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Purification and Properties of a Protein Which Binds Cytokinin-active 6-Substituted Purines[1]

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ABSTRACT

A protein which binds 6-substituted purines of the cytokinin type with relatively high affinity has been extensively purified from wheat germ. Conventional chromatographic techniques, as well as an affinity matrix to which a cytokinin was covalently coupled, were used in the purification. The wheat germ cytokinin-binding protein (CBF-1) has four unlike subunits and an apparent molecular weight of 183,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

CBF-1 is saturated at one cytokinin molecule per tetramer with a K_d for 6-benzylaminopurine of 5×10^{-7} molar. The protein exists both on the native wheat germ ribosome (1 molecule CBF-1 per 80S ribosome) and free in the cytosol with approximately three copies of the latter for each of the former. Data from affinity chromatography studies and cross-linking experiments strongly suggest that a specific binding site for CBF-1 occurs on the wheat germ ribosome.

Although cytokinins appear to regulate a wide variety of growth processes in higher plants, exhibiting activity in some instances at concentrations as low as 10^{-10} M (4), no satisfactory unifying concept has yet been proposed to account for their various activities. In recent years, attention has turned to the interaction of these low mol wt substances with large molecules in the cell which may be potential receptor sites (2, 10, 12). We described, in a preliminary report in 1975 (5), the first solubilization of a high-affinity, saturable cytokinin-binding moiety and presented evidence for the protein nature of the substance, which was isolated from wheat germ ribosomes. More recently (6), we have shown that the protein exists both loosely attached to ribosomes and as a soluble entity and is highly specific for 6-substituted purines of the cytokinin type. Some properties of what is apparently the same wheat germ protein have also recently been described by others (14–16). A very small protein with considerably lower affinities for cytokinins and quite different properties than the wheat germ protein has been reported from tobacco leaves (20). Here, we describe procedures for the complete isolation and purification of CBF-1[4], indicate some of its physical properties, and discuss the nature of its association with the ribosome.

EXPERIMENTAL PROCEDURES

Preparation of Ribosomal Salt Wash. Freshly milled wheat germ obtained locally was pulverized into a fine powder in a chilled mortar and subsequently ground in 1.5 volumes (w/v) grinding media (50 mM KOAc, 2 mM $CaCl_2$, 1 mM $Mg(OAc)_2$, and 1 mM DTT). The thick paste then was centrifuged at approximately 23,000*g* for 15 min at 4 C. The supernatant was collected and sufficient 1.0 M Tris-OAc (pH 7.6) and 0.1 M $Mg(OAc)_2$ were added to give final concentrations of 25 and 2.5 mM, respectively. The supernatant was centrifuged as before, and the resulting supernatant was decanted and centrifuged at 180,000*g* for 4 h at 4 C. The upper 80% of the postribosomal supernatant (S100) was recovered and stored at −80 C. The remaining supernatant was discarded and the underlying ribosomes were gently suspended at a concentration of approximately 500 A_{260} units/ml in a buffer [25 mM Tris-OAc (pH 7.6), 50 mM KOAc, 5 mM $Mg(OAc)_2$, and 3 mM 2-mercaptoethanol]. After a low speed centrifugation at 10,000*g* for 15 min to remove aggregates, the ribosomes were pelleted as before. The ribosomes then were resuspended in the same buffer, made 0.75 M in KCl, at 4 C and pelleted through this high salt wash at 180,000*g* for 4 h. The high salt extract was carefully collected so as not to disturb the pelleted material and was used as the source of CBF-1 protein in all the subsequent procedures.

Cytokinin-binding Assay. Radiolabeled cytokinins are obtainable commercially only at specific activities far too low to be of much use in receptor site studies. Accordingly, we have prepared for this work bzl^{6}Ade labeled in the benzene ring with tritium at a specific activity of 27 Ci/mmol. (7). In some of the routine assays, bzl^{6}Ade labeled with ^{14}C at position 8 of the purine moiety was used (12.8 mCi/mmol; Amersham/Searle). Cytokinin-binding activity was assayed by equilibrium dialysis. For routine assays of preparations made during the purification of CBF-1, a 0.6-ml aliquot of the sample was dialyzed 18 h against a buffer [24 mM Tris-OAc (pH 7.6), 50 mM KOAc, 5 mM $Mg(OAc)_2$, and 1 mM DTT] containing 3 nM [^{3}H]bzl^{6}Ade. Aliquots (0.4 ml) were removed from inside and outside the dialysis bag and measured for radioactivity. All fractions assayed from a chromatography run (30 to 50 dialysis bags) were dialyzed together in a single stirred container so that the final concentration of free ligand was identical for all fractions. Bound radioactivity was assumed to be that amount of radioactivity per 0.4-ml aliquot inside the dialysis bag (bound plus free) minus the amount of radioactivity per 0.4 ml outside (free) the dialysis bag.

For the determination of binding site concentrations and dissociation constants, a set of solutions containing increasing concentrations of [^{3}H]bzl^{6}Ade with proportionately decreasing radiolabel specific activity was made by dilution with unlabeled bzl^{6}Ade. The data were analyzed by the method of Scatchard (18).

