4$, 0.25% (v/v) 2-mercaptoethanol, 0.5% (v/v) dimethylsulphoxide and 0.1 nmol kinetin (specific activity of [8-^{14}C]kinetin: 15 mCi/mmol). After incubation with the 25 µl protein sample at 0° C in an ice bath for 10 min, 0.9 ml of 100% saturated $(NH_4)_2SO_4$ solution was added. After a further incubation for 5 min at 0° C, the resulting protein precipitate was collected by centrifugation at 0° C at 12,000 × *g* for 15 min in the SS-34 rotor of a Sorvall RC2-B centrifuge. The supernatant was removed carefully by aspiration. The pellet was solubilized in 1% (w/v) sodium dodecylsulphate in H_2O, added to 10 ml of scintillation fluid A, and counted. Assay counts were routinely corrected by subtraction of counts obtained in the same assay conducted in the presence of 10 nmol unlabelled kinetin to obtain a measure of specific kinetin binding. Specific kinetin binding was routinely 80% of total kinetin binding. Standard deviations in the binding assays were ca. 2.5% of the means.

Equilibrium dialysis was performed using a Bel-Art Multicavity Microdialysis Cell (Equilibrium Type, Model 373, cell volume 0.5 ml) obtained from Constantex, Los Angeles, Cal., USA. Equilibrium was achieved by rotating the dialysis cell at 50 rpm for 60 h at 4° C. In equilibrium-dialysis experiments the buffer (0.4 ml) on both sides of the dialysis membrane contained 25 mM Tris (Cl^-, pH 8.0), 0.05% (v/v) 2-mercaptoethanol, 0.5% (v/v) dimethylsulphoxide and 0.5 M $(NH_4)_2SO_4$.

Purification of the Cytokinin-binding Protein

All purification steps were carried out at 0–4° C. Specific kinetin binding was monifored using the standard assay containing 1.0 nmol [8-^{14}C]kinetin. Wheat germ (100 g) was suspended in 400 ml of an extraction medium (Buffer A) containing 50 mM Tris (Cl^-, pH 8.0) and 0.1% (v/v) 2-mercaptoethanol and homogenized for 1 min at full power using an Ultra-Turrax blender (Janke & Kunkel, Staufen, West Germany). The homogenate was filtered through 1 layer of muslin and then through Miracloth (Calbiochem, Richmond, Ca., USA). The filtered homogenate was then centrifuged at 45,000 × *g* for 90 min. The supernatant was brought to 50% $(NH_4)_2SO_4$ saturation, the resulting precipitate being collected by centrifugation and discarded. The supernatant was brought to 90% $(NH_4)_2SO_4$ saturation and the precipitate collected by centrifugation and dissolved in a minimum volume of Buffer B, containing 50 mM Tris (Cl^-, pH 8.0), 0.5 M $(NH_4)_2SO_4$ and 0.1% (v/v) 2-mercaptoethanol. This concentrated fraction was clarified by centrifugation at 30,000 × *g* for 15 min and the supernatant applied to an agarose A 0.5 m (50–100 mesh) column (3 cm × 85 cm) and eluted with Buffer B (Fig. 1). Active fractions (fractions 12–24 of Fig. 1) were pooled, concentrated by $(NH_4)_2SO_4$ precipitation, and dissolved in a minimum volume

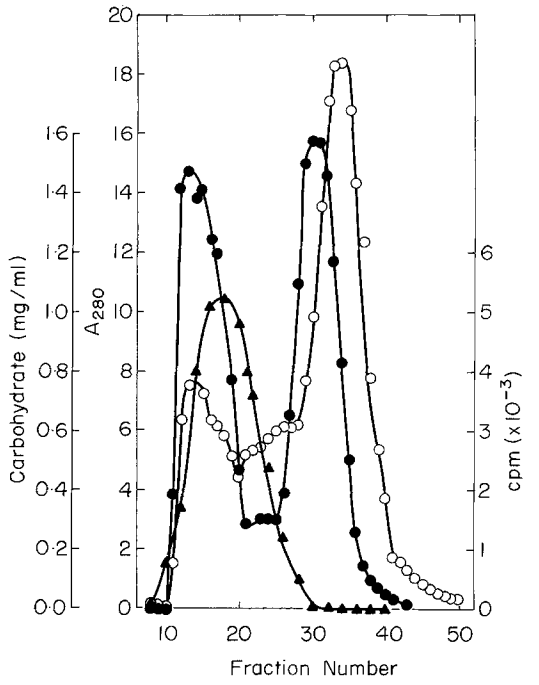

Fig. 1. Elution of the cytokinin-binding protein from an agarose A 0.5 m column in Buffer B: ▲ specific kinetin-binding (cpm); ● carbohydrate (mg glucose-equivalent/ml); ○ absorbance at 280 nm (A_{280}). Specific kinetin binding was determined in the standard assay as described in Materials and Methods

