Plant Physiol. (1981) 68, 577-584
0032-0889/81/68/0577/08/$00.50/0

Resolution and Properties of Two High Affinity Cyclic Adenosine 3':5'-monophosphate-Binding Proteins from Wheat Germ[1]

Received for publication June 18, 1980 and in revised form March 3, 1981

GIDEON M. POLYA AND JANETTE A. BOWMAN
Department of Biochemistry, La Trobe University, Bundoora, Victoria 3083, Australia

ABSTRACT

A high affinity cAMP-binding protein (cABP II) was purified to homogeneity from wheat germ. The apparent molecular weight of cABP II, as determined from gel exclusion chromatography, is 5.2×10^5 (at low ionic strength) and 2.8×10^5 (at high ionic strength). One polypeptide subunit (molecular weight, 80,000) was resolved by polyacrylamide gel electrophoresis of cABP II under subunit dissociating conditions. The purification protocol employed resolves cABP II from a distinct, less acidic cAMP-binding protein (cABP I). The K_d values for cAMP are about 10^{-6} molar and 10^{-7} molar for cABP II and cABP I, respectively. The cAMP-binding sites of cABP I and cABP II have a marked adenine-analog specificity, binding adenine, adenosine, adenine-derived nucleosides and nucleotides and a variety of adenine derivatives having cytokinin activity. While cABP II is phosphorylated in reactions catalyzed by endogenous protein kinases, there is no evidence for modulation of these cABP II-protein kinase interactions by cAMP.

Cyclic AMP has major amplifying regulatory functions in prokaryotes (24) and eukaryotes (12) but, despite considerable investigation (1, 20, 32), there is still no direct evidence for an explicit regulatory function for cAMP in higher plants (1). Reports and speculative interpretations of physiological responses of plants to exogenous cAMP have been criticized (20, 35), as has much of the evidence for the existence of cAMP in higher plants (1–3, 20). Adenylate cyclase has yet to be convincingly demonstrated in cell-free extracts from higher plants (18), and there is, as yet, no evidence for the presence in higher plants of cAMP-dependent protein kinases (1, 35). Nevertheless, possible key elements of a cAMP regulatory system have been demonstrated in higher plants, including the presence of low concentrations of cAMP of nonmicrobiological origin (3, 4), net synthesis and secretion of cAMP (3), and the presence of phosphodiesterases that catalyze the hydrolysis of cAMP (5, 7, 9, 21). Further, albeit circumstantial, evidence for a cAMP regulatory system in higher plants derives from the multiplicity of high affinity cyclic nucleotide-binding proteins in higher plants (8, 15, 16, 19, 25–27, 30). 5'-Nucleotidases that are competitively inhibited by cAMP and cGMP[2] are present in wheat seedlings (26, 27) and potato tubers (25). Cyclic GMP at μM concentrations promotes GTP binding to wheat embryo elongation factor 1 and promotes polypeptide synthesis in the cell-free translation system from wheat embryo (19). Cyclic AMP-binding activity has been resolved in *Phaseolus* (8), wheat embryo (30), and artichoke rhizomes (15, 16). Protein kinases present in partially purified preparations of cAMP-binding proteins from artichoke rhizome and wheat embryo were not activated by cAMP (15, 30). The present paper describes the resolution and properties of two cAMP-binding proteins from wheat germ and the interaction of one of these proteins with endogenous protein kinase.

MATERIALS AND METHODS

$(NH_4)_2SO_4$ Precipitation Assay for cAMP Binding. The $(NH_4)_2SO_4$-precipitation procedure employed to measure cAMP binding was similar to that described previously (30). In the standard cAMP binding assay, the assay medium (1 ml, total volume) contained 10^{-8} M cAMP (specific activity of [8-^{3}H]cAMP, 26 Ci/mmol; specific activity of [2,8-^{3}H]cAMP, 32 Ci/mmol), 50 mM Tris (Cl^-, pH 8.0), the protein sample being assayed (routinely added in 25 μl of solution) and 90% saturated $(NH_4)_2SO_4$. The final pH of tbe assays was 8.0, and the assays were conducted at 0 C. The order of additions was tritiated cAMP, protein, and, finally, 0.9 ml 100% saturated $(NH_4)_2SO_4$-50 mM Tris (Cl^-, pH 8.0). After incubation at 0 C for 10 min, the protein precipitate was collected by centrifugation at 16,000*g* for 10 min. The supernatant was removed carefully by aspiration. The pellet was solubilized in 1 ml 1% (w/v) SDS in H_2O, added to 10 ml scintillation fluid A (29), and counted at 32% efficiency. To obtain a measure of specific cAMP binding, assay counts were routinely corrected by subtraction of counts obtained in the same assay conducted in the presence of 0.1 μmol unlabeled cAMP. Specific cAMP binding was 93% of total cAMP binding and 97% of cAMP binding determined by subtraction of counts obtained when the assay was conducted in the absence of added protein. Standard deviations in the binding assays were about 4% of the means.

Equilibrium Dialysis. Dialysis tubing (size, 8/32) was washed prior to use, according to Brewer (6). Dialysis tubing sacs containing 0.5 ml cABP II solution were suspended in 10 ml solution containing 0.1 M Tris (Cl^-, pH 8.0), 10 mM 2-mercaptoethanol, 4×10^{-9} M labeled cAMP (specific activity of [8-^{3}H]cAMP, 26 Ci/mmol), 40 μM KCN, and various concentrations of unlabeled cAMP. The equilibrium dialysis experiments were conducted within capped 20-ml scintillation vials which were shaken at 120 oscillations per min at 30 C for 63 h in the dark. Samples (200 μl) were added with 300 μl H_2O to 5 ml scintillation fluid A (33) and counted.

Protein and Enzyme Assays. Protein was routinely determined by the Folin procedure (22) with crystalline BSA as a standard. For protein extinction coefficient determinations, protein was determined by the Biuret method (13) with crystalline BSA as a standard and by employing an extinction coefficient ($E_{280}^{0.1\%}$) for this protein of 0.66 (34). Phosphodiesterase (with cAMP or bis-*p*-nitrophenylphosphate as substrates), 5'-nucleotidase, glyceraldehyde-3-P dehydrogenase, and phosphofructokinase were assayed as described previously (10).

[1] This study was supported by a grant from the Australian Research Grants Committee.

[2] Abbreviations: cGMP, cyclic guanosine 3':5'-monophosphate; cABP I, cABP II, wheat germ cAMP-binding proteins I and II; CBP, wheat germ cytokinin-binding glycoprotein; PMSF, phenylmethylsulfonylfluoride.

Electrophoretic and Antibody Procedures. Electrophoresis of proteins in subunit dissociating conditions was conducted with 0.1% SDS-10% polyacrylamide disc gels, as described previously (25). The molecular weight standards used to calibrate disc gel mobilities were standards (mol wt ranges 14,300 to 71,500 and 53,000 to 265,000) obtained from BDH Chemicals, Poole, U. K. Densitometer tracings of disc gels (stained with Coomassie brilliant blue) were made at 600 nm. Isoelectric focusing was done with an LKB 1804-101 polyacrylamide-ampholyte gel plate and an LKB 2117 Multiphor apparatus (LKB Produkter A. B., Bromma, Sweden). Antibodies to cABP II (purified without use of PMSF) were raised in rabbits in a course of injections with a total of 0.5 mg cABP II in 50% (v/v) Freund's complete adjuvant injected subcutaneously in four spots at each of five injection times distributed over 6 weeks. Immunodiffusions involving immune, nonimmune, and preimmune sera and cABP II were conducted with agar immunodiffusion plates prepared on microscope slides.