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[4] Abbreviations: CBF-1, wheat germ cytokinin-binding protein; bzl^{6}Ade, 6-benzylaminopurine; bzl^{6}Ado, 6-benzylamino-9-β-D-ribofuranosylpurine; DMS, dimethylsuberimidate; OAc, acetate.

When the binding of ligand to a protein fraction of unknown molar concentration was determined, the results were graphed according to the form described by Rosenthal (17). Protein concentrations were determined by the method of Lowry *et al.* (11) as modified by Shatkin (19). One unit of binding activity was arbitrarily defined as that amount of protein which will bind 1 pmol bzl^{6}Ade when the concentration of free ligand is 1 μM.

Preparation of a Cytokinin Affinity Matrix. Bzl^{6}Ado was coupled to Sepharose 4B gel using a modification of the procedure of Lamed *et al.* (9). Sodium metaperiodate was added to a 50 mM solution of the cytokinin nucleoside in 10% aqueous pyridine (necessary because of the low solubility of the nucleoside in water at pH 7.0) so that a final concentration of 10 mM periodate was achieved. The oxidation reaction was allowed to proceed in the dark for 4 h at room temperature, after which the solution was made pH 5.5 by the addition of 50% (v/v) acetic acid. Sepharose 4B coupled to adipic acid dihydrazide was prepared by activation of Sepharose with CNBr (0.3 g/ml packed gel) as previously described (3). The activated gel was then added to an equal volume of adipic acid dihydrazide (90 g/l) and gently stirred with a mechanical stirrer for 16 h at 4 C. The gel then was washed with approximately 10 volumes 100 mM Na acetate buffer (pH 5.0) and subsequently incubated at 4 C for 16 h with an equal portion of the oxidized nucleoside reaction mixture. Finally, the gel was extensively washed with 100 mM Na acetate buffer (pH 5.0), followed by successive washes with 100 mM NaCl and water. Estimation of the amount of coupling was achieved by suspending a small aliquot of gel in 100 mM NaCl and immediately measuring the A at 271 nm against a blank containing an equal amount of underivatized gel. The amount of covalently coupled oxidized bzl^{6}Ado (bzl^{6}Ado$_{ox}$) ranged from 6.0 to 4.5 μmol/ml packed Sepharose in different preparations.

Radiolabeling of CBF-1. Highly purified CBF-1 was labeled with ^{14}C using the reductive alkylation procedure of Benne *et al.* (1). CBF-1 (100 mg) was dialyzed overnight against 100 mM potassium borate (pH 9.0), 0.1 mM EDTA, 90 mM KCl, and 4.5% glycerol. To the dialyzed protein in 1.0 ml was added 50 μCi [^{14}C]formaldehyde (49 mCi/mmol, New England Nuclear), followed by 0.17 μmol sodium borohydride in water (prepared immediately before use), which was added in 10 × 10-μl aliquots over 30 min. The reaction mix then was chromatographed on a Sephadex G-25 column (0.9 × 25 cm), and radioactive fractions eluting with the void volume were collected and pooled. Nearly all of the radioactivity in this preparation was precipitable with 10% (w/v) trichloracetic acid at 4 C, and the protein was estimated to have a specific radioactivity of 1.1 nCi/μg protein.

RESULTS

PURIFICATION OF RIBOSOMAL ASSOCIATED CBF-1

A number of chromatographic and salt precipitation fractionation procedures were attempted in preliminary experiments to isolate CBF-1. The procedure given below was found to provide a high yield of homogeneous material which was stable, as determined by cytokinin binding activity, when stored at −80 C. The purified protein was stored in small aliquots, as freezing and thawing tended to promote precipitation which resulted in the loss of activity. All purification steps were performed in the indicated sequence with no interruption between steps, as storage by freezing of pooled fractions from individual runs resulted in significantly lower final yields.

Phosphocellulose Chromatography. Ribosomal high salt wash protein was isolated from 32,000 A_{260} units of ribosomes derived from 160 g wheat germ with a yield of 250 mg protein. This material in a buffer containing 10 mM Tris-HCl (pH 7.6), 0.1 mM EDTA, 50 mM KCl, and 5 mM 2-mercaptoethanol at a concentration of 10 mg/ml was applied to a phosphocellulose column (Whatman P-11, 1.6 × 20 cm) equilibrated in the same buffer. The column was eluted with 50 ml buffer at a flow rate of approximately 30 ml/h, followed by a step gradient of the buffer made of 0.15, 0.5, 0.75, and 1.0 M KCl (50 ml at each step). About 75% of the cytokinin-binding activity eluted from the column immediately after the addition of 0.5 M KCl buffer. The remaining activity was distributed between the void peak, the 0.15 M KCl, and the 0.75 M KCl elution steps, with the 0.15 M KCl elution having the most binding activity. Rechromatography of the 0.15 M KCl-eluted protein on phosphocellulose resulted in a similar distribution of binding activity as was observed in the original experiment, indicating that binding activity eluting at 0.15 M KCl was identical to the material eluting at 0.5 M KCl. Attempts to chromatograph CBF-1 on phosphocellulose using a linear gradient resulted in the elution of binding activity in a broad asymmetrical peak with several shoulders, coupled with the loss of as much as 75% of the initial binding activity. Although this could be interpreted as evidence for different species of binding proteins, it is more likely to be the result of tertiary structure disruption, a commonly observed phenomenon with multisubunit proteins chromatographed on phosphocellulose.