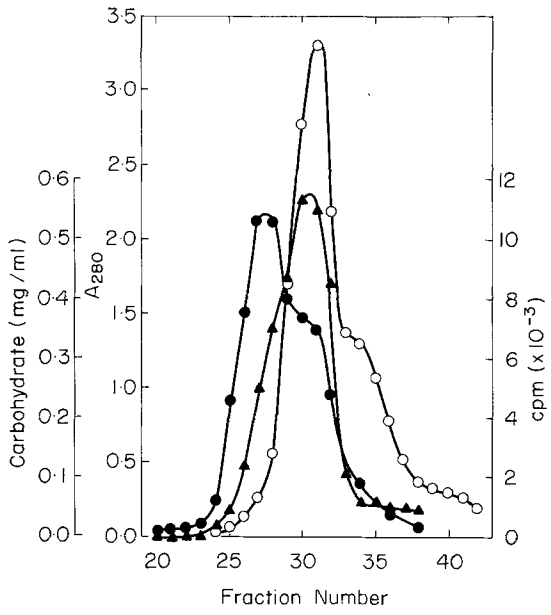

Fig. 3. Elution of the cytokinin-binding protein from a Sephacryl S-200 column in Buffer B. Other details as in legend to Figure 1

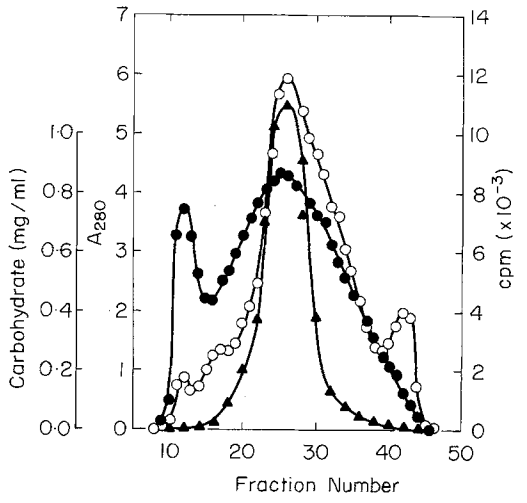

Fig. 2. Elution of the cytokinin-binding protein from an Ultrogel AcA 34 column in Buffer B. Other details as in legend to Figure 1

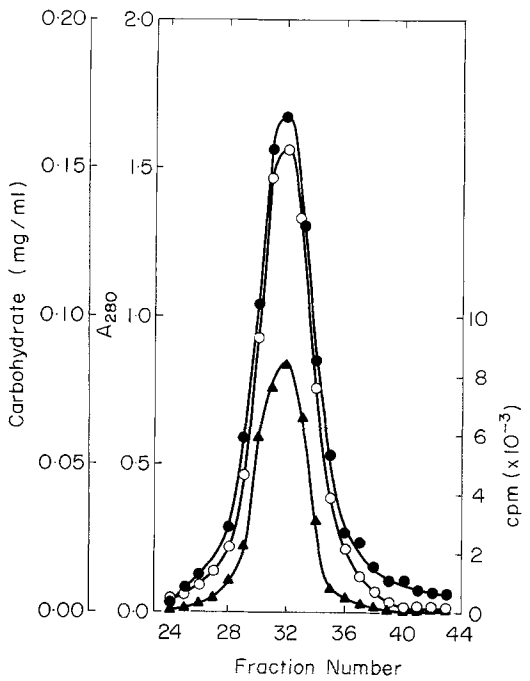

Fig. 4. Final elution of the cytokinin-binding protein from a Sephacryl S-200 column in Buffer B. Other details as in legend to Figure 1

of Buffer B. After centrifugation at 30,000 × *g* for 15 min to clarify, this concentrated solution was applied to an Ultrogel AcA 34 column (2 cm × 62 cm) and eluted with Buffer B (Fig. 2). Active fractions from this gel filtration procedure (fractions 20–31 of Fig. 2) were pooled, concentrated by $(NH_4)_2SO_4$ precipitation, dissolved in Buffer B, and applied to a Sephacryl S-200 column (3 cm × 72 cm) which was then eluted with Buffer B (Fig. 3). The peak fractions (fractions 30–32 of Fig. 3) were pooled, concentrated as before, re-applied. to the Sephacryl S-200 column, and eluted with Buffer B. This final chromatographic step yielded coincident peaks of carbohydrate, A_{280} and kinetin-binding activity (Fig. 4). The active fractions from this final step were pooled and used

