Plant Physiol. (1989) 89, 250–259
0032-0889/89/89/0250/10/$01.00/0

Received for publication May 6, 1988
and in revised form August 15, 1988

Purification and Identification of the Fusicoccin Binding Protein from Oat Root Plasma Membrane[1]

Albertus H. de Boer*[2], Bruce A. Watson, and Robert E. Cleland
University of Washington, Department of Botany KB-15, Seattle Washington 98195

ABSTRACT

Fusicoccin (FC), a fungal phytotoxin, stimulates the H^+-ATPase located in the plasma membrane (PM) of higher plants. The first event in the reaction chain leading to enhanced H^+-efflux seems to be the binding of FC to a FC-binding protein (FCBP) in the PM. We solubilized 90% of the FCBP from oat (*Avena sativa* L. cv Victory) root PM in an active form with 1% octyl-glucoside. The FCBP was stabilized by the presence of protease inhibitors. The FCBP was purified by affinity chromatography using FC-linked adipic acid dihydrazide agarose (FC-AADA). Upon elution with 8 molar urea, two major protein bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with molecular weights of 29,700 and 31,000 were obtained. Successive chromatography on DEAE Bio-Gel A, hexyl agarose, and FC-AADA resulted in the same two bands when the FC-AADA was eluted with sodium dodecyl sulfate. A direct correlation was made between ^{3}H-FC-binding activity and the presence of the two protein bands. The stoichiometry of the 29,700 and 31,000 molecular weight bands was 1:2. This suggests that the FCBP occurs in the native form as a heterotrimer with an apparent molecular weight of approximately 92,000.

The effects of growth regulators on plant growth and development have been studied extensively (29). However, the molecular mechanism of the primary action of the growth regulators is only poorly understood thus far. The primary action in hormonal signal transduction is usually the binding of the hormone to a specific receptor protein. For a number of reasons (low abundance, instability, low binding affinity, membrane localization) purification of hormone binding proteins from plants has proven to be difficult. Recently, auxin binding proteins have been purified from maize coleoptiles and maize shoots (18, 24).

Mammalian research has proven the value and power of toxins, such as snake venoms and bacterial neurotoxins, in the identification and purification of hormone receptors. For example, the acetylcholine receptor was identified and purified with the snake venom α-bungarotoxin, and turned out to be a cation channel (11). A parallel role for phytotoxins has yet to be demonstrated in plants, but a prime candidate for doing so seems to be FC.[3]

FC is a phytotoxin, produced by the fungus *Fusicoccum amygdali* L. It mimics the effects of auxin in many respects (19); hence, the interest of plant physiologists in FC. It is thought that FC, like auxin, has the PM-bound H^+-ATPase as its final physiological target. The H^+-ATPase plays a key role in the physiology of plant cells and it is likely that the activity of the enzyme is regulated in a number of different ways; FC probably interacts with one of the regulatory pathways.

More than 10 years ago, Dohrmann *et al.* (12) reported the presence of a binding protein for FC (FCBP) in a PM-enriched fraction of maize coleoptiles. The hypothesis that the FCBP and the H^+-ATPase were the same protein had to be abandoned after Stout and Cleland (26) separated these two proteins using gel filtration. It is believed now that the FCBP fulfills the role of a receptor which transduces the FC-signals across the PM. The key to understanding the steps between receptor activation and H^+-ATPase-stimulation lies in understanding the receptor itself. Therefore, purification and identification of the FCBP have become crucial for further progress in FC-research.

The first attempt to solubilize and purify the FCBP was made by Pesci *et al.* (21). Deoxycholate and Triton X-100 solubilized about 30% and 10%, respectively, of the FCBP in active form; using gel filtration they estimated that the mol wt of the FCBP was around 80 kD. Stout and Cleland (26) also used Triton X-100 to solubilize the FCBP from oat root microsomal membranes; they estimated the mol wt of the FCBP to be in the range of 60 to 100 kD. Aducci *et al.* (2) successfully solubilized the FCBP from spinach leaf microsomes using acetone, but the solubilized FCBP was characterized by poor stability (3); gel filtration indicated an apparent mol wt of 80 kD. Despite these successes in solubilization of the FCBP in active form, no one succeeded in purification of the FCBP.

We report here on the purification and identification of the FCBP using FC-affinity chromatography, after solubilization

[1] This work was supported by National Science Foundation Grant DMB 8502021.

[2] Current address: Department of Plant Physiology, Biological Center, University of Groningen, POB 14, 9750 AA Haren, The Netherlands.

[3] Abbreviations: FC, fusicoccin; AADA, adipic acid dihydrazide agarose; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; EG, ethylene glycol; FC-AADA, fusicoccin covalently linked to adipic acid dihydrazide agarose; FCBP, fusicoccin binding protein; ^{3}H-FC, dihydrofusicoccin; OG, octyl-β-D-glucopyranoside; PEI, polyethylinemine; PM, plasma membrane; PMSF, phenyl-methylsulfonyl fluoride.

of the FCBP in octyl-glucoside. A preliminary report of this work has been presented elsewhere.[4]

MATERIAL AND METHODS

Plant Material

Oat (*Avena sativa* L. cv Victory) seeds were rinsed in running tap water for 30 min, sown on cheesecloth covering a stainless steel screen 1 cm over 1 mM $CaSO_4$ solution (½ L). The solution was renewed daily. Roots were harvested after 5 d of growth in continuous dim red light at 25°C.

Extraction of Membrane Vesicles

The roots (200 g), cooled on ice, were ground in a Waring blender, with 200 mL of solution I: 10 mM Tris-HCl (pH 7.5), 250 mM sucrose, and 1 mM EDTA. The homogenate was filtered through four layers of cheesecloth and the material remaining on the cheesecloth was reextracted with 200 mL solution I. The pooled filtrates were centrifuged for 20 min at 10,000*g* in a fixed angle rotor. The resulting supernatant was centrifuged for 30 min at 100,000*g* to obtain the microsomal pellet.

Purification of Plasma Membranes

A very pure PM fraction was obtained following the aqueous phase partitioning procedure as described by Sandstrom *et al.* (22). Briefly, the microsomal pellet from 200 g of roots was resuspended in solution II (5 mM K^+-phosphate [pH 7.8], 250 mM sucrose, and 4 mM KCl) and the solution brought to a final weight of 5 g. Three tubes comprising the aqueous phase partition system were made as follows: tube A contained 15 g of a phase system that would contain, after addition of the 5 g microsomal homogenate, 6.5% (w/w) dextran (500,000 mol wt), 6.5% (w/w) polyethylene glycol (4,000 mol wt), 250 mM sucrose, 5 mM K^+-phosphate (pH 7.8), and 4 mM KCl. Tubes B and C contained 10 g of the lower phase obtained by partitioning a complete phase system identical in composition and final concentration to tube A. The 5 g microsomal homogenate was layered onto the phase system in tube A, mixed and centrifuged in a swinging bucket rotor at 1,000*g* for 5 min. The upper phase was transferred to tube B and the mixing and centrifugation were repeated. The procedure was repeated for tube C. To remove the polyethylene glycol, the upper phase from tube C was diluted into 10 mL solution III (10 mM Tris·HCl [pH 7.5] and 250 mM sucrose) and centrifuged for 30 min at 100,000*g*. The resulting pellet was resuspended in solution III to give a final concentration of 5 to 10 mg/mL and stored at −70°C.