DEAE/Bio-Gel A Chromatography. The 0.5 M KCl eluate from the phosphocellulose column (69 mg protein, approximately 4.3 mg/ml) was dialyzed against 1 liter of a buffer [10 mM Tris-HCl (pH 7.75), 25 mM KCl, 0.1 mM EDTA, and 5 mM 2-mercaptoethanol] for 12 h. The dialyzed protein was centrifuged at 10,000g to remove precipitated material, diluted 2:1 with distilled H_2O, and immediately applied to a 1.6 × 15-cm DEAE/Bio-Gel A column (Bio-Rad Laboratories) equilibrated in the same buffer. The column was subsequently eluted with 25 ml of this buffer followed by a linear gradient (25–250 mM KCl) in the buffer (150 × 150 ml). A relatively sharp peak of binding activity eluted from the column between 40 and 75 mM KCl, which also coincided with a major peak of protein. Absorption of CBF-1 to DEAE resin required very low salt concentrations, and binding activity was rapidly lost through precipitation if the protein was stored in this buffer for longer than 24 h. The amount of binding activity that eluted in the void peak was variable, depending on the length of dialysis. Dilution with water (after a 12-h dialysis period) was found to be the most effective method of lowering the salt concentration and avoiding loss of activity which accompanies extensive dialysis.

Sephadex G-200 Gel Filtration Chromatography. Fractions eluting between 40 and 75 mM KCl from the DEAE/Bio-Gel A column were pooled and concentrated with an Amicon ultrafiltration device (model 52, Amicon Corp.) using an Amicon, prewashed, 43 mm PM-10 membrane under 40 p.s.i. of nitrogen. The concentrated protein (25 mg, approximately 1.5 mg/ml) was applied directly to a G-200 Sephadex column (2.5 × 96 cm), equilibrated in a buffer [25 mM Tris-HCl (pH 7.5), 50 mM KCl, 0.1 mM EDTA, and 4 mM 2-mercaptoethanol]. The column was developed at a flow rate of 14 ml/h under a hydrostatic pressure of 15 cm. CBF-1-binding activity eluted as a single peak corresponding exactly with a symmetrical 280 nm absorption profile.

The eluted CBF-1 protein was concentrated by ultrafiltration and stored in small aliquots at −80 C. The over all purification of CBF-1 from the ribosomal salt wash by this three-column procedure was 4.6-fold, with a yield of 38%. This apparently low degree of purification is misleading in terms of the final product since the ribosomal wash used as starting material in this procedure contains relatively few proteins. The ribosome itself acts as a pelletable affinity matrix for CBF-1, and great purification of the protein is achieved simply by isolating ribosomes and removing CBF-1 with 0.75 M KCl.

PURIFICATION OF CBF-1 BY AFFINITY CHROMATOGRAPHY

Preliminary studies (5, 6) indicated that CBF-1 exists loosely bound to the wheat germ ribosome as well as in a free, soluble

state in the postribosomal supernatant. Attempts to purify the latter by the three-step chromatographic procedure described above results in a preparation which has a substantially lower specific activity than that isolated from ribosomes because of the much greater level of contaminating proteins present in the high speed supernatant fraction. Accordingly, an affinity chromatography procedure has been developed in which bzl^{6}Ado was cross-linked to an insoluble support medium. A column (0.9 × 3.0 cm) of this cytokinin-affinity matrix was equilibrated in a buffer containing 25 mM Tris-HCl (pH 7.6), 90 mM KOAc, 1.5 mM $Mg(OAc)_2$, and 1 mM DTT. An aliquot (36 mg in 1.0 ml of this buffer) of the postribosomal supernatant which had first been passed through a Sephadex G-25 column to eliminate low molecular weight impurities was applied to the column, and it was washed with 15 ml of the buffer. Subsequently, the column was eluted with 15 ml of the same buffer in which the K acetate was replaced with 2.5 M KCl, followed by a final 15 ml elution with 2.0 M KCl and 4.0 M urea in the same buffer. The bulk of the cytokinin-binding substance came off the column with 2.5 M KCl in the absence of urea (Fig. 1). Attempts to elute CBF-1 by competition elution with buffers containing free bzl^{6}Ade or bzl^{6}Ado were relatively ineffective as the protein eluted in a very broad and dilute peak. The effective concentration of the immobilized cytokinin on the column was approximately 10-fold greater than could be achieved in solution because of the low solubility of cytokinins in water. This fact no doubt accounted for our inability to elute CBF-1 by competition for the bound protein with a free ligand. The affinity chromatography procedure resulted in a better than 25-fold purification with a recovery of 81%.