Protein Kinase Assays. Protein kinase was assayed in a reaction medium (final volume 100 μl) containing 10 mM $MgCl_2$, 10 mM DL-DTT, 50 mM Pipes (Na^+, pH 7.0), 25 μM ATP (the specific activity of [γ-^{32}P]ATP in the assay was routinely about 50 mCi/mmol), substrate protein, and 25 μl protein kinase preparation in buffer A (50 mM Tris [Cl^-, pH 8.0]-10 mM 2-(mercaptoethanol). The reaction was initiated by addition of labeled ATP, and the reaction mixture was routinely incubated at 30 C for 30 min. The reactions were terminated by addition of 25 μl of a solution containing 50 mM unlabeled ATP and 0.25 M EDTA (Na^+, pH 7.5). Aliquots (100 μl) of the terminated reaction mixtures were spotted onto Whatman 3MM paper discs (diameter 2.0 cm), and the discs were dropped into 5% (w/v) trichloroacetic acid at 0 C. Discs were successively washed by suspension in 500 ml 5% (w/v) trichloroacetic acid at 0 C for 20 min (3 washes), 250 ml absolute ethanol for 5 min (2 washes), and 250 ml diethylether for 5 min (2 washes). Discs were dried and counted in 10 ml of scintillation fluid B (containing 0.5% [w/v] 2,5-diphenyloxazole and 0.03% [w/v] 1,4-bis-[4-methyl-5-phenyloxazol-2-yl]-benzene in toluene). Counts in the assays were corrected by subtraction of counts obtained in the absence of added protein kinase preparation. The assay measured all protein phosphorylations in the assay including possible self-phosphorylation of protein kinase or phosphorylation of other proteins in the protein kinase preparation added as well as phosphorylation of the substrate protein.

Analysis of Phosphorylated Protein. The protein products of the protein kinase-catalyzed reactions involving cABP II as substrate were precipitated with 2 ml 5% (w/v) trichloroacetic acid and recovered by centrifugation. The pellets were washed twice with 1 ml 5% (w/v) trichloroacetic acid at 0 C, and the washed pellets were suspended either in 200 μl 6 N HCl (for subsequent protein hydrolysis) or in 100 μl 1% (w/v) SDS (for subsequent electrophoretic resolution of phosphorylated peptides). Phosphorylated protein samples suspended in 6 N HCl were hydrolyzed by heating at 110 C in sealed ampoules for 4 h. Aliquots (20 μl) of protein hydrolysates were applied to Whatman 3MM paper (23 cm x 37 cm) together with 20-μl aliquots of a solution of standards composed of 10 mg/ml L-phosphoserine and 10 mg/ml DL-phosphothreonine in 50 mM phosphate (K^+, pH 7.5). High voltage electrophoresis was conducted at 2,500 v/180 mamp for 2 h in an electrophoresis buffer containing 2.5% (v/v) formic acid-7.8% (v/v) acetic acid (pH 1.9) (14) with a Shandon Southern Model L24 high voltage electrophoresis apparatus (Shandon Southern Instruments, Camberley, Surrey, U. K.). After electrophoresis, the paper was dried, and phosphoserine and phosphothreonine spots were detected by using a ninhydrin spray. Pi was detected by spraying the paper with a solution containing 3% $HClO_4$, 0.1 N HCl, and 1% (w/v) ammonium molybdate (14). The phosphoserine-, phosphothreonine-, and Pi zones were excised and counted in 10 ml scintillation fluid B. The phosphorylated protein that was dissolved in 1% (v/v) SDS was subjected to electrophoresis in 0.1% SDS-10% polyacrylamide disc gels, and the gels were stained. Phosphorylated polypeptide bands were located on disc gels by counting the radioactivity of gel slices. Gels were frozen on Dry Ice before slicing of the gels. Gel slices were incubated at 60 C for 4 h in a solution containing 1.0 ml NCS tissue solubilizer (Amersham/Searle, Arlington Heights, IL) and 50 μl H_2O before addition of 5 ml scintillation fluid B and ^{32}P counting. Phosphorylated polypeptides were also detected by autoradiography. Disc gels were placed in a Perspex holder, wrapped in a clear plastic wrapping (Glad Wrap, Union Carbide, Australia), and overlaid with Kodak X-Omat S x-ray film under a lead brick weight. After 2 to 4 days, the x-ray film was developed, and darkened zones on the autoradiograms were compared with underlying polypeptide bands on disc gels.

Plant Material and Chemicals. Raw wheat germ (percentage hydration, 13%) was purchased from Heidelberg Health Foods, Melbourne, Australia. [8-^{3}H]cAMP (26 Ci/mmol) and [2,8-^{3}H]-cAMP (32 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks, U. K.; the purity of tritiated cAMP was checked by chromatography on polyethyleneimine thin layer plates, as described previously (5). [γ-^{32}P]ATP (initial specific activity, 3200 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, U. K. [γ-^{32}P]ATP was prepared and purified by the method of Schendel and Wells (33), employing [^{32}P]orthophosphoric acid (2.6 Ci/mmol) obtained from the Australian Atomic Energy Commission, Lucas Heights, Sydney, Australia. The purity of [γ-^{32}P]ATP was checked by ascending chromatography in 0.1 M ammonium formate (pH 3.1) on Whatman DE-81 ion exchange paper (23). Dephosphorylated casein, histone, protamine, BSA, PMSF, and all nucleotides and nucleosides used were obtained from the Sigma Chemical Co. The histone used was a preparation of calf thymus histones catalogued by the Sigma Chemical Co. as preparation Type II-A (not to be confused with histone 2A [H2A]). DEAE-Sephacel and Sepharose 4B were obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden; DE-52 (DEAE-cellulose) was from Whatman, Maidstone, Kent, U. K., and Ultrogel AcA 34 was from LKB-Produkter AB, Bromma, Sweden. Cibacron Blue F3GA (CIBA-Geigy, Melbourne, Australia) was coupled to Sepharose 4B by the method of Heyns and De Moor (17). Zeatin was obtained from Calbiochem, San Diego, CA; all other compounds tested in ligand specificity studies were obtained from the Sigma Chemical Co.

Purification of cABP I and cABP II. All purification steps were carried out at 0 to 4 C. Wheat germ (500 g) was suspended in 2 liters buffer A (50 mM Tris Cl^- [pH 8.0], 10 mM 2-mercaptoethanol) containing 0.5 mM PMSF and 0.25% (v/v) ethanol. The suspension was homogenized for 2 min at full power using an Ultra-Turrax blender (Janke & Kunkel, Staufen, West Germany). The homogenized suspension was filtered two times through one layer of muslin, and 50 g Whatman DE52 (DEAE-cellulose) in 1 liter buffer A were added to the filtered homogenate. The DE52 was recovered by filtration through Miracloth (Calbiochem, Richmond, CA), washed three more times with 1 liter buffer A for each wash, and then suspended in 500 ml buffer A. The DE52 suspension was brought to 0.4 M KCl concentration and filtered through Miracloth, and the filtrate was retained. The filtrate was centrifuged at 30,000*g* for 10 min, and the supernatant was subjected to stepwise $(NH_4)_2SO_4$ precipitation. The fraction precipitating at 50% $(NH_4)_2SO_4$ saturation was retained for the resolution of protein kinases. The fraction precipitating between 50 and 100% $(NH_4)_2SO_4$ saturation was recovered by centrifugation at 30,000*g* for 10 min and dissolved in about 10 ml buffer A, and the resulting solution was dialyzed against 2 liters buffer A overnight. The dialyzed solution was applied to a column (7.1 cm^2 x 9.5 cm) of DEAE-Sephacel equilibrated with buffer A. The DEAE-Se-

phacel column was eluted with a linear gradient of increasing KCl concentration (from 0 to 0.4 M) in buffer A to resolve two cAMP-binding fractions denoted cABP I and cABP II. The cABP II fractions were pooled and concentrated by precipitation at 100% $(NH_4)_2SO_4$ saturation. The concentrated cABP II fraction in 2 ml buffer A was applied to a column (3.1 cm^2 x 73 cm) of Ultrogel AcA 34 equilibrated with buffer A, and the column was eluted with the same buffer. The peak cAMP-binding fractions (from half-peak height, upwards) were pooled, concentrated by precipitation at 100% $(NH_4)_2SO_4$ saturation, and rechromatographed through the Ultrogel AcA 34 column in buffer A. The cABP I fractions from the DEAE-Sephacel column were pooled and concentrated by precipitation at 100% $(NH_4)_2SO_4$ saturation. The concentrated cABP I fraction in 2 ml buffer A was applied to the Ultrogel AcA 34 column and eluted from the column in buffer A as one peak of cAMP-binding activity.