Solubilization of the FCBP with Octyl-Glucoside

PM vesicles, purified as described, were solubilized in solution B containing 150 mM NaCl (final protein concentration 1 mg/mL and OG: protein ratio 10:1). This solution was sonicated two times for 30 s (Sonifier Cell Disrupter, model W140 D, Neat Systems-Ultrasonics Inc., Plainview, NY) and left on ice for 30 min. Sonication did not improve the release of the FCBP from the membranes but it seemed to help maintain the solubilized receptor in an active form. The same phenomenon was observed by Stout and Cleland (26). Proteins not solubilized in 1% OG were pelleted by means of a 30 min centrifugation at 100,000*g*. About 90% of the FCBP was recovered in soluble and active form, along with 80% of the initial protein.

Acetone Solubilization of the FCBP

Twenty volumes of acetone (−20°C) were added to a 20 μL PM vesicle sample (1 mg/mL) while stirring. The precipitate was pelleted in a microfuge (15,000*g*), washed once with an equal amount of acetone, and dried under N_2-gas. The pellet was resuspended in 0.5 mL solution A plus 150 mM KCl and 0.1% Triton X-100 and sonicated for 1 min. The sample was centrifuged at 100,000*g* for 30 min and the supernatant was used to assay for ^{3}H-FC binding activity.

Trypsin Digestion

PM vesicles (0.15 mg protein/mL) were incubated at 25°C in Tris-HCl (100 mM, pH 8.0) and 125 mM sucrose, with 0.1 mg trypsin/mL (protein:trypsin = 1.6:1; w/w). The digestion was stopped with trypsin inhibitor; trypsin:trypsin inhibitor = 1:2 (w/w). In the control experiment the trypsin inhibitor was added at $t = 0$ before the trypsin. The concentration of Triton X-100, when used, was 0.0125% (w/v).

^{3}H-FC Binding

Binding Conditions

PM vesicles or solubilized PM proteins (5–10 μg protein/tube) were incubated in solution A (PM vesicles) or solution A + 0.2% OG (solubilized proteins) in the presence of 10^{-9} M ^{3}H-FC; total volume 1 mL. The incubation temperature was 30°C and incubation times as indicated. Addition of cold FC (10^{-6} M) showed that nonspecific binding was not significant at 10^{-9} M ^{3}H-FC. Dihydrofusicoccin (^{3}H-FC) is known to have similar binding characteristics and physiological effects as FC (5). Duplicate samples of 0.45 mL of each tube were assayed for ^{3}H-FC binding activity.

Binding Assay

Glass fiber filters (GF/B, Whatman 2.5 cm) were soaked for at least 2 h in 1% PEI. The filters were put on a filtration manifold (Millipore, 12 units) and washed with 5 mL water followed by 5 mL assay buffer (AB = 25 mM glycine/KOH, pH 9.5). Next, 5 mL AB was put on each filter and the protein/^{3}H-FC sample was added to the AB. The solution was slowly filtered through under vacuum and thereafter each filter was washed once with 10 mL AB. The filters were transferred to 5 mL Aquasol and radioactivity was counted after overnight extraction with a Beckman Scintillation Counter. Nonspecific binding of ^{3}H-FC to the filters was low (less than 0.5%) and was routinely subtracted.

This new assay method is very rapid and allows the assay of up to 50 samples per h. We tested the efficiency of the filters by comparing the results to the classical assay method

[4]International Symposium on "Physiology and Biochemistry of Auxins in Plants," Liblice, Czechoslovakia, 1987.

of gel filtration (26). ^{3}H-FC-labeled, solubilized, proteins were separated from free ^{3}H-FC on a Biogel P-6 column (1.5 × 17 cm). A small amount of each fraction was counted for the quantitation of ^{3}H-FC and the remainder of each fraction assayed on PEI-coated GF/B filters. The filters bound all ^{3}H-FC-labeled proteins present in the first peak of the P-6 column and none of the free ^{3}H-FC present in the second peak. A further disadvantage of the time consuming gel filtration method is that some of the ^{3}H-FC-labeled proteins remained bound in the column.

Preparation of FC-Linked Adipic Acid Dihydrazide Agarose

FC is a glycoside and thus may be coupled to a solid support using a method analogous to that used for nucleotides (17). Aducci *et al.* (1) showed that FC coupled to BSA through aldehydes generated by the oxidative opening of the glucose ring retained binding activity as measured by a competition assay with ^{3}H-FC.

The O-acetyl group on the glucose moiety was cleaved to render FC susceptible to periodate oxidation. FC (6.85 mg = 10^{-5} mol), carrying a trace of ^{3}H-FC was solubilized in 138 μL (95%) ethanol. NaOH (138 μL, 0.1 N in 95% ethanol) was added to make the solution alkaline and this mixture was left for 1 h in the dark at room temperature. The solution was neutralized with 13 μL HCl (1 N), the ethanol was evaporated with N_2-gas and 1 mL of chloroform was added. The chloroform was washed once with 1 mL of H_2O, followed by 2 mL of H_2O. The water-phase was back extracted with 2 mL chloroform and the chloroform fractions were pooled. The chloroform was evaporated with N_2-gas and pumped dry under vacuum for 2 h.

The di-aldehyde was prepared by periodate oxidation. The dried, saponified FC was solubilized in 414 μL (95%) ethanol. Na-meta-periodate (161 μL, 100 mg/mL in H_2O) was added and the solution buffered at pH 4.9 with 46 μL Na-acetate (0.1 M). The reaction was allowed to proceed in the dark at room temperature with stirring for 2 d. The formation of FC-aldehyde was followed using the Purpald-assay (see below). After 2 d the remaining periodate was neutralized with 6.9 μL ethylene glycol (100%).

Di-deacetyl FC was extracted with 3 aliquots (0.5 mL) of acetone (−20°C). The acetone was evaporated with N_2-gas and 0.5 mL H_2O was added to solubilize remaining salts. FC was extracted from the aqueous-phase with three aliquots (1 mL) of methylene chloride. The methylene chloride was dried over Na_2SO_4 and the Na_2SO_4 reextracted once with 1 mL methylene chloride. The methylene chloride was pooled and evaporated to dryness with N_2-gas.

The di-deacetyl FC was coupled to AADA. Three g of wet AADA were washed three times with 5 mL Na-acetate buffer (0.1 M, pH 4.5). To the settled gel was added the di-deacetyl FC, solubilized in 0.5 mL (95%) ethanol plus 0.5 mL Na-acetate buffer (0.1 M, pH 4.5). The beads were kept in suspension by use of a tube inverter and the reaction allowed to proceed at room temperature. The extent of coupling of FC to the agarose was followed by sampling the supernatant at intervals and counting the ^{3}H-FC. Coupling was complete in 2 h. Approximately 9% of the initial amount of FC was coupled to the AADA. The FC concentration in the settled gel in this experiment was 0.27 mM. The FC-AADA was washed extensively in 2 M NaCl and stored in 0.5 M NaCl + 0.05% Na-azide. The binding was stable over time and not affected by ethanol, butanol, urea (8 M), SDS (2%) or high salt (2 M NaCl).