CHARACTERIZATION AND PROPERTIES OF CBF-1

Mol Wt Determinations. Purified samples of CBF-1 prepared from either the ribosomal high salt wash or from the postribosomal supernatant were chromatographed on a Sephadex G-200 column precalibrated with mol wt standards. Samples were co-chromatographed with several mol wt standards to increase the accuracy of estimating the elution volume of CBF-1. The data (Fig. 2) indicate that the mol wt of native CBF-1 is approximately 155,000. Both ribosome associated and postribosomal supernatant derived CBF-1 gave identical mol wt values by this procedure. Other attempts at determining the mol wt or homogeneity of purified native CBF-1 included polyacrylamide gel electrophoresis in basic and acidic systems. At basic pH, in 7.5% acrylamide gels using the method of Maziel (13), CBF-1 only partially entered the gel and appeared as a single very diffuse band after staining. In acidic pH gels, CBF-1 was apparently unstable and appeared as diffuse bands.

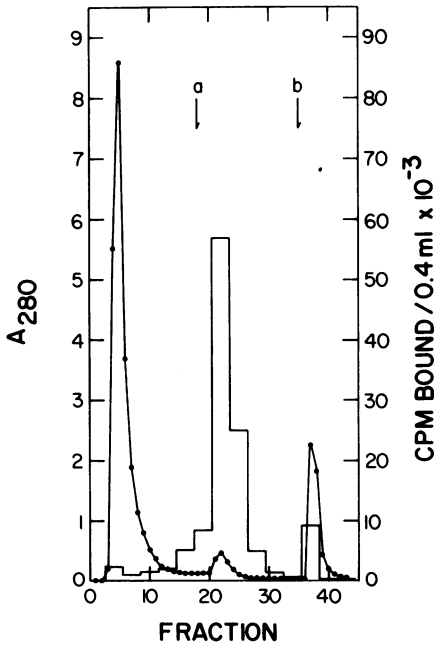

FIG. 1. Affinity chromatography of CBF-1. Wheat germ postribosomal supernatant protein (36 mg in 1.0 ml) was applied to the top of a Sepharose 4B column (0.9 × 3.0 cm) containing covalently attached bzl^{6}Ade. The column was eluted with a buffer [25 mM Tris-OAc (pH 7.6), 90 mM KOAc, 1.5 mM $Mg(OAc)_2$, and 1 mM DTT] followed at (a) with a high salt buffer [25 mM Tris-HCl (pH 7.6), 2.5 M KCl, 1.5 mM $Mg(OAc)_2$, 1 mM DTT]. At b, the latter buffer was made 2.0 M in KCl and 4.0 M in urea. Binding of bzl^{6}Ade (bar graph) was determined on 0.4-ml aliquots from three combined 0.9-ml fractions subjected to equilibrium dialysis (concentration of free bzl^{6}Ade was 1 nM).

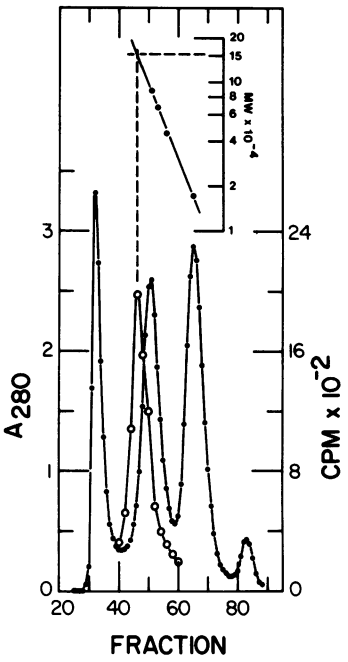

FIG. 2. CBF-1 mol wt determination by gel filtration chromatography. One-half mg CBF-1 purified through the three column procedure (see text and Figs. 1-3) was chromatographed on a column of Sephadex G-200 (2.5 × 96 cm) along with dextran blue (mol wt, greater than 2,000,000), 10 mg conalbumin (mol wt, 86,000), 10 mg ovalbumin (mol wt, 43,000), 10 mg myoglobin (mol wt, 18,000), and 0.1 mg phenol red as an elution volume indicator. Fractions (O——O) were assayed for bzl^{6}Ade binding as described in the legend to Figure 1.

SDS-Gel Electrophoresis. This procedure was carried out essentially as described by Laemmli (8) in 10% acrylamide gels. The apparent subunit patterns of CBF-1 from the ribosomal wash purified by the three column chromatography procedure are compared with those of CBF-1 prepared from the postribosomal supernatant by affinity chromatography in Figure 3. A lightly stained band in the ribosomal-derived material with a mol wt about 50,000 is missing in the affinity chromatography-purified material. Likewise, a faint band with a mol wt of about 20,000 in the latter is absent from the ribosomal material. Since the two preparations have about the same specific activity for cytokinin binding, it is concluded that these two bands are contaminants not essential for cytokinin binding. Faintly staining, low mol wt (12,000–15,000) bands appear in some gels, but with great variability among preparations, and are probably contaminants or degradation products of CBF-1. The protomer of CBF-1 would thus appear to be made up of four subunits with mol wt on SDS-polyacrylamide gel electrophoresis of 34,000, 39,000, 53,000, and 57,000 to give a total mol wt of 183,000, some 18% greater than that estimated by Sephadex-G200 chromatography of the native molecule.