Partial Purification of Wheat Germ Protein Kinases. The fraction eluted from DE52 in 0.4 M KCl-buffer A in the purification protocol for cABP I and cABP II (see above) was subjected to stepwise $(NH_4)_2SO_4$ fractionation. The fraction precipitating at 50% $(NH_4)_2SO_4$ saturation was recovered by centrifugation at 30,000*g* for 10 min, the pellet dissolved in about 20 ml buffer A, and the resulting solution dialyzed against 2 changes of 2 liters buffer A overnight. The dialyzed solution was clarified by centrifugation at 30,000*g* for 10 min, and the clarified solution was added to 50 ml (bed volume) of Cibacron F-3GA-Sepharose 4B that had previously been well washed with H_2O and then resuspended in buffer A. The gel was washed with 2 liters buffer A before elution of protein kinase in 1.0 M KCl-buffer A. The protein kinase-containing fraction was concentrated by precipitation at 50% $(NH_4)_2SO_4$ saturation, dissolved in about 5 ml buffer A, and applied to a column (3.1 cm^2 x 72 cm) of Ultrogel AcA 34 equilibrated with buffer A. Protein kinase components partially resolved by elution from the Ultrogel AcA column in buffer A were further purified by rechromatography on this column in buffer A.

RESULTS

Purification of Wheat Germ cAMP-Binding Proteins. Two wheat germ cAMP-binding proteins, denoted cABP I and cABP II, were resolved by elution from DEAE-Sephacel by a gradient of increasing KCl concentration (Fig. 1a). The KCl concentrations for elution of the peak fractions of cABP I and cABP II were 0.09 M and 0.15 M, respectively (Fig. 1a). The serine protease inhibitor, PMSF, was routinely included during homogenization of wheat germ. When PMSF was not included, the amount of cABP I (the cAMP-binding protein eluting at lower ionic strength from DEAE-Sephacel [Fig. 1a]) was very low, and, in some preparations, no peak of cAMP-binding activity corresponding to cABP I was evident. Subsequent gel filtration of cABP I fractions from DEAE-Sephacel chromatography yielded one peak of cAMP-binding activity. The schedule for the partial purification of cABP I is presented in Table I. The overall purification was only 4.3-fold with respect to the homogenate, and the overall yield of cABP I was only 0.6% of cAMP-binding activity in the homogenate or 3.2% of activity retained after the DE52 ion exchange step (Table I). The cAMP binding/protein ratio was not constant for the cABP I fractions from the final gel filtration, indicating heterogeneity of cABP I preparations. Dissociating 0.1% (w/v) SDS-10% (w/v) polyacrylamide gel electrophoresis of the final preparations of cABP I revealed a multiplicity of polypeptide bands. Further purification of cABP I was not achieved because of the lability of the protein; 50% of cAMP-binding activity was lost on storage of cABP I in buffer A at 4 C over 11 days.

The more acidic cAMP-binding protein, cABP II, was purified to homogeneity by repeated gel filtration of the cAMP-binding fractions resolved by chromatography on DEAE-Sephacel (Figs. 1a–c). The final gel filtration step yielded one peak of cAMP-binding activity coinciding with the one major peak of A_{280} (Fig. 1c). The overall purification of cABP II was 42-fold with respect to the homogenate and 16-fold with respect to the cAMP-binding

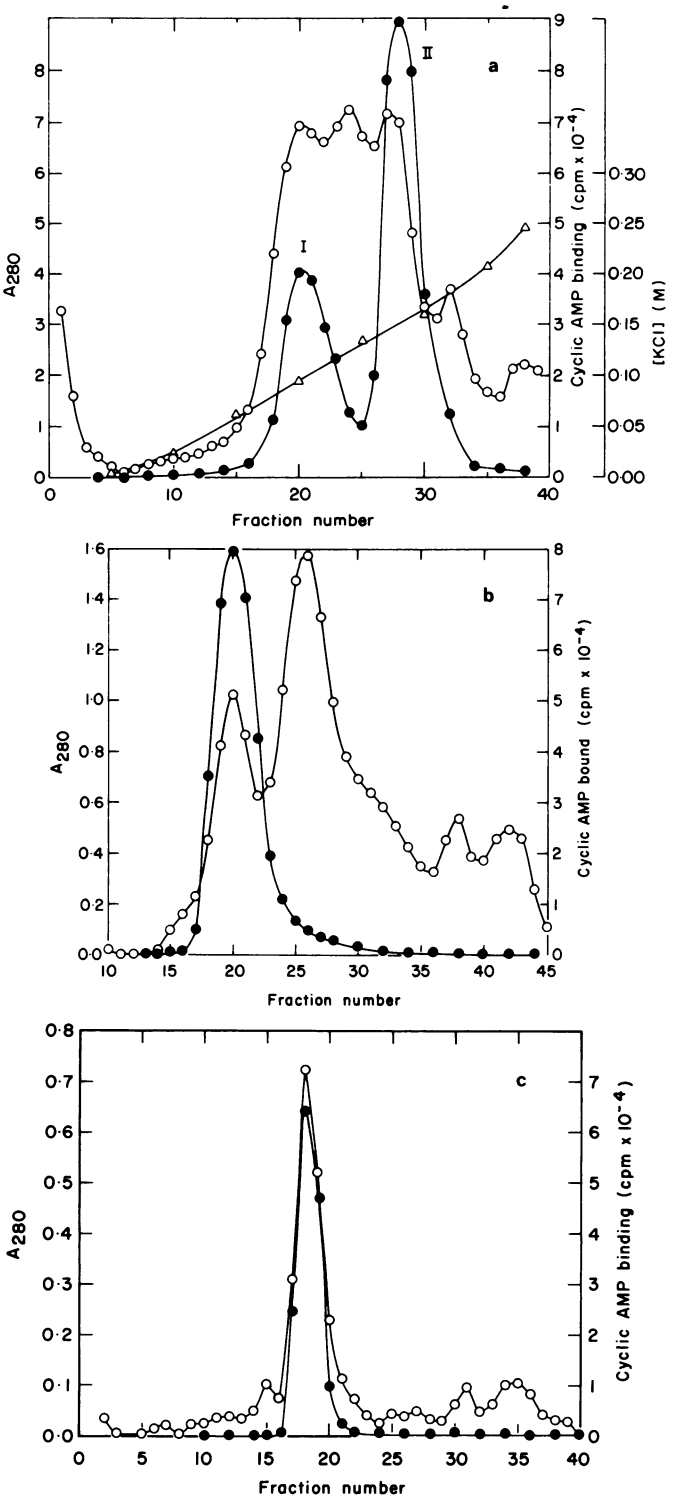

FIG. 1. Sequential purification of cABP II by column chromatography. A DEAE-Sephacel column (7.1 cm^2 × 9.5 cm) eluted with a linear gradient of increasing KCl concentration in buffer A (a); gel filtration of cABP II fractions from (a) on an Ultrogel AcA 34 column (3.1 cm^2 × 73 cm) in buffer A (b); and final gel filtration of cABP II on the Ultrogel AcA 34 column in buffer A (c). (Δ), KCl concentration; (○), A_{280}; and (●), cAMP binding in the standard assay.

Table I. *Partial Purification of cAMP-Binding Protein I*

Cyclic AMP binding was determined in the standard assay containing 10^{-8} M cAMP (specific activity of [2,8-^{3}H]cAMP, 32 Ci/mmol). Wheat germ (500 g) was extracted, and the yields at each stage were corrected for losses due to sampling for analysis.