Table 1. Purification of the cytokinin-binding protein from wheat germ

100 g wheat germ was extracted; the yields at each stage have been corrected upwards for losses caused by sampling for analysis. Specific kinetin binding was determined as described in Materials and Methods but with 1.0 nmol [8-^{14}C kinetin in the assay. Specific kinetin binding was calculated from the [8-^{14}C]kinetin displaced in the assay by inclusion of 10 nmol unlabelled kinetin.

Fractionation stage	Protein (mg)	Carbohydrate (mg glucose equivalent)	Specific binding (nmol kinetin)	Specific binding (nmol kinetin bound/mg protein)
Homogenate	7,584	20,192	1,564	0.2
High-speed supernatant	6,568	10,744	1,801	0.3
50–90% $(NH_4)_2SO_4$ cut	1,613	528	1,800	1.1
Agarose A 0.5 m	1,328	175	1,818	1.4
Ultrogel AcA34	309	45	870	2.8
Sephacryl S200 (1)	155	7.2	665	4.3
Sephacryl S200 (2)	111	5.0	512	4.6

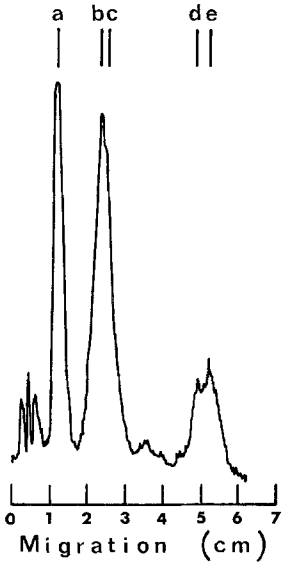

Fig. 5. Dissociating 0.1% sodium dodecylsulphate – 10% polyacrylamide gel electrophoresis of the cytokinin-binding protein. The densitometer trace of the disk gel was obtained as described in Materials and Methods. The positions of the major discrete polypeptide bands (a to e) are indicated. The closely-migrating bands (b) and (c) were incompletely resolved by the gel scanner. 20 µg of the final cytokinin-binding protein preparation was applied for this electrophoresis

in the experiments described in this paper. The overall purification schedule is given in Table 1. Non-dissociating polyacrylamide gel electrophoresis and 0.1% sodium dodecylsulphate-polyacrylamide gel electrophoresis of the final protein preparations were conducted as described previously (Polya, 1975). Densitometer tracings of disk gels at 600 nm were made using a Gilford Model 2520 Gel Scanner attached to a Gilford Model 240 Spectrophotometer (Gilford Instrument Labs., Oberlin, O., USA).

Results

Purification of the Kinetin-binding Protein

The purification procedure resulted in a 20-fold purification of the kinetin-binding activity on a protein basis (30-fold on a carbohydrate basis relative to the $(NH_4)_2SO_4$ cut) and an overall yield of 30%. The specific kinetin-binding activity of the final preparation (as measured in the standard assay with 10^{-6} M final [8-^{14}C]kinetin) was 4.6 nmol kinetin bound/mg of protein. Non-dissociating 10% polyacrylamide gel electrophoresis of the kinetin-binding protein at pH 9 in 0.1 M Tris-glycine yielded only one broad zone of material staining with Coomassie Blue and migrating to the anode. However 10% polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecylsulphate (Fig. 5) gave a major sharp band (a) (molecular weight 60,000 ± 5,000 daltons), two closely-migrating bands (b and c) (MW's 46,000 and 43,000, respectively) and two much more weakly staining bands (d and e) (MW's 14,000 and 13,000, respectively) (Fig. 5). Accordingly the final preparations may not be homogeneous. Attempts at dissociation and reassociation of subunits to resolve this matter have been unsuccessful to date.