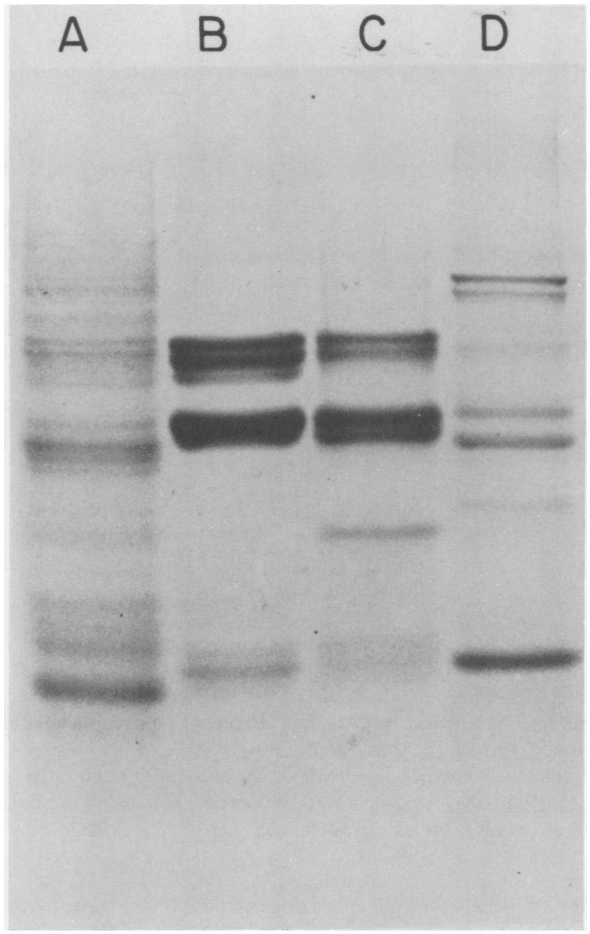

FIG. 3. SDS-polyacrylamide gel electrophoresis of CBF-1. Samples are: A, 25 μg crude postribosomal supernatant from wheat germ; B, 20 μg CBF-1 purified through the three column procedure; C, 15 μg CBF-1 purified by affinity chromatography (see text and Fig. 1), D, 5 μg each conalbumin (mol wt, 86,000), BSA (mol wt, 68,000), ovalbumin (mol wt, 43,000), DNase I (mol wt, 34,000), and RNase A (mol wt, 12,600). Samples were run at 50 v constant voltage for 4 h in 10% SDS-polyacrylamide gels.

Cytokinin-binding Characteristics of CBF-1. In preliminary studies (5, 6), we have shown that Scatchard plot analysis (18) of concentration-dependent binding of cytokinins to wheat germ ribosomes yields a biphasic curve which was interpreted as the interaction of a single, relatively high affinity site ($K_d = 5 \times 10^{-7}$ M for bzl^{6}Ade) together with multiple low affinity ($K_d > 10^{-4}$ M) sites. Only the high affinity site was removed by 0.75 M KCl and it was saturated at approximately 1 molecule ligand/ribosome. We analyzed the purified protein for concentration-dependent binding in the same manner, and our data indicate that, at ligand saturation, 6.1 nmol bzl^{6}Ade is bound/mg purified protein. Assuming a mol wt for CBF-1 of 183,000 as determined by summing the apparent subunit mol wt found on SDS gels, we calculate a ratio of 1.12 mol cytokinin bound/mol CBF-1. Using a mol wt of 155,000 as determined by Sephadex G-200 chromatography, the corresponding ratio is 0.95 mol cytokinin bound/mol protein. Since 1 molecule of cytokinin is bound to the high affinity site of each ribosome (5, 6), we conclude that native wheat germ ribosomes contain an average of one copy of CBF-1 per ribosome. With regard to specificity of the binding we have previously demonstrated (6) that only 6-substituted purines with cytokinin activity exhibit high affinity binding to CBF-1. However, certain hydroxylated cytokinins, including zeatin [6(4-hydroxy-3-methyl-2-butenylamino)-purine], which have cytokinin activity in tissue culture bioassays exhibit very much less affinity for CBF-1 than for bzl^{6}Ade (6, 14, 16). This point will be discussed further elsewhere. Polya and Bowman (15) report high affinity binding of a number of structurally disparate compounds having no cytokinin activity to a wheat embryo protein. The significance of this finding awaits further clarification of the purity of their preparation which is probably not homogeneous (16). Apparently, the intact CBF-1 molecule is required for binding. Subunits of CBF-1 isolated from gels and from columns by a variety of procedures exhibit no cytokinin-binding capacity either singly or in various combinations. Pretreatment of CBF-1 with 6 M urea similarly abolishes all cytokinin binding capacity.

Table I. *Amino Acid Composition of CBF-1*

One-half mg acetone-precipitated CBF-1 was hydrolyzed in 1 ml 5.7 N HCl at 110 C under vacuum for 24, 48, or 72 h. Values are corrected for degradation or slow release during hydrolysis.