Purification Stage	Protein	Total cAMP Binding	cAMP Binding
	mg	*nmol*	*pmol/mg protein*
Homogenate	74,060	54.4	0.7
DE52	6,460	10.6	1.6
50 to 100% $(NH_4)_2SO_4$	1,110	4.9	4.4
DEAE-Sephacel (cABP I)	395	1.2	3.1
Ultrogel AcA 34	106	0.34	3.2

Table II. *Purification of cAMP-Binding Protein II*

Cyclic AMP binding was determined in the standard assay containing 10^{-6} M cAMP (specific activity of [8-^{3}H]cAMP in the assay, 26 mCi/mmol). Wheat germ (500 g) was extracted, and the yields at each stage were corrected for losses due to sampling for analysis.

Purification Stage	Protein	Total cAMP Binding	cAMP Binding
	mg	*nmol*	*nmol/mg protein*
Homogenate	57,100	4,770	0.08
DE52	2,020	446	0.22
50 to 100% $(NH_4)_2SO_4$	502	146	0.29
DEAE-Sephacel	78	108	1.4
Ultrogel AcA 34 (1)	22	65	3.0
Ultrogel AcA 34 (2)	11	38	3.5

fraction retained by DE52 at low ionic strength (Table II). The overall yield of purified cABP II was only 0.8% of total homogenate cAMP binding or 8.5% of activity retained by DE52 at low ionic strength (Table II). If one assumes that 50% of the cAMP-binding activity retained by DE52 is cABP II (*cf.* Fig. 1a), one can estimate from Table II that the amount of cABP II in wheat germ is at least 0.13 mg/g fresh weight. The purity of cABP II preparations was assessed at greater than 95% on the basis of constant specific cAMP binding activity on gel filtration (Fig. 1c) and from gel electrophoresis under subunit dissociating conditions. Electrophoresis of cABP II (purified with or without the use of PMSF) in 0.1% (w/v) SDS-10% (w/v) polyacrylamide gels yielded only one major polypeptide band. Challenging purified cABP II with rabbit immune serum yielded only one precipitin line, and no precipitin line was observed with nonimmune control sera.

Subunit Composition, Isoelectric Point and Molecular Weight of cABP II. Electrophoresis of purified cABP II in 0.1% (w/v) SDS-10% (w/v) polyacrylamide gels (*i.e.* in subunit dissociating conditions) yielded one major polypeptide band with a mol wt of 81,000 ± 3,000 (5 determinations); the subunit mol wt estimate for cABP II isolated in the absence of PMSF was 79,000 ± 3,000 (9 determinations). Isoelectric focusing of cABP II yielded one polypeptide at a position corresponding to an isoelectric point at pH 6.0. The apparent molecular size of cABP II (isolated from homogenates containing PMSF) was determined by elution from an Ultrogel AcA 34 column calibrated with protein standards of known molecular weight. At low ionic strength (buffer A), cABP II has an apparent mol wt of 515,000 ± 45,000 (mean of 2 separate determinations) eluting between rabbit muscle phosphofructokinase (mol wt, 360,000) and bovine thyroglobulin (mol wt, 670,000). However, at high ionic strength (0.5 M $[NH_4]_2SO_4$ in buffer A), cABP II behaves as a 270,000-dalton species eluting between yeast alcohol dehydrogenase (mol wt, 141,000) and rabbit muscle phosphofructokinase (mol wt, 360,000). The apparent mol wt of cABP II (prepared in the absence of PMSF) at high ionic strength (0.5 M $[NH_4]_2SO_4$ in buffer A) is 290,000 ± 30,000 (mean of 4 determinations). The apparent mol wt of cABP I at low ionic strength, as inferred from chromatography on Ultrogel AcA 34 in buffer A, is 185,000 ± 15,000 (mean ± deviation from 2 determinations).

Cyclic AMP Binding to cABP I and cABP II. Binding of cAMP to cABP II is optimal in the assay pH range 6.0 to 9.0, and no distinct pH optimum was evident within this range. Binding of cAMP to cABP II at pH 4.1 and 5.2 is 3.7% and 33.5%, respectively, of cAMP binding at pH 8.0 in the standard assay. The $(NH_4)_2SO_4$ precipitation assay measured the amount of tritiated cAMP bound to the protein at equilibrium under the conditions used, *i.e.* the insolubilized cAMP-cABP II complex was pelleted through a solution containing the equilibrium concentration of free cAMP. This type of assay has been applied previously to cAMP-binding proteins (*e.g.* see Refs. 10, 11, 30) and to plant hormone-binding proteins (*e.g.* see Refs. 28, 29). Changing the order of additions to the assay (*i.e.* cABP II added last or saturated $[NH_4]_2SO_4$ solution added last) or prolongation of the preincubation time (to 20 min) did not substantially alter cAMP binding. When 0.1 μmol unlabeled cAMP was added after addition of 100% saturated $(NH_4)_2SO_4$ to the standard assay, the [8-^{3}H]cAMP displaced was the same as that obtained when 0.1 μmol unlabeled cAMP was included before precipitation of the protein. Inasmuch as very small amounts (about 20 μg) of cABP II were added to the assays, the protein pellet was too small to be seen, and its location was established by appropriate marking of the assay test tubes prior to centrifugation, as previously described (29). Cyclic AMP binding was a linear function of added cABP II in the standard assay (up to 60 μg protein added). The radioactive compound bound to cABP II in the standard assay is cAMP as determined by successive ascending chromatography in H_2O and then in 0.5 M ammonium acetate, pH 7.5, on poly(ethyleneimine)-cellulose thin layer plates (see Ref. 5).

The apparent dissociation constant (K_d) of cABP II for cAMP was estimated by determining the amount of bound and free cAMP at various cAMP concentrations and constructing a Scatchard plot. One high-affinity cAMP-binding component was apparent (Fig. 2a); the apparent K_d for cAMP binding is 7×10^{-7} M. At saturation, 6.2 nmol cAMP is bound to high-affinity sites per mg of cABP II ($E_{280}^{0.1\%} = 0.82$). This stoichiometry corresponds to 0.5 binding sites per molecule of 80,000-dalton cABP II subunit. The apparent K_d for cAMP of soluble cABP II was $3.0 \pm 1.5 \times 10^{-6}$ M, as determined by equilibrium dialysis; the cAMP-binding stoichiometry at saturation (corrected for low affinity cAMP binding) was about 0.9 mol per mol cABP II subunit. Successive ascending chromatography of the equilibrium dialysis medium in H_2O and then 0.5 M ammonium acetate (pH 7.5) on poly(ethyleneimine)-cellulose thin layer plates (see Ref. 5) revealed no significant degradation of cAMP during equilibrium dialysis. Thus, the affinities and stoichiometries determined for the cAMP-cABP II interaction by either the $(NH_4)_2SO_4$ precipitation procedure or by equilibrium dialysis are similar. The binding of cAMP to cABP II, prepared in the absence of PMSF, was also examined under the conditions of the $(NH_4)_2SO_4$ precipitation assay; the apparent K_d was 1.0 ± 0.1 μM (mean ± deviation from duplicate determinations) with a stoichiometry of 0.6 sites/subunit.