Regeneration of FC-AADA and Hexyl Agarose

With the gel still in the column, the column was washed with one column volume of distilled water, followed by one column volume of ethanol, two column volumes of *n*-butanol, again one column volume of ethanol, and finally one column volume of distilled water. The agaroses were stored in 0.5 M NaCl + 0.05% Na-azide.

Purpald-Assay

Purpald is a reagent for the determination of aldehydes (13). To an assay-mixture consisting of 0.25 mL Purpald stock solution (= 1 mg Purpald per mL of 1 N NaOH) plus the sample of aldehyde to be assayed, H_2O was added to yield a final volume of 0.5 mL. Glutaraldehyde was used as a standard (from 2 mM stock solution) to generate a standard curve from 0 to 0.2 μmol. The tubes were vortexed frequently during a 30 min incubation period at room temperature. Absorbance (553 nm) was measured with a Gilford spectrophotometer.

Protein-Assay

Proteins were measured according to Bradford (9).

Chemicals

Radioactive dihydrofusicoccin (^{3}H-FC, specific activity 35.2 Ci/mmol) was prepared by Amersham, U.K.; Trypsin (porcine pancreas, EC 3.4.21.4.), trypsin inhibitor, leupeptin, pepstatin, adipic acid dihydrazide agarose, and hexyl agarose were obtained from Sigma; DEAE Biogel A (100–200 mesh) came from BioRad and OG was purchased from Calbiochem.

Solutions

Solution A = 25 mM Mes/Tris (pH 7.5), 1 mM $MgCl_2$, 0.5 mM EDTA, 25 mM KCl, 10% glycerol (w/v), 10^{-4}% leupeptin, 10^{-4}% pepstatin, 0.2 mM PMSF. *Solution B* = solution A + 1% OG and 4 mM DTT. *Solution C* = solution A + 0.2% OG and 4 mM DTT. *Solution D* = solution C + 1 M $(NH_4)_2SO_4$ and a total glycerol concentration of 25% (w/v). *Solution E* = solution A + 1 M NaCl, 40% EG, and a total glycerol concentration of 25% (w/v). *Solution F* = solution C + 0.5 M NaCl and 0.6 M $(NH_4)_2SO_4$.

RESULTS

Solubilization and Stabilization of the FCBP

When associated with the plasma membrane, the FCBP is rather stable. However, Aducci *et al.* (3) showed that the stability of the FCBP was poor after solubilization from microsomal membranes. In order to diminish the presence of deactivating enzymes (proteases, phosphatases, α-mannosidases) we used highly purified plasma membranes as starting

material for the solubilization of the FCBP. Right-side out PM vesicles were purified from microsomal membranes with the dextran/PEG two-phase system; due to this purification step the specific activity of the FCBP (using 1 nM ^{3}H-FC in the assay) increased from about 3 pmol ^{3}H-FC/mg protein in the microsomal extract to around 25 pmol ^{3}H-FC/mg protein in the purified PM vesicles.

We tested seven different detergents and an acetone-wash for their ability to solubilize the FCBP in active form (Table I). The acetone solubilization as described by Aducci *et al.* (2) worked well for oat root FCBP but only when some Triton X-100 was added to release the FCBP from the acetone powder. Of all the detergents tested, only OG retained full activity of the receptor and it solubilized 90% in active form. Despite the fact that the acetone solubilization worked well, we choose to work with OG because of its good characteristics for further purification of the FCBP; it is nonionic and has a high critical micelle concentration (25 mM). The delipidation of the FCBP in the acetone wash was also seen as a potential problem in subsequent chromatography steps because the FCBP, being an integral membrane protein, is probably hydrophobic in nature.

It is essential to maintain the FCBP stable in soluble form during the time-consuming purification steps. In view of the problems Aducci *et al.* (3) encountered with endogenous phosphatases, all the solutions used had 25 mM KF. The importance in inhibiting proteases after solubilization of the FCBP is shown in Figure 1. While trypsin degrades the FCBP activity to a limited extent in intact PM vesicles, it causes a greater loss of activity after partial delipidation with Triton X-100 and rapid and total inactivation after solubilization of the FCBP in OG. It is not surprising, then, that the FCBP was still unstable if stored at room temperature or at 4°C without protease inhibitors (Table II). PMSF had the strongest effect in preventing loss of activity, although all three protease inhibitors combined (PMSF, leupeptin, and pepstatin) gave the best results.

Binding-Characteristics

The concentration of OG used for the solubilization (1%) was too high for optimal binding of FC to the FCBP (Fig. 2);

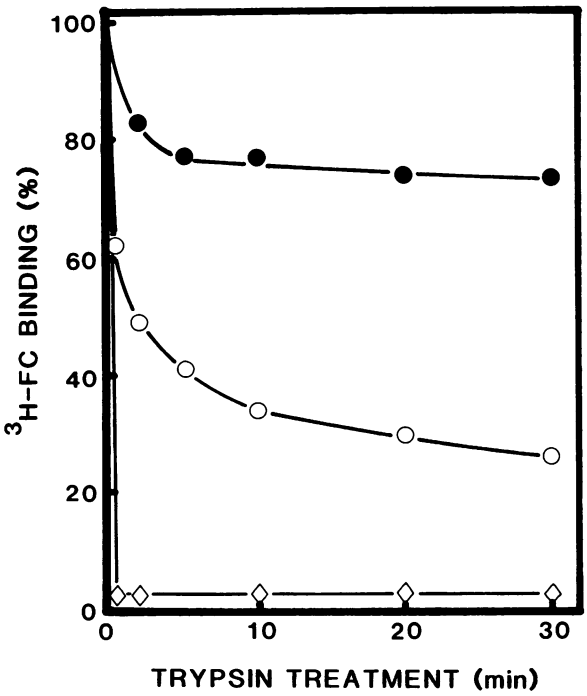

Figure 1. Sensitivity of the FCBP to trypsin. (●), FCBP in intact, right-side out PM vesicles (100% = 18 pmol ^{3}H-FC/mg protein); (○), FCBP in vesicles, opened with 0.0125% Triton X-100 (100% = 7 pmol ^{3}H-FC/mg protein); (◇), FCBP solubilized in 1% OG (100% = 13 pmol ^{3}H-FC/mg protein). Duration of the binding-assay after the trypsin treatment was 1 h.

Table I. *Solubilization of the FCBP with Different Detergents and Acetone*

The FCBP was solubilized from PM vesicles with 1% detergent or with acetone (see "Materials and Methods"). The detergent:protein ratio was 125:1. ^{3}H-FC binding activity was measured in the soluble fraction and in the pellet. Detergent levels in the assay are those that give maximal FC-binding. Control is ^{3}H-FC binding to native vesicles; 100% = 12.2 pmol ^{3}H-FC/mg protein.