Amino Acid	Amino Acid in CBF-1	Amino Acid Composition
	mol/mol	%
Cysteic acid	10	0.7
Asp	107	7.6
Thr	58	4.1
Ser	105	7.5
Glu	242	17.2
Pro	49	3.5
Gly	169	12.0
Ala	93	6.6
Val	107	7.6
Met	8	0.6
Ile	37	2.6
Leu	76	5.4
Try	40	2.8
Phe	62	4.4
Lys	48	3.4
His	48	3.4
Arg	149	10.6

Ultraviolet Spectrum and Amino Acid Composition of CBF-1. The protein has a notably complex UV absorption spectrum which allows it to be readily identified. The absorption maximum occurred at 277.5 nm with shoulders (as determined by the first derivative spectrum) occurring at 297, 290.5, 287.5, 283, 270, 268, 266, 264, and 254 nm. Points of inflection are found at 291.5, 289.5, 286, 280, 271, 269.5, 267.5, 265.5, 263, 259.5, and 256.6 nm. The amino acid composition of CBF-1 (Table I) indicates it to be rich in the basic amino acid arginine and deficient in sulfur-containing amino acids.

Removal of CBF-1 from Ribosomes by Sucrose Density Gradient Fractionations. Preliminary attempts to purify ribosomes by centrifugation through sucrose gradients resulted in the loss of the high affinity cytokinin-binding site from the ribosomes. Cytokinin-binding activity appeared in the gradient above the ribosomes as a skewed curve with maximal activity corresponding to a sedimentation coefficient of 7.5S (Fig. 4). A considerable amount of cytokinin binding was detected in the gradient in regions as high as 15S, suggesting that the binding protein is being partially dragged through the gradient because of its interaction with the ribosome.

Affinity Chromatography of CBF-1-depleted Ribosomes. The dissociation of CBF-1 from ribosomes as a result of sucrose gradient centrifugation suggests that the protein is very weakly bound to the ribosome. Whether or not the association is real or due to co-precipitation of the protein along with the ribosome in low-ionic strength buffers, nonspecific binding, ionic interactions because of the highly basic nature of CBF-1, or some other nonspecific association has been called into question (16). To test

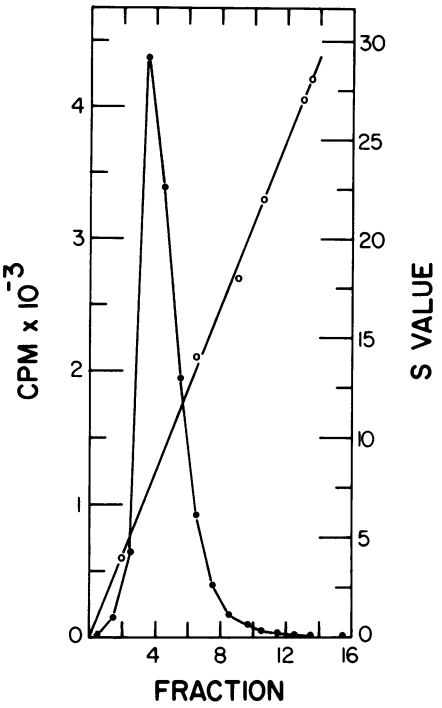

FIG. 4. Sedimentation of CBF-1 on calibrated sucrose gradients. Wheat germ ribosomes (55 A_{260} units) isolated in a low salt buffer and which therefore contained saturating levels of CBF-1 were layered on a 12-ml gradient of sucrose (5–20%) in buffer and centrifuged for 6.2 h at 39,000 rpm in an IEC SB-283 rotor. Standards for the S value determination (○——○) were crude wheat germ RNA (2.3 A_{260} units containing 4, 18, and 28S RNA) and Brome Mosaic virus RNA (27S RNA). Gradients were fractionated into 0.75-ml aliquots and UV absorbance at 254 nm was monitored continuously. Fractions were assayed for bzl^{6}Ade binding (●——●) as described in the legend to Figure 1.

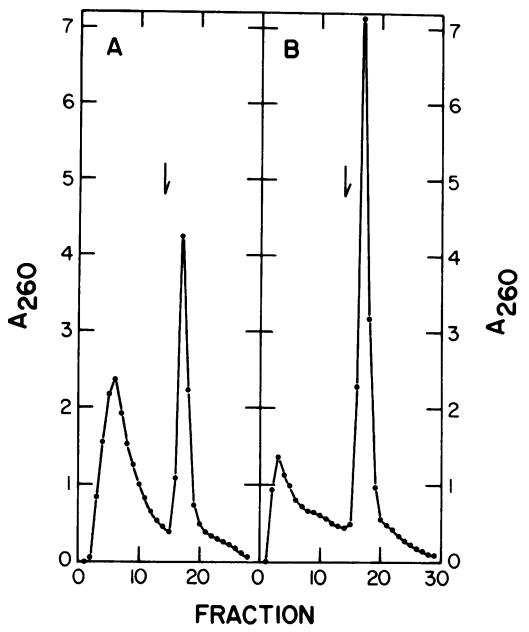

FIG. 5. Chromatography of wheat germ ribosomes depleted of CBF-1 by sucrose gradient centrifugation on a cytokinin affinity column. Thirty A_{260} units CBF-1-depleted ribosomes were added to a 0.9 × 3.0-cm column of Sepharose 4B to which bzl^{6}Ado had been covalently coupled either in the absence (Panel A) or presence (Panel B) of 80 μg highly purified CBF-1. The columns were developed with 12 ml of a buffer [10 mM Tris-OAc (pH 7.6), 50 mM KOAc, 5 mM $Mg(OAc)_2$, and 1 mM DTT]. The arrows indicate the point at which the buffer was made 0.75 M in KCl.