Binding of cAMP to cABP I was optimal in the assay pH range of 6.0 to 9.0; binding of cAMP to cABP I at pH 4.1 and pH 5.2 is 12.4% and 20.3%, respectively, of cAMP binding at pH 8.0. No cAMP phosphodiesterase was detected associated with partially purified cABP I. The apparent dissociation constant (K_d) of the cABP I-cyclic AMP complex in the conditions of the standard binding assay at pH 8.0 was estimated by determining the amount of bound and free cAMP at various cAMP concentrations and

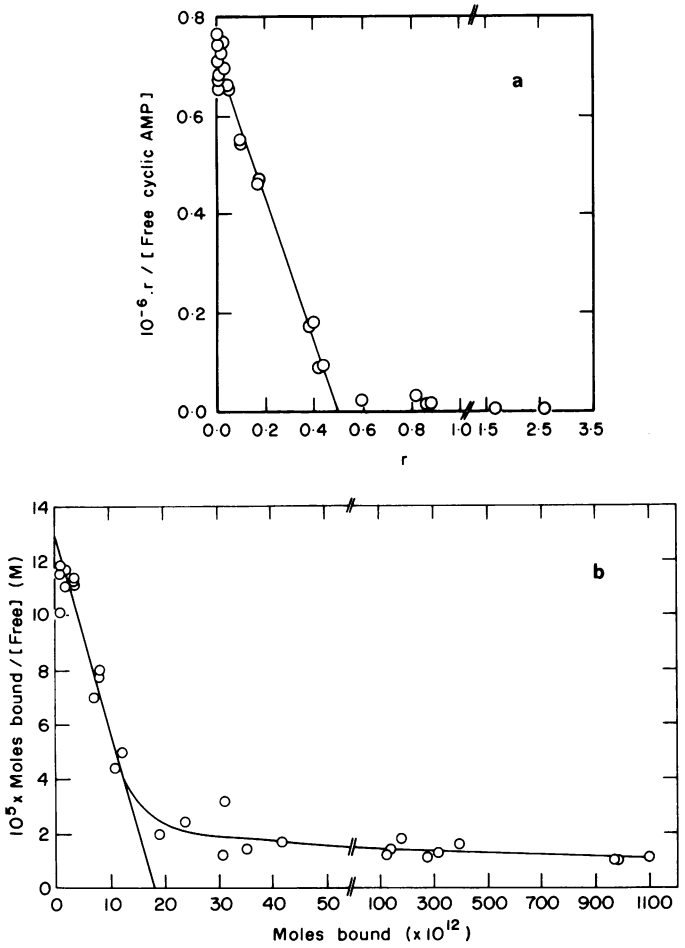

FIG. 2. Scatchard plots for cAMP binding to cABP I and cABP II under the conditions of the standard $(NH_4)_2SO_4$ precipitation assay. Cyclic AMP binding was determined radiochemically at a variety of cAMP concentrations and was corrected by subtraction of counts obtained in the standard assay in the absence of added protein. Plot for cAMP-cABP II binding, r = mol cAMP bound per mol cABP II subunit (a); and plot for cAMP-cABP I binding, mol cAMP bound per M cAMP concentration in the final 1-ml assay volume is plotted *versus* mol cAMP bound (b).

constructing a Scatchard plot. High- and low-affinity binding components were apparent (Fig. 2b). The apparent K_d for high-affinity cAMP binding was $1.4 \pm 0.2 \times 10^{-7}$ M (mean ± SD from three separate determinations). The highest stoichiometry measured at saturation in such analyses (and corrected for low affinity binding) was 130 pmol cAMP bound to high-affinity sites per mg protein, corresponding to only 0.02 binding sites per 185,000-dalton protein if the preparations were homogeneous. Assuming one cAMP binding site per cABP I molecule present, this very low observed stoichiometry indicated a minimum purity of about 2% of the cABP I preparations examined. This estimate of the low purity of the cABP I preparations was qualitatively confirmed by 0.1% SDS-10% polyacrylamide gel electrophoresis of samples of the partially purified cABP I; a multiplicity of polypeptide bands was observed. A minimum estimate of the amount of cABP I in wheat germ can be made from the data in Table I by using the relationship between high-affinity binding at saturation and binding in the conditions of the standard assay at 10^{-8} M cAMP concentration (Fig. 2b). Assuming one high-affinity cAMP binding site per cABP I, the amount of cABP I resolved at the DEAE-Sephacel stage (Table I) corresponds to 37 nmol/kg fresh weight.

Adenine Analog Ligand Specificity of cABP I and cABP II. The cAMP-binding sites of cABP I and cABP II bind a variety of adenine derivatives. While a range of adenine-derived nucleosides and nucleotides displace cAMP from cABP II (Table III), the following nonadenine nucleosides or nucleotides cause less than 20% inhibition of cAMP binding to cABP II when included in the standard assay at 10^{-4} M concentration: 3',5'-cyclic UMP and 3',5'-cyclic CMP; 2',3'-cyclic GMP, 2',3'-cyclic CMP, and 2',3'-cyclic UMP; 2'-GMP, 2'-UMP, and 2'-CMP; 3'-GMP, 3'-IMP, and 3'-UMP; 5'-UMP, 7-methyl-5'-GMP, 5'-IMP, 5'-CMP, 5'-GMP, and 6-mercaptopurine riboside-5'-monophosphate; 5'-dTMP, 5'-dUMP, 5'-dGMP, 5'-dCMP, and 5'-dIMP; 5'-ITP, 5'-GTP, 5'-UTP, and 5'-CTP; 5'-UDP; and cytidine, 2',3'-isopropylideneguanosine, 2'-deoxyinosine, guanosine, 8-mercaptoguanosine, 2'-deoxyguanosine, 2'-deoxyuridine, 6-mercaptopurine-9-β-D-ribofuranoside, and inosine. In addition, the following nonadenine nucleosides or nucleotides at 10^{-4} M cause less than 50% inhibition of specific cAMP binding to cABP II: 3',5'-cyclic IMP; 3',5'-cyclic dTMP; 5'-XTP; 5'-CDP; xanthosine; and uridine.

A similar specificity for adenosine derivatives was displayed by the cAMP-binding site of cABP I. A range of adenine-derived nucleosides and nucleotides at 10^{-4} M concentration displace cAMP from cABP I (Table III); 3',5'-cyclic IMP was the only nonadenine nucleotide we have found to inhibit specific cAMP binding to cABP I by more than 50% when included in the standard assay at 10^{-4} M (Table III). The following nonadenine nucleosides and nucleotides cause less than 20% inhibition of specific cAMP binding to cABP I when included in the standard assay at 10^{-4} M: 3',5'-cyclic dTMP and 3',5'-cyclic CMP; 2',3'-cyclic CMP and 2',3'-cyclic GMP; 2'-GMP, 2'-UMP, and 2'-CMP; 3'-IMP, 3'-UMP, 3'-GMP, and 3'-CMP; 6-mercaptopurine riboside-5'-monophosphate, 7-methyl-5'-GMP, 5'-GMP, 5'-UMP, 5'-IMP, and 5'-CMP; 5'-dIMP, 5'-dGMP, 5'-dUMP, 5'-dCMP, and 5'-dTMP; 5'-XTP, 5'-GTP, 5'-ITP, 5'-CTP, and 5'-UTP; 5'-UDP,

Table III. *Displacement of cAMP from cABP I and cABP II by Nucleosides and Nucleotides*

Specific cAMP binding was determined in triplicate (control assays, in the absence of added unlabeled nucleotides, with 5-fold replication) in the standard assay conditions at pH 8.0 in the presence or absence of 0.1 mM test ligand in the final 1-ml assay volume. Inhibition of specific [^{3}H]cAMP binding is expressed as percentage of the effect of inclusion of 0.1 mM unlabeled cAMP (100%).

Added Compound	Inhibition	
	cABP I	cABP II
	% control	
3',5'-Cyclic AMP	100	100
Adenosine	100	94
3',5'-Cyclic dAMP	98	100
N^6-2-*O*'-Dibutyryl-3',5'-cyclic AMP	98	94
N^6-Monobutyryl-3',5'-cyclic AMP	95	86
5'-dAMP	94	52
2'-Deoxyadenosine	93	96
3'-Deoxyadenosine	93	97
3',5'-Cyclic IMP	78	32
2',3'-Cyclic AMP	74	105
NADH	73	75
Adenosine-5'-diphosphoribose	73	65
3'-AMP	68	87
NAD^+	58	50
5'-ADP	49	55
7-Deazaadenosine	48	53
5'-AMP	43	51
2'-AMP	39	85
NADPH	37	34
5'-ATP	35	30
$NADP^+$	34	19

5'-CDP, and 5'-XDP; and 2',3'-isopropylideneguanosine, 6-mercaptopurine-9-β-D-ribofuranoside, xanthosine, 8-mercaptoguanosine, guanosine, cytidine, 2'-deoxy-D-ribose, 2'-deoxyinosine, 2'-deoxyguanosine, 6-mercaptoguanosine, uridine, 2'-deoxyuridine, and 2'-deoxycytidine. Thymidine, 3',5'-cyclic UMP, 3',5'-cyclic GMP, and inosine at 10^{-4} M cause less than 50% inhibition of specific cAMP binding to cABP I.