Detergent	Detergent in Assay	Initial Activity		
		Solubilized	In pellet	Inactivated
	%		%	
CHAPS	0.2	13	21	66
Na-cholate	0.4	15	28	57
Digitonin	0.1	18	35	47
Lubrol PX	0.2	26	17	57
Octyl-glucoside	0.2	91	9	0
Triton X-100	0.1	27	21	52
Zwittergent 3-14	0.1	3	3	94
Acetone (+ Triton)	0.1	82	16	2

Table II. *Effect of Storage Temperature and Protease Inhibitors Upon Degradation of the FCBP*

After solubilization of the FCBP in 1% OG the ^{3}H-FC-binding activity was immediately measured in the presence of protease inhibitors (assay time 2 h; 30°C). The rest of the sample was stored as indicated and assayed 75 and 165 h later. Protease inhibitors (final concentration): leupeptin (1 μg/mL), pepstatin (1 μg/mL), PMSF (0.2 mM). 100% = 6.3 pmol ^{3}H-FC bound per mg protein.

Treatment	Storage Temperature					
	Room temperature		4°C		−70°C	
	75 h	165 h	75 h	165 h	75 h	165 h
	% of control					
− Protease inh.	4	2	14	3	101	75
+ Protease inh.	45	15	88	45	113	84

optimal binding occurred at 0.2% OG. For this reason OG was reduced to 0.2% in the binding assays and during coupling of the FCBP to the FC-AADA beads. There is a considerable difference in the rapidity of binding of FC to the FCBP in its native environment and in solubilized state (Fig. 3). In membrane bound form, the binding of ^{3}H-FC is relatively rapid and complete in 1 h. The binding was much slower after solubilization of the FCBP and saturation was only reached after 4 h. As reported previously (21), the binding is very temperature dependent: at 0°C the amount of ^{3}H-FC bound to the FCBP was only 20% compared to the experiment which was carried out at 30°C. The time and temperature

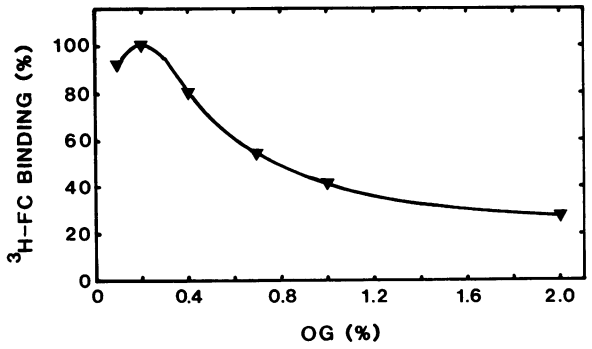

Figure 2. Effect of OG concentration in the assay-buffer upon binding of ^{3}H-FC to the FCBP. The FCBP was solubilized in 1% OG. The optimum OG concentration for binding was 0.2% and the binding activity was 14 pmol ^{3}H-FC/mg protein (= 100%).

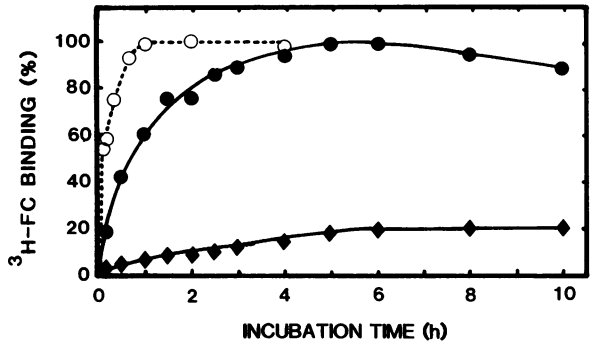

Figure 3. Time course of ^{3}H-FC binding to the FCBP in native vesicles at 30°C (O, dotted line) and to the solubilized FCBP (solid lines) at 30°C (●) and at 0°C (◆). Native vesicles: 100% = 8.9 pmol ^{3}H-FC/mg protein at 30°C. Solubilized FCBP: 100% = 13.0 pmol ^{3}H-FC/mg protein at 30°C.

dependency of the binding were important for the affinity chromatography experiment described below.

We observed a 50% increase in binding activity when the solubilized FCBP was incubated with $(NH_4)_2SO_4$ (Fig. 4). The activation was maximal at 1 M $(NH_4)_2SO_4$; the FCBP precipitates between 1 and 2.2 M $(NH_4)_2SO_4$. The FCBP can be recovered in active form from the $(NH_4)_2SO_4$ precipitate. The mechanism of the activation is not clear (removal of a natural ligand or an inhibitory protein?) but the activation also occurred when the FCBP was mixed with the AADA (see below). It is of interest that Aducci *et al.* (4) found that a preincubation of corn coleoptiles with auxin enhanced *in vitro* ^{3}H-FC binding with 50%.

Ideally, in affinity chromatography the protein of interest is eluted from the column with the free ligand. This was not feasible with the FC-AADA due to the slow reversibility of the binding and the high concentration of FC-linked to the AADA (0.27 mM). Therefore, we tested a number of compounds to see whether they could break the ^{3}H-FC/FCBP binding (Fig. 5). Urea, SDS, methanol, and acetone were all effective in breaking the binding, whereas EG, NaCl, and $MgCl_2$ had virtually no effect. We choose to elute the FC-affinity column with 8 M urea, as urea appeared to be the only compound that selectively eluted the FCBP (see next section).

$HgCl_2$, a reagent known to modify sulfhydryl groups, was

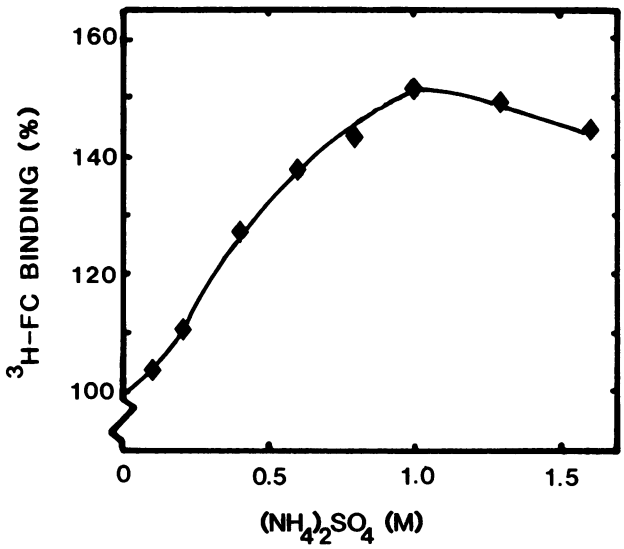

Figure 4. Effect of $(NH_4)_2SO_4$ upon ™inding of ^{3}H-FC to the FCBP during the binding assay; assay time was 2 h. Control (100%) is binding in the absence of $(NH_4)_2SO_4$ (21 pmol ^{3}H-FC/mg protein).

reported (6) to inhibit FC-binding. We found that 30 min preincubation of the FCBP in 0.1 and 0.5 mM $HgCl_2$ reduced subsequent binding of ^{3}H-FC by 25 and 80%, respectively. The presence of DTT or mercaptoethanol (10 mM) prevented the inactivation of the FCBP by $HgCl_2$. However, $HgCl_2$ had no effect once the binding between ^{3}H-FC and FCBP had been formed (Fig. 5); thus, FC-binding protects the $HgCl_2$ sensitive SH-group(s). Whether this SH-group plays a role in the regulation of binding is unknown, but the presence of sulfhydryl groups in or near the binding site of receptor proteins is not unprecedented, as for example the acetylcholine receptor has a SH-group near the ACh-binding sites (16).