this point, ribosomes depleted of CBF-1 by sucrose density gradient centrifugation as described above were passed through a column of Sepharose 4B to which a cytokinin had been coupled. In the absence of added CBF-1 (Fig. 5, panel A), the majority (approximately 70%) of the ribosomes elute from the column in the void volume. If the chromatography is done in the presence of CBF-1 supplied as an equal molar ratio of the highly purified product, then the bulk of the ribosomes (75%) are retained on the column and require the addition of high salt concentrations to the buffer to elute them from the affinity matrix (Fig. 5, panel B). The 30% background retention of CBF-1-depleted ribosomes in the absence of added binding protein may be a result of the multiple low affinity cytokinin-binding sites previously described (5) interacting with the very high effective concentration of bzl^{6}Ado on the affinity column. Alternatively, the association may result from hydrophobic interactions of the ribosomes with non-cytokinin portions of the gel, such as the adipic acid dihydrazide spacer used in attachment of the cytokinin to the gel matrix. A similar study, in which ribosomes depleted of CBF-1 by washing in 0.75 M KCl were used, demonstrated no stimulation of added CBF-1 on ribosome retention to the affinity column. The high salt wash has presumably irreversibly altered the binding site on the ribosome with which CBF-1 interacts perhaps by removing other proteins in the vicinity of the site.

Chemical Cross-linking of CBF-1 to Depleted Ribosomes. Although the affinity chromatography experiments indicate that CBF-1 associates with 80S ribosomes, these experiments do not clarify the specificity of the interaction. A specific interaction is defined here as one involving a finite number of sites, topographically locatable, and of sufficient affinity to have a significant number of sites occupied at endogeneous concentrations of the interacting groups. Even though the affinity of CBF-1 for ribosomes is probably low, determination of the above conditions is possible through the use of cross-linking reagents which covalently link associated proteins. Here, attempts to cross-link CBF-1 to ribosomes were performed using DMS and glutaraldehyde in the presence of wheat germ ribosomes depleted of CBF-1 by sucrose gradient centrifugation and radiolabeled CBF-1.

In preliminary runs, cross-linking with glutaraldehyde at concentrations from 0.025 to 0.125% resulted in CBF-1-ribosome complexes but, at high levels, a substantial amount of 80S ribosomal dimers formed, which indicates nonspecific cross-linking. At 0.05% glutaraldehyde, a near-maximal amount of CBF-1-ribosome cross-linking was observed with a minimal amount of 80S dimer formation. Attempts to cross-link ribosomes and CBF-1 using DMS at concentrations from 3 to 50 mM failed to yield any positive results. The ineffectiveness of the dimethylsuberimidate may be a result of its narrow spatial requirements, or a deficiency of lysine residues in the protein as suggested by the amino acid analysis. Further investigation with *bis*-alkyl-imidates of varying chain lengths would be necessary to resolve these possibilities.

Considerably more glutaraldehyde-mediated covalent cross-linking of [^{14}C]CBF-1 was observed to ribosomes depleted by sucrose gradient centrifugation than to native wheat germ ribosomes which had a full complement of CBF-1 as determined by equilibrium dialysis (Fig. 6). Some incorporation of radioactivity was observed in the latter ribosomes, however, probably due to exchange between free [^{14}C]labeled CBF-1 and bound CBF-1 during equilibration which may have occurred during the 5-min incubation period before glutaraldehyde was added.

As a further check on the specificity of CBF-1 binding to the ribosomes, competition studies were run in which glutaraldehyde-mediated cross-linking of [^{14}C]CBF-1 to CBF-1-depleted ribosomes was carried out in the presence of unlabeled CBF-1 or