Solutions of the cytokinin-binding protein (1–20 mg/ml) become turbid at low ionic strength (at 0–4° C) but remain clear in the presence of 0.5 M $(NH_4)_2SO_4$. Overnight dialysis at 4° C of solutions of the protein (2–20 mg/ml in ca. 5 ml of Buffer B) against ca. 4 l of Buffer A results in a gelatinous precipitate. This behaviour at low ionic strength complicated attempts at purification by ion-exchange chromatography. The protein washed through DEAE-cellulose at low ionic strength (in Buffer A) and no activity was subsequently eluted by Buffer B (Buffer A containing 0.5 M $(NH_4)_2SO_4$). The protein is retained by phosphocellulose (equilibrated with Buffer A) and can be subsequently eluted at high ionic strength in Buffer B. However, chromatography on DEAE-cellulose or phosphocellulose columns resulted in losses of over 90% of cytokinin-binding activity. The cytokinin-binding protein is retained by cellulose (Avicel PH-101) in a buffer containing 50 mM Tris (Cl^-, pH 8.0) and 2.7 M $(NH_4)_2SO_4$ and can be eluted as a turbid solution at low ionic strength by the same buffer containing no $(NH_4)_2SO_4$. This latter hydrophobic chromatography procedure was discarded be-

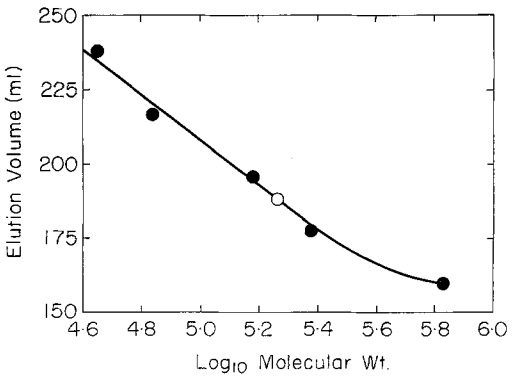

Fig. 6. Estimation of the molecular size of the cytokinin-binding protein by gel filtration in Buffer B through a calibrated Sephacryl S-200 column (3 cm × 72 cm). The elution volumes of proteins of known molecular weight (solid circles) were determined. In decreasing order of elution volume the protein standards were rabbit muscle phosphoglycerate kinase, bovine serum albumin, yeast alcohol dehydrogenase, rabbit muscle pyruvate kinase and bovine thyroglobulin. The elution volume of the cytokinin-binding protein is indicated by the open circle

Table 2. Effect of various pretreatments on kinetin binding

The kinetin-binding protein (2.0 mg/ml) was preincubated in Buffer B in the conditions indicated before determination in triplicate of specific kinetin binding in the standard assay system. Specific [^{14}C]kinetin binding is expressed as percent of the specific binding of the control stored at 0° (1238 cpm).

Pretreatment	Specific kinetin-binding (% of control)
None	100
30° C/30 min	102
30° C/180 min	92
100° C/5 min	9
1% sodium dodecylsulphate/0° C/15 min	0
1 mg/ml DNase, 5 mM $MgCl_2$/30° C/30 min	92
1 mg/ml RNase/30° C/30 min	105
1 mg/ml lysozyme/30° C/30 min	112
1 mg/ml pronase/30° C/30 min	80
2 mg/ml pronase/30° C/180 min	14

cause of variable retention of the protein in conditions of high ionic strength.

Since the cytokinin-binding protein co-purified with carbohydrate (Figs. 1–4) and the final preparation contained ca. 5% carbohydrate by weight, affinity chromatography using concanavalin A-Sepharose 4B was attempted. The protein was bound to a column of concanavalin A-Sepharose 4B (1.5 cm × 6 cm) in buffer B containing 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 1 mM $MgCl_2$, and was eluted by the same buffer containing 0.5 M α-methylglucoside. However the large amounts of the cytokinin-binding protein (ca. 3 mg/g wheat embryo; see Table 1) rendered this procedure infeasible for large-scale purifications.

The final large-scale purification procedure adopted relied on successive gel-filtration steps to purify the protein from higher- and lower-molecular-weight carbohydrate and protein contaminants (Figs. 1–4, Table 1). Precipitation of the protein at low ionic strength (encountered in ion-exchange chromatography experiments) was avoided by conducting gel filtration elutions in Buffer B (which contained 0.5 M $(NH_4)_2SO_4$). The cytokinin-binding protein behaved as a soluble protein with an apparent molecular size of 180,000 ± 20,000 daltons on gel filtration through calibrated Sephacryl S-200 columns in Buffer B, eluting between yeast alcohol dehydrogenase and rabbit-muscle pyruvate kinase (Fig. 6).