Purification on FC-AADA

FC-linked AADA was used to purify the FCBP in one step from the mixture of solubilized PM-proteins. Typically, 2 to 5 mg of membrane protein was solubilized as described in "Materials and Methods." The supernatant was diluted with solution C to bring the OG concentration to 0.2% and concentrated to the start volume in a stirred Amicon cell with a membrane cutoff of 50,000 mol wt. The filtrate from the cell contained no FCBP activity and very little total protein. Loss of activity on the membrane was small (less than 10%) after the membrane had been used once. The protein solution was batch loaded on the FC-AADA (3 mL), equilibrated in solution C. The extent of binding of the FCBP to FC linked to the beads was determined by assaying samples taken from the free solution for FCBP activity. A control tube with underivatized AADA (no FC, reactive groups blocked with formaldehyde) was treated exactly like the tube with FC-AADA. The kinetics of binding were more complex than expected (Fig. 6). Apparently, there were three processes going on simultaneously, the first two of which occur in both tubes and the last one only in the tube with FC-AADA: (a), a rapid activation of the FCBP due to the presence of the agarose, (b) a slow decrease in FCBP-activity due to its presence at room temperature, and (c) a decrease in FCBP-activity due to

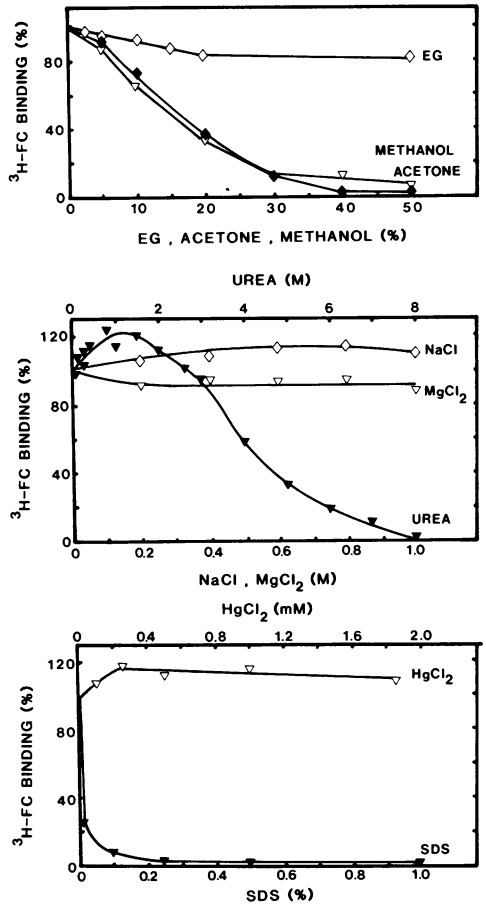

Figure 5. Effectiveness of different compounds in breaking the ^{3}H-FC/FCBP binding. Binding was allowed to proceed for 4 h, then the different compounds were added and left for 1 more h whereafter binding of ^{3}H-FC to the FCBP was determined. Control is the sample that received H_2O instead of one of the compounds (100% = 21 pmol ^{3}H-FC/mg protein).

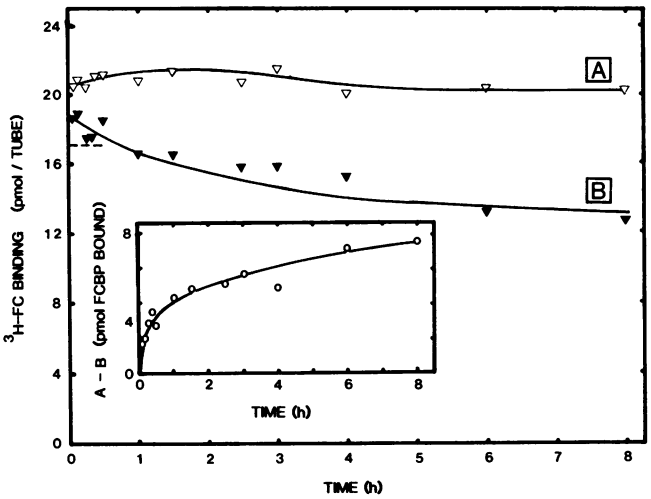

Figure 6. Change of FCBP-activity of the free solution during batch-loading of solubilized PM proteins on AADA, blocked with formaldehyde (A) and on FC-AADA (B) at room temperature. The inset shows the difference between A and B and represents the amount of FCBP bound to the FC-AADA. The assay time for ^{3}H-FC binding in the free solution was 2 h. Control = ^{3}H-FC binding activity per tube at the start of the experiment in the absence of agarose (=17.2 pmol ^{3}H-FC per tube).

binding to the FC-linked beads. The latter phenomenon became obvious when we subtracted the FCBP-activity of the free solution of tube B (= FC-AADA) from that in tube A (= AADA). The inset in Figure 6 shows that the binding kinetics were then very similar to the binding kinetics of free ^{3}H-FC and the solubilized FCBP (Fig. 3). In this particular experiment around 7 pmol of FCBP was bound to the FC-AADA, which represents 35% of the total amount present at the start. The activation of the FCBP by the AADA here was 25% but sometimes was as much as 40%.

After batch-loading overnight, the agarose beads were transferred to a column (1 cm diameter) and washed with 10 column volumes of solution C followed by 1 column volume of solution C + 100 mM NaCl. There was a lot of nonspecific binding to the FC-AADA and the AADA; even when the columns were washed with 0.5 M NaCl and 30% EG there were still many proteins bound to the FC-AADA which could be eluted with 2% SDS (data not shown). The FCBP was eluted from the FC-AADA with 8 M urea in 50 mM Tris/Mes buffer (pH 7.5). The fractions at the urea front from the FC-AADA column showed significant FCBP binding activity (assayed in the presence of 0.4 M urea) which shows that the FCBP is not irreversibly denatured by urea. Urea could not be removed through dialysis or ultrafiltration as then also all protein was lost. The urea was removed by binding eluted proteins to a DEAE Biogel-A column (1 × 5 cm). Bound proteins were eluted in 100 mM (NH_4)-acetate (pH 3.5) and the eluate lyophilized. For still unknown reasons, no FCBP-activity could be detected in the (NH_4)-acetate eluate. However, when the lyophilized sample was run on SDS-PAGE, two major bands were seen with a M_r of 29,700 and 31,000 (Fig. 7). In some experiments a band of M_r 67,000 was visible as well. Urea did not elute proteins in the control experiment where only the AADA matrix was used.

Anion-Exchange and Hydrophobic Interaction Chromatography

The final yield of the FCBP in the one-step purification was usually not very high and we were unable to recover active FCBP from the urea fractions. To overcome these problems we devised a three step purification scheme where the FCBP was prepurified on anion-exchange and hydrophobic interaction chromatography. The partially purified FCBP was then subjected to affinity chromatography on FC-AADA. We will first describe the two prepurification steps separately.