In addition to adenosine derivatives, adenine and a variety of N^6-substituted adenine derivatives active as cytokinins displace cAMP from cABP I and cABP II (Table IV). Cytokinins and 3',5'-cyclic AMP derivatives are the better adenine analog ligands of cABP I and cABP II (Table IV), and the 3',5'-cyclic AMP derivatives are more effective than other adenine nucleotides in displacing cAMP from cABP I or cABP II (*cf.* Tables III and IV).

Interaction of cABP II with Endogenous Protein Kinases. Three protein kinase fractions (denoted protein kinases I, II, and III) were resolved by the procedure described in "Materials and Methods," involving batchwise adsorption to DEAE-cellulose, stepwise $(NH_4)_2SO_4$ precipitation, chromatography on Cibacron F3GA-Sepharose 4B, and repeated gel filtration on an Ultrogel AcA 34 column (Figs. 3a, b, c). Four protein kinase components were distinguishable after the first gel filtration step (Fig. 3a). In addition to a peak of protein kinase eluting in the void volume of the column and associated with cloudy fractions, three forms of soluble protein kinase (I, II, and III) were partly resolved by this gel filtration procedure (Fig. 3a). Fractions in these separate zones were carefully pooled (from fractions at half peak height and upwards with respect to protein kinase activity) for subsequent gel filtration. The separate pooled fractions I and II were concentrated by precipitation at 50% $(NH_4)_2SO_4$ saturation and rechromatographed on the Ultrogel AcA 34 column in buffer A (Figs. 3b, c). Protein kinase I (the protein kinase with the highest apparent molecular weight) was resolved on rechromatography as a peak of approximately constant specific activity with a minor peak of

Table IV. *Titration of the cAMP-Binding Sites of cABP I and cABP II with Adenine Derivatives*

Specific cAMP binding was determined in triplicate in the standard assay at pH 8.0 in the presence of various concentrations (routinely, a range of about 6 to 7 different concentrations) of each test ligand. Specific cAMP binding in the absence of test ligand was determined for each titration curve with 5-fold replication. The concentration of test ligand for 50% inhibition of specific cAMP binding (C_{50}) was estimated from plots of specific cAMP binding *versus* test ligand concentration.

Ligand	C_{50}	
	cABP I	cABP II
	M	
Kinetin	6×10^{-8}	3×10^{-5}
Zeatin (mixed isomers)	1×10^{-7}	7×10^{-6}
N^6-Dimethylallyladenine	1×10^{-7}	1×10^{-6}
N^6-Benzyladenine	2×10^{-7}	3×10^{-6}
N^6-2-*O'*-Dibutyryl-3',5'-cyclic AMP	2×10^{-7}	4×10^{-6}
3',5'-Cyclic AMP	2×10^{-7}	3×10^{-7}
Zeatin (*trans* isomer)	4×10^{-7}	2×10^{-6}
3',5'-Cyclic dAMP	8×10^{-7}	2×10^{-7}
N^6-Monobutyryl-3',5'-cyclic AMP	9×10^{-7}	2×10^{-7}
Adenosine	1×10^{-6}	5×10^{-6}
N^6-Dimethyladenine	1×10^{-6}	1×10^{-6}
3'-Deoxyadenosine		8×10^{-7}
2'-Deoxyadenosine	1×10^{-6}	3×10^{-6}
Adenine	2×10^{-6}	4×10^{-5}
N^6-Benzyladenosine	2×10^{-6}	7×10^{-5}
Kinetin riboside	5×10^{-6}	5×10^{-5}
5'-dAMP	5×10^{-6}	
N^6-Dimethylallyladenosine	8×10^{-6}	5×10^{-5}
3'-AMP	2×10^{-5}	2×10^{-5}
NADH	4×10^{-5}	6×10^{-5}
NAD^+	5×10^{-5}	1×10^{-4}
2',3'-cAMP	6×10^{-5}	1×10^{-5}
2'-AMP		9×10^{-5}
N^6-Dimethyladenosine	$>10^{-4}$	$>10^{-4}$

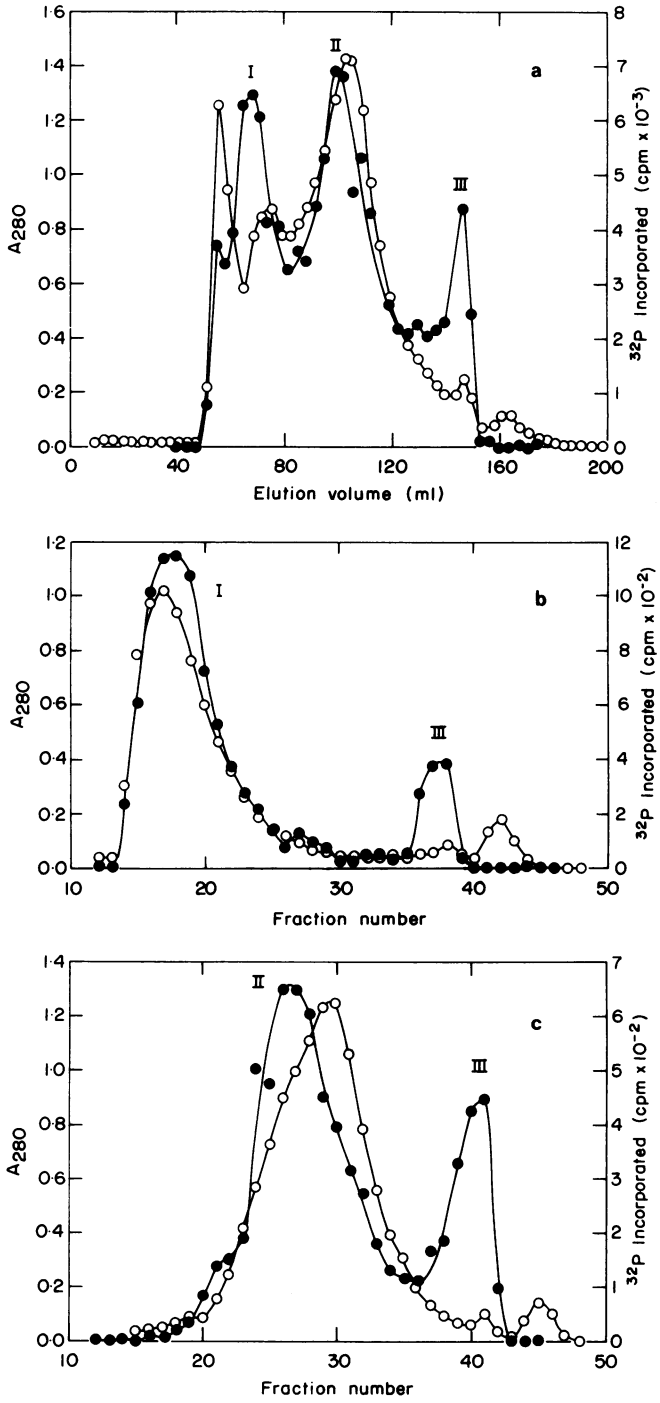

FIG. 3. Resolution of protein kinases I, II, and III by gel filtration on an Ultrogel AcA 34 column. The protein kinase fraction binding to Cibacron F3GA-Sepharose 4B was applied to a column (3.1 $cm^2 \times 72$ cm) of Ultrogel AcA 34 and eluted in buffer A (a); subsequent resolution of protein kinases I and III by gel filtration in buffer A (b); and further resolution of protein kinases II and III by gel filtration in buffer A (c). (○), A_{280}; and (●), protein kinase assayed, as described in "Materials and Methods," with 100 μg dephosphorylated casein as substrate.