Inactivation of the Cytokinin-binding Protein

The cytokinin-binding material is heat-labile and is inactivated by 1% sodium dodecylsulphate (Table 2). Incubation with deoxyribonuclease, lysozyme and ribonuclease has little effect on cytokinin-binding activity but pretreatment with pronase largely abolished cytokinin binding (Table 2). Thus the kinetin-binding material appears to be a protein. While $HgCl_2$ and N-ethylmaleimide at 1 mM concentration inhibited kinetin binding by 34% and 55%, respectively, 1 mM iodoacetamide, Cd acetate and $Pb(NO_3)_2$ were not inhibitory.

Kinetin Binding to the Cytokinin-binding Protein

The $(NH_4)_2SO_4$ precipitation assay measures the amount of [^{14}C]kinetin bound to the insolubilized binding protein at equilibrium in the conditions used. The insolubilized kinetin-protein complex was pelleted through a solution containing the equilibrium concentration of free kinetin. Prolongation of the incubation period at 0° C after addition of 100% saturated $(NH_4)_2SO_4$ (to 20 min) did not increase kinetin binding. When 10 nmol unlabeled kinetin was added to the standard assay after addition of 100% saturated $(NH_4)_2SO_4$, the [^{14}C]kinetin displaced was 92% of that obtaining when 10 nmol unlabelled kinetin was included before precipitation of the protein. Since only 10–50 μg of cytokinin-binding protein was normally added in the assays, the pellet formed after centrifugation was too small to be seen. Each test tube (3 ml) was therefore marked and appropriately placed in the angle-head SS-34 rotor prior to centrifugation, to permit subsequent aspiration from the pellet-free side of the test tube. The smallness of the pellet also meant that very little of the equilibrium solution was occluded in the pellet and accordingly

Table 3. pH-dependence of specific kinetin binding

The pH-dependence of specific kinetin binding was determined by including 50 mM acetate (Na^+) (pH 4.0, 4.5 or 5.0), 50 mM maleate ($tris^+$) (pH 6.0, 6.3 or 6.7), 50 mM Tris (Cl^-) (pH 7.0, 7.5, 8.0, 8.5 or 9.0) or 50 mM Tris-glycine (pH 9.2) in the standard assay. The specific binding at these different assay pH values is given as a percent of control specific binding at pH 8.0 (997 cpm)

Assay pH	Specific kinetin binding (% of control)
4.0	25
4.5	53
5.0	82
5.5	92
6.0	93
6.3	105
6.7	105
7.0	109
7.5	95
8.0	100
8.5	110
9.0	90
9.2	95

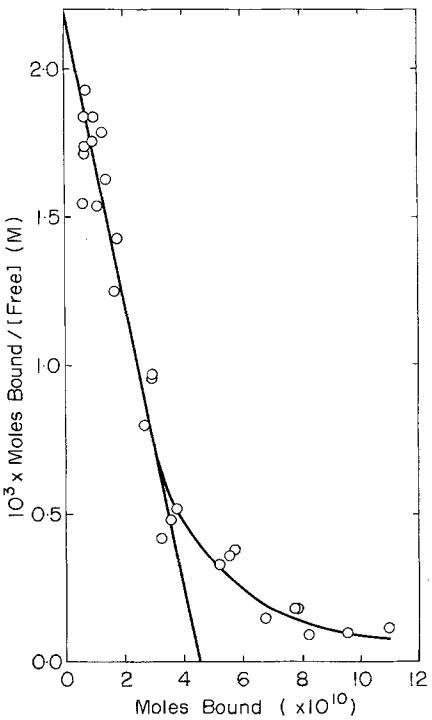

Fig. 7. Scatchard plot for kinetin binding to the cytokinin-binding protein in the conditions of the standard assay. Kinetin-binding was determined radiochemically at a variety of kinetin concentrations and was corrected by subtraction of counts obtained in the standard assay in the absence of added protein. The ratio mol kinetin bound/concentration of free kinetin in the final 1-ml assay volume is plotted versus moles kinetin bound. The apparent high-affinity dissociation constant (K_d) of the protein for kinetin was calculated from the negative reciprocal of the slope

carrier protein was not included in the assays. Note that the cytokinin-binding protein does not lose activity even on repeated precipitation with $(NH_4)_2SO_4$ (Table 1). Kinetin binding and specific kinetin binding were linear functions of the concentration of the kinetin-binding protein preparation (up to 2.5 mg/ml) added to the standard assay.