The ^{3}H-FC binding activity eluted from DEAE Bio-Gel A as an asymmetric peak (Fig. 8). The main peak eluted at 150 mM NaCl while a small shoulder on the peak was found at a slightly higher ionic strength. The total recovery of the binding activity was 40% and two-thirds of the amount of protein loaded onto the column passed through the column unbound. The increase in specific activity was variable; from 2- to 15-fold. On SDS-PAGE, two bands were pronounced in the fractions from the shoulder with an apparent mol wt of 29,400 and 30,700, although these bands were less pronounced in the fractions with the highest binding activity.

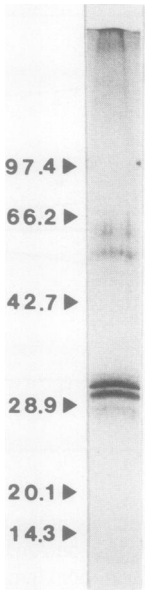

Figure 7. SDS-PAGE (7.5–15%) of proteins eluted from the FC-affinity agarose (FC-AADA) with 8 M urea and 50 mM Tris/Mes (pH 7.5); stained with silver. The mol wt of the two bands was estimated as 29,700 and 31,000, respectively. Mol wt markers and their mol wt according to the amino acid sequence: phosphorylase B (97,400), bovine serum albumin (66,200), ovalbumin (42,700), carbonic anhydrase (28,900), soybean trypsin inhibitor (20,100), lysozyme (14,300).

The FCBP, an integral membrane protein, is likely to have hydrophobic areas. Thus, we tested a series of hydrophobic agaroses for their capacity to bind the FCBP. When the protein was loaded in 1 M $(NH_4)_2SO_4$, binding of the FCBP increased with the length of the hydrophobic side chain from C2 to C6; the latter agarose bound virtually all of the FCBP loaded on the column. Lowering the $(NH_4)_2SO_4$ concentration was not effective in eluting the FCBP. However, the FCBP could be eluted in a decreasing gradient of $(NH_4)_2SO_4$ and an increasing gradient of EG, NaCl, and OG (Fig. 9). We used a convex gradient because this resulted in a better separation of the protein peak from the ^{3}H-FC binding peak. The recovery of ^{3}H-FC binding activity was 30 to 40% of the amount loaded onto the column.

Three-Step Purification

For the 3-step purification (anion exchange, hydrophobic interaction, and affinity chromatography) we started with 40 mg PM-proteins. After solubilization and concentration of the sample to 10 mL in a stirred Amicon cell (mol wt cut off 50,000), the solubilized proteins were loaded on a DEAE Bio-Gel A column (1.5 × 11 cm) and the FCBP was eluted in a linear NaCl gradient. The fractions containing binding activity were pooled, brought to 1 M $(NH_4)_2SO_4$ with saturated ammonium sulfate, and loaded on a hexyl agarose column (1.5 × 8 cm). Binding activity was eluted with a convex gradient of EG, NaCl, OG, and $(NH_4)_2SO_4$ (see Fig. 9). The fractions containing binding activity were pooled and diluted five times with buffer F. This dilution was necessary because EG concentrations higher than 3% have a negative effect upon the binding activity (at 6 and 12% EG, the binding activity is reduced with one-third and one-half, respectively).

The diluted sample from the hydrophobic column was batch-loaded onto 2 mL FC-AADA and rotated overnight at room temperature. Samples from the free solution were measured for binding activity at the start and end of the experiment and compared with a control tube which contained only protein and solution F. The control tube lost 13% of its free binding activity overnight, whereas the free binding activity in the tube with FC-AADA dropped by 60%. In this experiment the loss in binding activity in the free solution, due to binding of the FCBP to the FC-linked beads, amounted to 10 pmol ^{3}H-FC.

After overnight binding, the FC-AADA was transferred to a 1 cm column and washed with 5 column volumes of solution F, followed by 10 column volumes of solution C. Since SDS breaks the FC-FCBP binding at very low concentrations (Fig. 5) the FCBP (and possibly other, nonspecifically bound proteins) was eluted with a linear SDS gradient, 0 to 0.1% (Fig. 10). The SDS fractions (20 times diluted in the binding-assay) contained ^{3}H-FC binding activity with a peak at 0.05% SDS (Fig. 10). The same fractions were run on SDS-PAGE and fractions 7 and 8, having the highest binding-activity, contained two prominent bands with a M_r of 29,700 and 30,800. The amount of protein in each of the two bands was estimated by densitometry, using BSA as a standard, after Coomassie blue staining. The amount of protein in each of the two bands in fraction B was 176 ng for the 29,700 and 370 ng for the 30,800; *i.e.* the stoichiometry of these two bands was close to 1:2. The profile of the densitometer scan of lane 8 is shown alongside Figure 10. It can be seen that there is a band at approximately 67,000 mol wt and some bands of higher mol

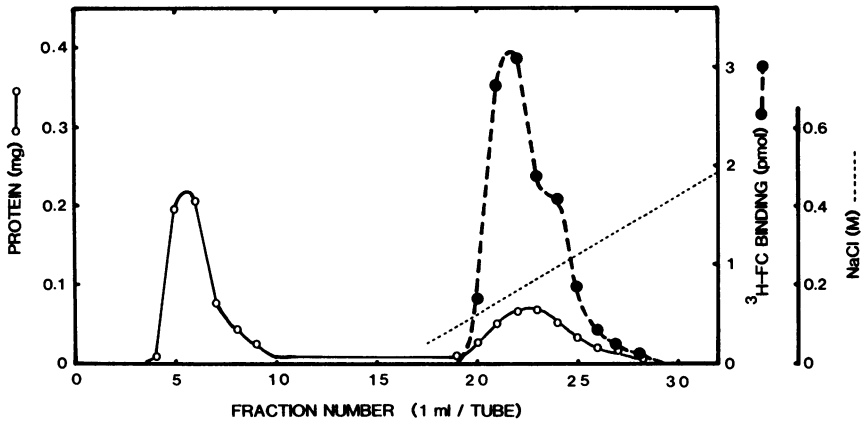

Figure 8. DEAE Biogel-A chromatography of PM proteins solubilized from 2.5 mg purified PM vesicles with 1% OG. After solubilization the sample was diluted three times with solution B and the volume was reduced to 3 mL in an Amicon cell (mol wt cut off 50,000). After loading of the protein in solution B plus 50 mM NaCl on the column (1 × 3 cm) and washing the column with 5 column volumes of the same solution, the column was eluted with a linear gradient of 50 to 600 mM NaCl in solution B.