low molecular weight, high specific activity protein kinase eluting just before the salt peak of the column (protein kinase III) (Fig. 3b). Protein kinase II was not resolved from a major peak of copurifying protein on rechromatography, but a further component of apparently low molecular weight, high specific activity protein kinase (protein kinase III), was resolved from protein kinase II in this second gel filtration (Fig. 3c). The form III protein kinase preparations used in subsequent analyses were derived from the first gel filtration (Fig. 3a) or from rechromatography of protein kinase II (Fig. 3c). The generation of low apparent molecular weight protein kinase III from repeated gel filtration of protein kinase II (*cf.* Figs. 3a, c) suggests identity of the catalytic moieties of protein kinases II and III. No cAMP-binding activity is associated with the protein kinases resolved by this procedure. The apparent molecular weights of protein kinases I, II, and III were determined by elution in buffer A from an Ultrogel AcA 34 column (3.1 cm^2 x 72 cm) calibrated with protein standards of known molecular weight. Protein kinases I and II have apparent mol wt of 7.3 ($\pm$0.3) $\times 10^5$ and 2.5 ($\pm$0.3) $\times 10^5$, respectively (these values are the means of duplicate estimations). The apparent mol wt of protein kinase III is 7.0 ($\pm$0.5) $\times 10^4$ (mean $\pm$ SD from three determinations). The apparent molecular weight of protein kinase III was also estimated from gel filtration in buffer A through a calibrated Sephadex G-75 column (3.1 cm^2 x 71 cm), the estimate of mol wt by using this procedure being 7.2 ($\pm$1.5) $\times 10^4$ (mean of duplicate estimations). Protein kinase preparations II and III yielded a multiplicity of polypeptide bands on polyacryalmide gel electrophoresis under subunit dissociating conditions, indicative of lack of homogeneity. A major polypeptide with a mol wt of 92,000 $\pm$ 7,000 (mean $\pm$ SD from 16 determinations) is present in protein kinase I preparations, but this is not necessarily a protein kinase subunit. Preparations of protein kinases I and II catalyze a significant incorporation of label from [γ-^{32}P]ATP into the acid-precipitable fraction in the absence of added protein substrate; protein kinase III preparations exhibit an absolute dependence on added protein substrate for protein kinase activity (Table V). While dephosphorylated casein and cABP II are substrates for all three protein kinase preparations, an histone fraction and protamine are not substrates for protein kinase III and are relatively poor substrates (if substrates at all) for protein kinases I and II (Table V). The phosphorylation of cABP II by endogenous protein kinase was confirmed by analysis of electrophoretic resolutions of acid-insoluble reaction products in 0.1% SDS-10% polyacrylamide gels, as described in "Materials and Methods." Figure 4 shows the distribution of ^{32}P-labeled material after such electrophoresis of the protein fraction from incubation of cABP II with protein kinase I in the standard protein kinase assay. The one major peak of radioactivity is associated with the cABP II subunit band, as determined by gel slicing (Fig. 4A) or by autoradiography. Since

Table V. *Substrate Specificity of Wheat Germ Protein Kinases*

Protein kinase was assayed in triplicate under standard assay conditions but with the inclusion of the test substrate proteins at the final concentrations indicated. No significant protein kinase was detected in any of the substrate preparations used except for cABP II (9.7 pmol [^{32}P]phosphate incorporated per 30 min with 59 μg cABP II in the standard assay). For details of the protein substrates used, see "Materials and Methods."

Added Protein	Protein Kinase		
	I	II	III
	pmol phosphate incorporated per 30 min		
None	34	16	0
Histone, 100 μg	17	31	0
Protamine, 100 μg	52	20	0
Casein, 100 μg	221	65	7
cABP II, 59 μg	197	71	29

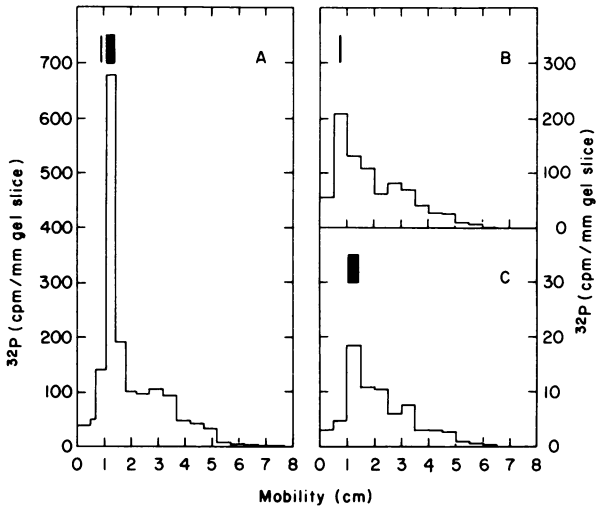

FIG. 4. Phosphorylation of cABP II catalyzed by protein kinase I. The products of the standard assay were resolved by electrophoresis in 0.1% (w/v) SDS-10% (w/v) polyacrylamide disc gels, and the disc gels were sliced and ^{32}P was counted, as described in "Materials and Methods." The *vertical bars* indicate the positions of the major polypeptide bands on the disc gels. The cABP II preparation used was isolated in the absence of PMSF. A, cABP II and protein kinase I both present in reaction mixture; B, protein kinase I, only, added to reaction mixture; and C, cABP II, only, added to reaction mixture.

phosphorylation is observed in the absence of added substrate with both cABP II and protein kinase I in the standard assay (see Table V), the products of reactions with either cABP II or protein kinase I added alone to the reaction medium were examined (Figs. 4b, c); in these situations, the peaks of ^{32}P-labeling coincide with the cABP II band (Fig. 4c) or the major band of the protein kinase I preparation (Fig. 4b), respectively. After high voltage electrophoresis of the protein hydrolysate from the complete reaction mixture with cABP II and protein kinase I present, 1, 14, and 68% of total applied radioactivity were in the phosphothreonine, phosphoserine, and Pi spots, respectively. Autoradiography of disc gel resolutions of acid-insoluble products of reactions involving cABP II and protein kinases II and III confirmed phosphorylation of the cABP II subunit in these reactions. The major ^{32}P-labeled product of the phosphorylation reaction involving protein kinase III and purified cABP II exactly copurifies with native cABP II on gel filtration through an Ultrogel AcA 34 column in buffer A. Similarly, native cABP II and protein kinase substrate activity (analyzed employing protein kinase III) exactly copurify on gel filtration in these conditions. A large number of experiments employing purified cABP II and protein kinase preparations I, II, and III failed to provide any evidence for activation of protein kinase by cAMP in this system.

DISCUSSION

A minimum estimate of the amount of cABP I in wheat germ is about 40 pmol/g fresh weight. This estimate is of the same order of magnitude as well substantiated upper estimates of cAMP levels in higher plants, *e.g.* 14 pmol/g fresh weight in *Lolium* endosperm cells (3). The K_d of cABP I for cAMP (1.4×10^{-7} M) is similar to critical estimates of cAMP concentration in higher plants (3, 4). Thus, a cAMP receptor function can be more plausibly entertained for cABP I than for cABP II, of which there are at least 1,600 pmol subunit per g fresh weight wheat germ. The catalytic functions (if any) of cABP I and cABP II are unknown. The cABP I and cABP II preparations were devoid of 5'-nucleotidase, adenosine deaminase, and phosphofructokinase activities. Cytokinins and cAMP (ligands of cABP I and cABP II) bind to the active site

of mammalian cAMP phosphodiesterase and to a site on yeast glyceraldehyde-3-P dehydrogenase (11). However, neither cAMP phosphodiesterase nor glyceraldehyde-3-P dehydrogenase activity was associated with cABP I or with cABP II preparations.

Cytokinins and cAMP are the better ligands found for cABP I and cABP II; with respect to both proteins, cAMP is the best ligand of the naturally occurring nucleotides tested (Tables III and IV). While neither the naturally occurring cytokinin, zeatin, nor cAMP are good ligands of the major wheat germ cytokinin-binding protein CBP (28, 29), zeatin at low concentrations displaces cAMP from cABP I and cABP II (Table IV). The interactions of cytokinins with the cAMP-binding sites of cABP I and cABP II, while of potential physiological significance, can presently be regarded only as further instances of the overlapping of sets of compounds known to interact with other cytokinin- or cAMP-binding proteins (11, 28).