The radioactive compound bound to protein in the assays was kinetin (rather than a kinetin degradation product) as determined by chromatographic analysis of bound kinetin. The bound radioactivity measured in the standard binding assay comigrated with [8-^{14}C]kinetin on ascending chromatography on both poly(ethyleneimine)-cellulose thin layers (developed with Solvent A or solvent B) and cellulose thin layers (developed with solvent C) (see Materials and Methods). No evidence for other radioactive compounds was found in these analyses. The pH-dependence of specific kinetin binding was examined over a range of pH 4.0 to pH 9.2. While specific kinetin binding at pH values 4.0, 4.5 and 5.0 was only 25%, 53% and 82% of binding at pH 8.0, respectively, specific kinetin binding was relatively pH-independent in the pH range of 5.5 to 9.2 (Table 3).

The apparent dissociation constant (K_d) of the cytokinin-binding protein for kinetin in the conditions of the standard binding assay was estimated by determining the amount of bound and free kinetin at various kinetin concentrations and constructing a Scatchard plot (Fig. 7). High- and low-affinity binding of kinetin is apparent. The apparent K_d for high-affinity binding is $2.1 \pm 0.5 \times 10^{-7}$ M. At saturation 10.0 nmol of kinetin were bound to high affinity sites per mg of protein (protein concentration was determined for this purpose by the modified biuret-Folin procedure of Dorsey et al. (1977) which gives equal absorbance with different proteins). This ratio would correspond to ca. 1.7 binding sites per 180,000 daltons glycoprotein if the preparations were homogeneous. The apparent K_d for kinetin of the soluble protein in the presence of 0.5 M $(NH_4)_2SO_4$ was $7.5 \pm 2.0 \times 10^{-6}$ M as determined by equilibrium dialysis; at saturation in these conditions there were ca. 1.6 high affinity sites per 180,000-dalton glycoprotein.

Displacement of Kinetin from the Kinetin-binding Protein by Cytokinins and Cyclic Nucleotides

If the kinetin-binding protein described above is a physiological cytokinin receptor one would expect that other cytokinins should displace kinetin from the protein. Inclusion of low concentrations of a variety of N^6-substituted adenine and adenosine derivatives having cytokinin activity inhibited kinetin bind-

Table 4. Displacement of [8-^{14}C]kinetin from the binding protein by cytokinins

Kinetin-binding was determined in the standard assay. All additions were made to give the indicated final concentrations in the 1-ml assay volume. Inhibition of [8-^{14}C]kinetin binding is expressed as percent of the inhibition produced by inclusion of 10^{-5} M unlabelled kinetin in the assay (1236 cpm displaced).

Addition	Concn. (M)	Inhibition (% control)
Kinetin	10^{-6}	50
	10^{-5}	100
N^6-Benzyladenine	10^{-7}	9
	10^{-6}	95
N^6-Benzyladenosine	10^{-7}	6
	10^{-6}	99
N^6-Dimethylallyladenine	10^{-7}	6
	10^{-6}	93
N^6-Dimethylallyladenosine	10^{-7}	2
	10^{-6}	94
Kinetin riboside	10^{-7}	2
	10^{-6}	88
trans-Zeatin	10^{-6}	6
	10^{-5}	33
Zeatin (mixed isomers)	10^{-5}	28
	10^{-4}	84
Zeatin riboside	10^{-6}	3
	10^{-5}	45
N^6-Dimethyladenine	10^{-6}	−1
	10^{-5}	20
N^6-Dimethyladenosine	10^{-6}	7
	10^{-5}	21
Adenine	10^{-6}	−7
	10^{-5}	4
	10^{-4}	4
Adenosine	10^{-6}	−6
	10^{-5}	1
4-Aminopyrazolo-(3,4d)-pyrimidine	10^{-5}	6
	10^{-4}	8
4-Aminopyrazolo-(3,4d)-pyrimidine-2′-deoxyriboside	10^{-5}	7
	10^{-4}	9