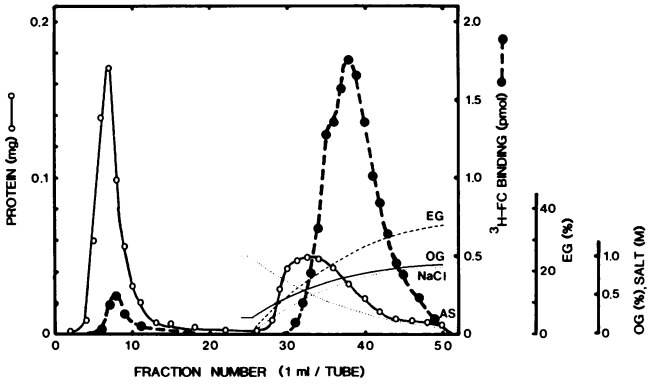

Figure 9. Hexyl-agarose chromatography of PM proteins solubilized from 2 mg purified PM vesicles in 1% OG. The solubilized protein sample was brought to 1 M $(NH_4)_2SO_4$ with saturated ammonium sulfate and loaded on the column (1 × 4 cm) that had been equilibrated with solution D. After sample loading the column was washed with 7 column volumes of solution D and then eluted with a convex gradient which was created by pumping buffer E into 12 mL of solution D at a pump rate of 8 mL/h. The pump rate out of the mixing chamber into the column was also 8 mL/h. AS = ammonium sulfate.

wt. We think that these latter bands are not related to the FCBP but that the 67,000 band may be related as it was sometimes seen in the one-step purification as well.

DISCUSSION

The results presented here show that the plasma membrane from oat roots is a good source of stable FCBP. Treatment of the PM with 1% OG allowed the recovery of almost all of the FCBP in soluble and active form. Earlier attempts to purify the FCBP probably failed due to the lack of a suitable detergent, the instability of the FCBP in soluble form, and the lack of a rapid assay method (3, 21). This is the first report that shows that affinity chromatography can be successfully applied for the purification of the FCBP. A problem with the FC-linked adipic acid dihydrazide agarose column was the nonspecific binding of other PM proteins which could not be washed away easily. The high density of FC in the agarose (0.27 mM) might give the column undesired hydrophobic properties, thus causing hydrophobic proteins to stick.

Fortunately, urea at concentrations above 3 M was effective in breaking the ^{3}H-FC/FCBP binding (Fig. 5) and when used on the FC-AADA it appeared to be selective; *i.e.* virtually no other proteins co-eluted with the FCBP even though they were still present in the column. The urea eluate contained considerable binding activity (up to 5 pmol was measured with a 20-fold dilution during the assay). Since SDS-PAGE showed that the eluate contained only two protein bands (Fig. 7) it is likely that the ^{3}H-FC-binding activity is correlated with one (or both) of these bands, even though binding activity was lost during removal of the urea on an anion-exchange column. More work still needs to be done on the recovery of the FCBP in active form from the urea eluate.

The fact that the FCBP eluted in a peak and shoulder from the anion-exchange column (Fig. 8) may indicate that the FCBP occurs as a heterogeneous population. It will be interesting to see whether the peak and shoulder fractions result in different FCBPs on the FC-affinity column. From the

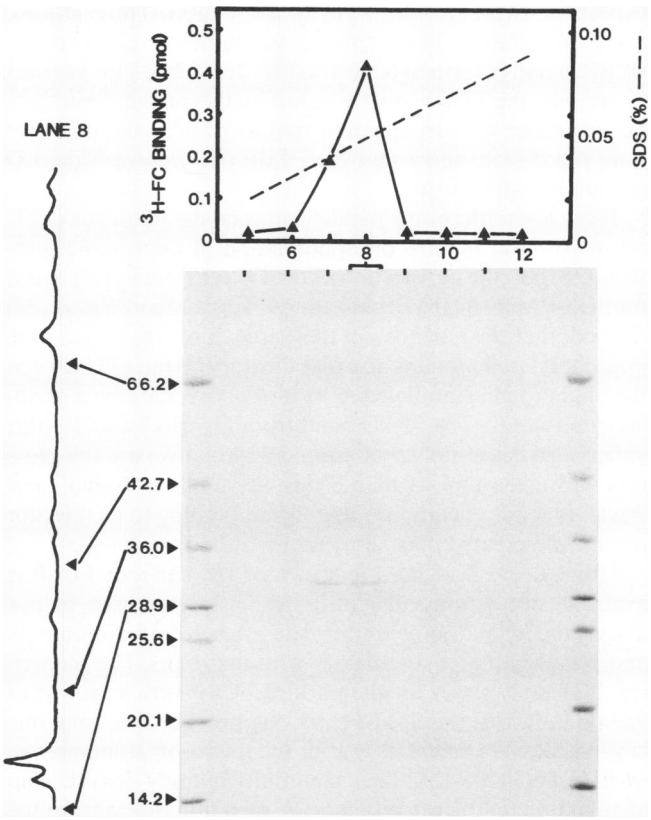

Figure 10. SDS-PAGE (7.5–15%) of the purified FCBP, stained with Coomassie brilliant blue. The protein was purified in three steps: (a) anion exchange chromatography, (b) hydrophobic interaction chromatography, and (c) FC-affinity chromatography. In the last step the FCBP was eluted from the FC-AADA with a shallow SDS-gradient; fraction 7 and 8 had considerable ^{3}H-FC binding activity (top part of the figure). The same two fractions showed protein bands when run on the SDS-PAGE, the most prominent of which had a mol wt of 29,700, 30,800, and 66,800. A densitometer scan made of lane 8 shows the relative amounts of protein in each band; 80% of each fraction was loaded in the respective lanes. Mol wt markers and their mol wt according to the amino acid sequence: bovine serum albumin (66,200), ovalbumin (42,700), glyceraldehyde 3-phosphate dehydrogenase (36,000), carbonic anhydrase (28,900), trypsinogen (25,600), soybean trypsin inhibitor (20,100), α-lactalbumin (beef milk) (14,200).

elution pattern on the hexyl-agarose it can be concluded that the FCBP is one of the more hydrophobic proteins in the plasma membrane (Fig. 9). Prepurification of the FCBP on anion-exchange and hydrophobic interaction columns greatly reduced the problem of nonspecific binding of PM proteins to the FC-affinity column. Despite the considerable loss in activity on both columns the advantage of the three-step purification was that it enabled a direct correlation of FCBP-activity in two fractions eluted with SDS with the presence of the 29,700 and 31,000 mol wt proteins as the major bands in the same fractions (Fig. 10).

Important additional information came from the densitometer scan of the Coomassie blue stained gel. The estimated amount of protein in the 31,000 band was twice that in the 29,700 band. It seems unlikely that the two proteins are proteolysis products from the 67,000 mol wt protein that coeluted in several experiments as, in that case, one would

expect the two bands to occur in a 1:1 ratio. Three groups, using gel filtration, have reported an estimated mol wt of the FCBP in native form of 60 to 100 kD (3, 21, 26). This suggests to us that the FCBP might occur in the native form as a hetero-trimer with an apparent mol wt of 92,000. So far, we were not able to show that the FCBP occurs as a 92,000 D protein in native form.

There is an interesting parallel between the influence of FC on *in vivo* and *in vitro* phosphorylation of microsomal proteins (28) and the purification of the FC-receptor as two bands on SDS-PAGE in the 30 kD range. Tognoli and Basso (28) showed that two bands in the same mol wt range were phosphorylated proteins and that the upper band (33 kD) was the only protein stimulated in its phosphorylation by a preincubation in FC. The 31 kD band from Figures 7 and 10 (this paper) also has a mol wt of around 33 kD if we use the same mol wt for the mol wt markers as was used by Tognoli and Basso. It is not uncommon that ligand binding to its receptor results in increased phosphorylation of the receptor (25).