If cAMP has a regulatory function in higher plants as in other eukaryotes, one expects to find high affinity cAMP-binding proteins in higher plants. In *Triticum*, there is a multiplicity of such proteins, namely the cyclic nucleotide-binding 5'-nucleotidase of seedling leaves (26, 27) and the wheat germ cAMP-binding proteins cABP I and cABP II. The physiological significance of these cAMP-protein interactions has yet to be established, and the functions of cABP I and cABP II are unknown. The interaction of cABP II with the active sites of the wheat germ protein kinases has limited analogy with the interaction of the regulatory subunit (R) with the catalytic subunit (C) of the mammalian form II cAMP dependent protein kinase (subunit composition, R_2C_2). R has a high-affinity for cAMP, binds to the active site of a protein kinase (C), and is phosphorylated in a reaction catalyzed by C (31). While we have been unable to demonstrate cAMP-dependent protein kinase in experiments involving cABP II and wheat germ protein kinases, it would be premature to exclude the possibility that cABP II may regulate the activities of these protein kinases under certain conditions.

LITERATURE CITED

1. AMRHEIN N 1977 The current status of cyclic AMP in higher plants. Annu Rev Plant Physiol 28: 123–132
2. AMRHEIN N 1974 Evidence against the occurrence of adenosine-3':5'-monophosphate in higher plants. Planta 118: 241–258
3. ASHTON AR, GM POLYA 1978 Cyclic adenosine 3':5'-monophosphate in axenic rye grass endosperm cell cultures. Plant Physiol 61: 718–722
4. ASHTON AR, GM POLYA 1977 Adenosine 3':5'-cyclic monophosphate in higher plants. Isolation and characterization of adenosine 3':5'-cyclic monophosphate from *Kalanchoë* and *Agave*. Biochem J 165: 27–32
5. ASHTON AR, GM POLYA 1975 Higher plant cyclic nucleotide phosphodiesterases. Resolution, partial purification and properties of three phosphodiesterases from potâto tuber. Biochem J 149: 329–339
6. BREWER JM 1974 General techniques: preparation of materials. *In* JM Brewer, AJ Pesce, RB Ashworth, eds, Experimental Techniques in Biochemistry. Prentice-Hall, Englewood Cliffs, NJ, pp 1–9
7. BREWIN NJ, DH NORTHCOTE 1973 Partial purification of a cyclic AMP phosphodiesterase from soybean callus. Isolation of a non-dialysable inhibitor. Biochim Biophys Acta 320: 104–122
8. BROWN EG, T AL-NAJAFI, RP NEWTON 1979 Adenosine 3':5'-cyclic monophosphate, adenylate cyclase and a cyclic AMP binding-protein in *Phaseolus vulgaris*. Phytochemistry 18: 9–14
9. BROWN EG, MJ EDWARDS, RP NEWTON, CJ SMITH 1980 The cyclic nucleotide phosphodiesterases of spinach chloroplasts and microsomes. Phytochemistry 19: 23–30
10. BROWNLEE AG, DR PHILLIPS, GM POLYA 1980 Purification and characterization of two high-affinity adenosine 3':5'-cyclic monophosphate-binding proteins from yeast. Identification as multiple forms of glyceraldehyde-3-phosphate dehydrogenase. Eur J Biochem 109: 39–49
11. BROWNLEE AG, GM POLYA 1980 The ligand specificity of the 3',5'-cyclic AMP-binding site of yeast glyceraldehyde-3-phosphate dehydrogenase. Interaction with adenosine derivatives and pharmacologically-active compounds. Eur J Biochem 109: 51–59
12. COHEN P 1978 The role of cyclic AMP-dependent protein kinase in the regulation of glycogen metabolism in mammalian skeletal muscle. Curr Top Cell Regul 14: 117–190
13. DAWSON RMC, DC ELLIOTT, WH ELLIOTT, KM JONES (eds.) 1969 Data for Biochemical Research, 2nd ed. Oxford University Press, London, p 618
14. EIL C, IG WOOL 1973 Phosphorylation of liver ribosomal proteins. Characteristics of the protein kinase reaction and studies of the structure of phosphorylated ribosomes. J Biol Chem 248: 5122–5129
15. GIANNATTASIO M, G CARRATU, GF TUCCI 1974 Presence of a cyclic-AMP-binding protein in Jerusalem artichoke rhizome tissues. FEBS Lett 49: 249–253
16. GIANNATTASIO M, G CARRATU, GF TUCCI, AM CARAFA 1979 Cyclic AMP binding protein from Jerusalem artichoke rhizome tissues. Phytochemistry 18: 1613–1616
17. HEYNS W, P DE MOOR 1974 A 3(17)β-hydroxysteroid dehydrogenase in rat erythrocytes. Conversion of 5α-dihydrotestosterone into 5α-androstane-3,17β-diol and purification of the enzyme by affinity chromatography. Biochim Biophys Acta 358: 1–13
18. HINTERMANN R, RW PARISH 1979 Determination of adenylate cyclase activity in a variety of organisms: evidence against the occurrence of the enzyme in higher plants. Planta 146: 459–461
19. LANZANI GA, M GIANNATTASIO, LA MANZOCCHI, R BOLLINI, AN SOFFIENTINI, V MACCHIA 1974 The influence of cyclic GMP on polypeptide synthesis in a cell-free system derived from wheat embryos. Biochem Biophys Res Commun 58: 172–177
20. LIN P P-C 1974 Cyclic nucleotides in higher plants? Adv Cyclic Nucleotide Res 4: 439–461
21. LIN P P-C, JE VARNER 1972 Cyclic nucleotide phosphodiesterase in pea seedlings. Biochim Biophys Acta 276: 454–474
22. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
23. MORRISON JF 1968 Chromatographic separation of nucleoside phosphates on diethylaminoethylcellulose paper. Anal Biochem 24: 106–111
24. PASTAN I, S ADHYA 1976 Cyclic adenosine 5'-monophosphate in *Escherichia coli*. Bacteriol Rev 40: 527–551
25. POLYA GM 1975 Purification and characterization of a cyclic nucleotide-regulated 5'-nucleotidase from potato. Biochim Biophys Acta 384: 443–457
26. POLYA GM 1974 Regulation of a plant 5'(3')-ribonucleotide phosphohydrolase by cyclic nucleotides and pyrimidine, purine and cytokinin ribosides. Proc Natl Acad Sci USA 71: 1299–1303
27. POLYA GM, AR ASHTON 1973 Inhibition of wheat seedling 5'(3')-ribonucleotide phosphohydrolase by adenosine 3',5'-cyclic monophosphate. Plant Sci Lett 1: 349–357
28. POLYA GM, JA BOWMAN 1979 Ligand specificity of a high affinity cytokinin-binding protein. Plant Physiol 64: 387–392
29. POLYA GM, AW DAVIS 1978 Properties of a high-affinity cytokinin-binding protein from wheat germ. Planta 139: 139–147
30. POLYA GM, JPH SIA 1976 Properties of an adenosine 3',5'-cyclic monophosphate-binding protein from wheat embryo. Plant Sci Lett 7: 43–50
31. ROSEN OM, R RANGEL-ALDAO, J ERLICHMAN 1977 Soluble cyclic AMP-dependent protein kinases: review of the enzyme isolated from bovine cardiac muscle. Curr Top Cell Regul 12: 39–74
32. SACHAR RC, SR TANEJA, K SACHAR 1975 Cyclic AMP—its biological role in higher plants. J Sci Ind Res 34: 54–64
33. SCHENDEL PF, RD WELLS 1973 The synthesis and purification of [γ-^{32}P]-adenosine triphosphate with high specific activity. J Biol Chem 248: 8319–8321
34. SCOPES RKS 1974 Measurement of protein by spectrophotometry at 205 nm. Anal Biochem 59: 277–282
35. TREWAVAS A 1976 Post-translational modification of proteins by phosphorylation. Annu Rev Plant Physiol 27: 349–374