ing to the protein. In contrast, adenine and adenosine and the adenine-antimetabolite 4-aminopyrazolo-(3,4-d)-pyrimidine and its 2′-deoxyriboside were not inhibitory (Table 4). The inhibitory effectiveness of the various cytokinins tested was in the order N^6-benzyladenine, N^6-benzyladenosine, N^6-dimethylallyladenine, N^6-dimethylallyladenosine > kinetin riboside > kinetin > zeatin, zeatin riboside > N^6-dimethyladenine, N^6-dimethyladenosine. An apparent anomaly is the relative ineffectiveness of *trans*-zeatin, zeatin (mixed isomers) and zeatin riboside with respect to N^6-dimethylallyladenine in displacing kinetin from the protein (Table 4). While *trans*-zeatin at 10^{-5} M inhibited kinetin binding by 33%, no significant inhibition was observed when *trans*-zeatin was included in the assay at 10^{-6} M final concentration at assay pH values over a range from pH 4.0 to pH 9.0.

Table 5. Effect of adenine nucleotides on kinetin binding

Kinetin binding was determined in the standard assay containing an additional 5 mM Tris (Cl^-, pH 8.0) in the absence and presence of 0.1 mM adenine nucleotide in the final 1 ml assay volume. Inhibition of [8-^{14}C]kinetin binding is expressed as percent of the effect of 10^{-5} M unlabelled kinetin (1542 cpm displaced).

Addition	Inhibition (% control)
Kinetin (10^{-5} M)	100
5′-AMP	7
3′-AMP	8
2′-AMP	8
5′-ADP	3
5′-ATP	2
3′,5′-cyclic AMP	7
2′,3′-cyclic AMP	43
N^6-Monobutyryl-3′,5′-cyclic AMP	26
N^6,2-O′-Dibutyryl-3′,5′-cyclic AMP	27

Elliott and Murray (1975) have advanced evidence for the action of N^6-,2-O′-dibutyryl-3′,5′-cyclic AMP as a cytokinin and against involvement of 3′,5′-cyclic AMP in induction of betacyanin synthesis by cytokinins in *Amaranthus* seedlings. In the kinetin-binding assay, both N^6-,2-O′-dibutyryl-3′,5′-cyclic AMP and N^6-monobutyryl-3′,5′-cyclic AMP were inhibitory. In contrast, 3′,5′-cyclic AMP and a variety of other adenine nucleotides did not displace kinetin from the cytokinin-binding protein (Table 5). An intriguing observation in this analysis was the inhibition of kinetin-binding by 2′,3′-cyclic AMP – this being the only inhibitory non-N^6-substituted adenine derivative found in this study. The possibility that the cytokinin-binding protein might have a 2′,3′-cyclic AMP phosphodiesterase activity was tested since cytokinins are competitive inhibitors of mammalian 3′,5′-cyclic AMP phosphodiesterase (Hecht et al., 1974) and such enzymes from higher plants also catalyze the hydrolysis of 2′,3′-cyclic AMP (Ashton and Polya, 1975). However no 2′,3′-cyclic AMP phosphodiesterase activity was detected (less than 1 nmol min^{-1} mg^{-1} protein) when the protein was assayed at either pH 5.0 or pH 8.0. The catalytic activity of the cytokinin-binding protein (if any) is as yet unknown.

Discussion

The soluble, high-affinity kinetin-binding protein from wheat germ satisfies some of the major criteria for a physiological, high-affinity cytokinin receptor.

This protein has an apparent K_d of 2×10^{-7} M for the synthetic cytokinin kinetin. Kinetin is specifically displaced from the protein by low concentrations of biologically active synthetic and naturally-occurring cytokinins but not by non-cytokinin adenine derivatives (Tables 4, 5). However from this primary evidence we clearly cannot conclude that this protein is a cytokinin receptor mediating the biological effects of cytokinins in vivo for reasons discussed below.

An apparent anomaly is that zeatin, a naturally-occurring and potent cytokinin, is much less effective than N^6-dimethylallyladenine in displacing kinetin from the protein (Table 4). This would not have been expected from the comparable biological activities of these compounds (Skoog et al., 1967; Skoog and Armstrong, 1970; see Leopold and Kriedemann, 1975, pp. 155–168). However, since we do not know the location and in-vivo chemical milieu of this protein, this anomaly could prove experimentally useful in defining in-vitro simulations of in-vivo conditions (if in fact this protein is a cytokinin receptor).