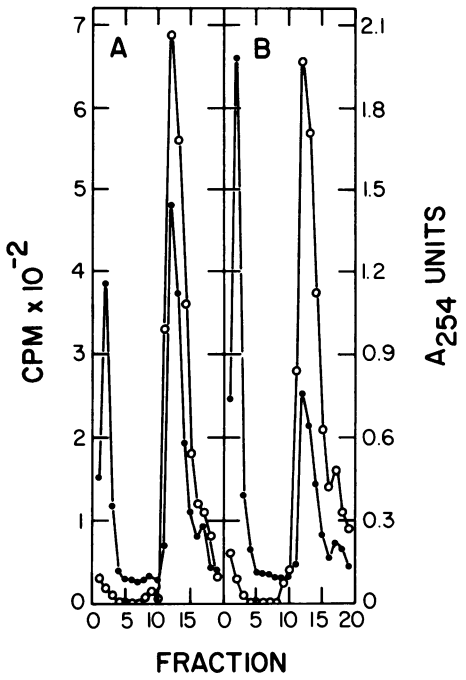

FIG. 6. Glutaraldehyde-mediated cross-linking of radiolabeled CBF-1 to CBF-1-depleted and native ribosomes. [^{14}C]CBF-1 was prepared as described. Ribosomes were either isolated in a low salt buffer as described under "Experimental Procedures" and were thus saturated with CBF-1 or depleted of CBF-1 by sucrose gradient centrifugation (see Fig. 4). Each ribosome preparation was incubated for 5 min at 1 C in a buffer [10 A_{260} units in 250 μl 50 mM Hepes-KOH (pH 7.5), 25 mM KOAc, 7.5 mM $Mg(OAc)_2$, and 5 mM 2-mercaptoethanol] containing [^{14}C]CBF-1 (4000 dpm). Glutaraldehyde then was added to a final concentration of 0.05% (previously determined to give maximal yield) and the reaction mixture was incubated at 4 C for 1 h. The reaction mixture then was layered on linear sucrose gradients (5–25%) in buffer and centrifuged for 2 h at 39,000 rpm in an IEC SB-283 rotor. Gradients were fractionated into 0.5-ml aliquots and monitored for UV absorbance at 254 nm. To each fraction was added 100 μg BSA as carrier, and the mixture was precipitated with 2 ml 10% trichloracetic acid. Precipitates were collected on glass fiber filters and assayed for radioactivity by liquid scintillation spectrometry. Top of the gradient is at the left margin in each panel. The peak of radioactivity near the top of the gradient represents free CBF-1, the second peak is CBF-1 covalently cross-linked to 80S ribosomes, and the third small peak is believed to represent 80S dimers. Panel A, experiment performed with CBF-1-depleted ribosomes; Panel B, experiment performed with native ribosomes. (○), A_{254} units; (●), cpm.

equal concentrations of BSA. Cross-linking of CBF-1 to the ribosome in the presence of BSA was the same as in the control but substantially reduced in the presence of unlabeled CBF-1, indicating competition for the same binding site which was limiting.

DISCUSSION

The purification procedures described here yield a basic protein of 183,000 daltons having four subunits, two smaller ones (34,000 and 39,000 daltons) and two larger (53,000 and 57,000 daltons). We have considered the possibility that the protein is a tetramer consisting of two identical small subunits and two equal large ones partially modified by proteolysis during isolation. However, four distinct subunits invariably appear, even in samples prepared by the most rapid affinity chromatography procedures. Moore (14) reported that this protein contains a complex of several subunits including a low mol wt entity at 15,000 daltons. A pattern not unlike that described by Moore can be generated in CBF-1 by aging it for several weeks in solution at 4 C. Polya and Davis (16), although reporting five bands on electrophoresis gels, point out that their wheat germ cytokinin-binding protein preparation may not be homogeneous.

CBF-1 isolated from the high speed supernatant appears to be identical in every respect with material recovered from the ribosomal high salt wash. In a preliminary report (6), we described a 30,000 dalton entity from the cytosol which bound cytokinins with high affinity. More recent work (unpublished data) reveals that this moiety cross-reacts with an antibody prepared against CBF-1 and we now believe it to be partially degraded CBF-1. Thus, wheat germ has only a single high-affinity cytokinin-binding factor; we estimate that there exist 3 molecules CBF-1 free in the cytosol for each copy bound to the ribosome.

The evidence presented here suggests that the association of CBF-1 with the ribosome is the result of a specific interaction. Our data indicate that wheat germ ribosomes isolated in a low salt buffer contain 1 molecule CBF-1/80S ribosome. Although this apparent relationship may be coincidental, it indicates at the very least that CBF-1 does not bind in a nonspecific way to a multiplicity of ribosomal sites. Further evidence in favor of this view is provided by the study illustrated in Figure 5. Here, CBF-1 reassociates with ribosomes gently depleted of the protein by centrifugation through sucrose gradients but, as noted under "Results," not with ribosomes more harshly depleted by washing in 0.75 M KCl, a procedure which removes several proteins, some of which may be important in binding of CBF-1 to the ribosome. Studies with glutaraldehyde-mediated cross-linking of CBF-1 (Fig. 6) indicate that a site specific for CBF-1 probably exists on the ribosome. The covalent attachment of CBF-1 occurs to a far greater degree on CBF-1-depleted ribosomes than on CBF-1 saturated ribosomes. Nonspecific proteins, such as BSA, do not compete with CBF-1 for the site, whereas unlabeled CBF-1 itself very effectively dilutes the counts incorporated when [^{14}C]CBF-1 is provided in the reaction mixture.

One of the most surprising features reflected in the purification of CBF-1 is the large amount of the protein present in wheat germ. From 1 g of starting material, 300 A_{260} units of ribosomes saturated with CBF-1 could be recovered by extracting the homogenates several times. Inasmuch as we calculate that three copies of CBF-1 occur free in the cytosol for each one on the ribosome, the total yield is about 15 nmol (2.2 mg) CBF-1/g fresh weight of wheat germ. Polya and Davis (14) calculate a similar level of CBF-1 in wheat embryo and note that such concentrations argue against a receptor function for this protein analogous to the amplifying function of animal steroid receptors or adenylate cyclase-modifying hormone receptors. This argument, however, ignores the fact that the wheat embryo is a specialized storage organ in a quiescent state. Preliminary work in our laboratory with antibodies to CBF-1 demonstrates that, immediately upon germination, CBF-1 falls to very low levels and reappears in large amounts only during the formation of female reproductive tissues. Thus, the seed may be a reservoir of CBF-1 which becomes quickly diluted during germination by cell division. However, whether or not CBF-1 actually does mediate one or more of the biological roles of the cytokinins cannot be answered on the basis of present knowledge.

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