A hormone-receptor function for a protein implies a functional consequence of hormone binding to the protein and at present we have no knowledge of such a consequence. However the observation of displacement of kinetin from the protein by 2′,3′-cyclic AMP (Table 5) may provide a useful clue as to the function of the protein. Although no catalytic function as a 2′,3′-cyclic AMP phosphodiesterase could be ascribed to this protein, phosphodiesterases have been implicated as sites of action of cytokinins in animal (Hecht et al., 1974; Johnson et al., 1974) and plant systems (Anderson and Cherry, 1969; Cherry and Anderson, 1972; see review by Letham, 1973). The effectiveness of 10^{-4} M N^6-,2-O′-dibutyryl-3′,5′-cyclic AMP (similar to that of 10^{-4} M N^6-monobutyryl-3′,5′-cyclic AMP) in displacing kinetin from the cytokinin-binding protein (Table 5) correlates well with its biological cytokinin activity (Elliott and Murray, 1975).

The cytokinin-binding protein preparations obtained in this study appear homogeneous on the basis of final Sephacryl S-200 gel filtration profiles (Fig. 4). However, the multiplicity of bands on 0.1% sodium dodecylsulphate-polyacrylamide gel electrophoresis (Fig. 5) indicates inhomogeneity of the final preparations. The major 60,000-dalton polypeptide (band (a) of Fig. 5) is further implicated as part of the native cytokinin-binding protein by its disappearance from non-active fractions during purification (data not shown). The subunit composition of the cytokinin-binding protein remains to be determined. There is a large amount of the cytokinin-binding protein in wheat germ (ca. 2.7 mg/g fresh weight; 27 nmol high-affinity kinetin-binding sites/g fresh weight, i.e. a concentration of at least 27 μM for such sites). This is in marked contrast to the very low incidence of hormone receptors in animal systems (Cuatrecasas, 1973, 1974; Kuhn et al., 1975; Yamamoto and Alberts, 1976) and thus argues against a receptor function for this protein analogous to the amplifying functions of animal steroid-hormone receptors or adenylate-cyclase-modifying hormone receptors. This same comment applies to the high-affinity ribosomal and soluble cytokinin-binding proteins of wheat germ described by Fox and Erion (1975, 1977) – the concentration of these proteins can be estimated to be greater than 10^{-6} M. The amount of the relatively low-affinity cytokinin-binding protein obtained from tobacco leaves is 0.5 mg/g fresh weight (Yoshida and Takegami, 1977), corresponding to an intracellular concentration of at least 0.1 mM for this 5,000-dalton protein.

Fox and Erion (1977) have extensively purified a ca. 100,000-dalton protein (CBF-1) from the ribosomal fraction from wheat germ. A very similar protein (possibly identical to CBF-1) and a ca. 30,000-dalton high-affinity cytokinin-binding protein (CBF-2) have also been resolved from the post-ribosomal supernatant fraction from wheat germ (Fox and Erion, 1977). CBF-1 is similar to the soluble cytokinin-binding protein described in the present paper in having a cytokinin-binding site that has a high affinity for biologically-active cytokinins but a low affinity for adenine and adenosine (Fox and Erion, 1977, cf. Table 4); however the two preparations show apparent differences in molecular size, subunit complexity and behaviour on ion-exchange chromatography. The two preparations may accordingly represent two distinct cytokinin-binding proteins or, alternatively, represent different macromolecular complexes containing the same cytokinin-binding polypeptide. Thus while the binding protein described in the present paper co-purifies on gel filtration with a peak of carbohydrate and is eluted from concanavalin A-Sepharose 4B by α-methylglucoside, it is not yet known if the cytokinin-binding polypeptide is glycosylated, is complexed with a glycoprotein, or simply co-purifies with a glycoprotein.

The evidence for an in-vivo ribosomal location of CBF-1 (Fox and Erion, 1977) is equivocal since this protein may have bound to ribosomes in cell-free conditions. In addition to possible non-electrostatic interactions, CBF-1 can bind to both anion- and cation-exchangers (Fox and Erion, 1977). The (intracellular) locations and functions of the high-affinity cytokinin-binding proteins of wheat germ remain to be unambiguously determined. Despite the relatively low affinity for zeatin of this system, the qualified use of the soluble wheat germ cytokinin-binding protein for in-vitro cytokinin detection is suggested.

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