Although no Scatchard analysis for the oat root FCBP is available, we assume that at 1 nM ^{3}H-FC (as used in our assays) the high affinity binding site is labeled predominantly and is half saturated, in analogy with maize (6). The theoretical binding capacity for the purified FCBP with a mol wt of 92,000 is 10,870 pmol ^{3}H-FC per mg protein, assuming one binding site. We calculate that in the microsomal membrane fraction the FCBP with the high affinity binding site made up about 0.04% of the total protein. Purification of PM from the microsomal fraction with the dextran/PEG two-phase system greatly increased the specific activity of the FCBP. In the purified PM vesicles the binding activity was 50 pmol ^{3}H-FC per mg protein; *i.e.* about 0.4% of all PM proteins is made up by the FCBP.

Trypsination of functional membrane proteins in vesicles with a known orientation has been used as a technique to determine the location of functional enzyme domains. The small (20%) loss in FC-binding activity after trypsin treatment of the intact right-side out PM vesicles (Fig. 1) can be explained in two ways: (a) the binding site is located at the inside of the membrane, or (b) the trypsin sensitive site(s) is protected by the membrane. We think that the latter is true as experiments with macromolecular conjugates of FC to BSA clearly indicate that the binding site of the FCBP is located at the apoplastic side of the membrane (1, 14). Feyerabend and Weiler (14) could remove virtually all FC-binding sites from right-side out PM vesicles from *Vicia faba* by a trypsin digest. This discrepancy with our experiments may result from the use of different plants: oat (a monocot) *versus* bean (a dicot). Also, the source of trypsin used in these kinds of experiments may be of importance. We used porcine trypsin instead of the commonly used bovine trypsin as the latter produced inactivation of the (Na, K)-ATPase from the outside in right-side out sealed vesicles from dog kidney outer medulla (15).

Nothing is known thus far about the mode of action of the FC-receptor. We will discuss a few possibilities that might be important for determining the direction of future research. One possibility, suggested by Blatt (8), is that FC affects an outwardly rectifying K^+-channel. Blocking of the channel opening by FC would reduce K^+-efflux and result in an increase in net K^+-influx. Such a sharp drop in K^+-efflux (10-fold) after FC addition has indeed been observed (10). Although it is not immediately clear how the plugging of a K^+-leak would lead to hyperpolarization, this hypothesis points out once more the crucial role that K^+ plays in the FC response (20). The question whether the FC-receptor acts as a K^+-channel will be further pursued with the patch-clamp technique, studying the purified FC-receptor after reconstitution in artificial liposomes.

Another possibility is that the activated FC-receptor stimulates the H^+-ATPase through phosphorylation. It was recently reported (23) that the PM H^+-ATPase is phosphorylated by a Ca^{2+}-dependent protein kinase. Evidence that the H^+-ATPase is regulated in its activity by phosphorylation came from Bidwai and Takemoto (7). They showed that the bacterial phytotoxin syringomycin induced a protein kinase mediated phosphorylation of the H^+-ATPase in a Ca^{2+}-dependent manner and also stimulated H^+-extrusion by the H^+-ATPase. If FC activates the H^+-ATPase in a fashion similar to syringomycin, it might do so by initiating the phosphatidylinositol pathway. In that case, the first step after activation of the FC-receptor could be the coupling to G-proteins, which in turn activate the phospholipase C. Little is known about G-proteins in plants, but with antibodies raised against G-proteins in the PM of animal cells, it seems worthwhile to look for the presence of G-proteins in the plant PM.

With the purification technique described here it should now be possible to raise monoclonal antibodies against the FCBP which will enable the purification of the FCBP in larger quantities. Then, the protein can be further characterized and the mechanism by which the receptor transduces the FC-signal into the cell elucidated.

ACKNOWLEDGMENTS

Fusicoccin was a kind gift from Prof. A. Ballio. We furthermore thank Prof. A. Ballio, Dr. M. H. Gelb, and Dr. R. G. Stout for their valuable suggestions and discussions.

LITERATURE CITED

1. **Aducci P, Federico R, Ballio A** (1980) Interaction of a high molecular weight derivative of fusicoccin with plant membranes. Phytopathol Mediterr **19**: 187–188
2. **Aducci P, Ballio A, Federico R** (1982) Solubilisation of fusicoccin binding sites. *in* D Marmé, E Marrè, R Hertel, eds, Plasmalemma and Tonoplast: Their Functions in the Plant Cells. Elsevier Biomedical Press, Amsterdam, pp 279–284
3. **Aducci P, Ballio A, Fiorucci L, Simonetti E** (1984) Inactivation of solubilised fusicoccin-binding sites by endogenous plant hydrolases. Planta **160**: 422–427
4. **Aducci P, Ballio A, Marra M** (1986) Incubation of corn coleoptiles with auxin enhances *in vitro* fusicoccin binding. Planta **167**: 129–132
5. **Ballio A** (1977) Fusicoccin: structure-activity relationships. *In* E Marrè, O Ciferri, eds, Regulation of Cell Membrane Activities in Plants, Elsevier North Holland, Amsterdam, pp 217–223
6. **Ballio A, Federico R, Pesci A, Scarlorbi D** (1980) Fusicoccin binding sites in subcellular preparations of spinach leaves. Plant Sci Lett **18**: 39–44
7. **Bidwai AP, Takemoto JY** (1987) Bacterial phytotoxin, syringomycin, induces a protein kinase-mediated phosphorylation of red beet plasma membrane polypeptides. Proc Natl Acad Sci USA **84**: 6755–6759
8. **Blatt MR** (1988) Mechanisms of fusicoccin action: a dominant role for secondary transport in a higher plant cell. Planta **174**: 187–200

9. **Bradford MM** (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. Anal Biochem **72**: 248
10. **Clint GM, MacRobbie EAC** (1984) Effects of fusicoccin in "isolated" guard cells of *Commelina communis* L. J Exp Bot **35**: 180–192
11. **Conti-Tronconi BM, Raftery MA** (1982) The nicotenic cholinergic receptor: correlation of molecular structure with functional properties. Annu Rev Biochem **51**: 491–530
12. **Dohrmann U, Hertel U, Pesci P, Cocucci SM, Marrè E** (1977) Localization of "*in vitro*" binding of the fungal toxin fusicoccin to plasma-membrane-rich fractions from corn coleoptiles. Plant Sci Lett **9**: 291–299
13. **Dupont-Durst H, Gokel GW** (1978) A classification test for aldehydes involving phase transfer catalysis. J Chem Educ **55**: 206
14. **Feyerabend M, Weiler EW** (1988) Characterization and localization of fusicoccin-binding sites in leaf tissues of *Vicia faba* L. probed with a novel radioligand. Planta **174**: 115–122
15. **Forbusch B III** (1982) Characterization of right-side out membrane vesicles rich in (Na,K)-ATPase and isolated from dog kidney medulla. J Biol Chem **257**: 12678–12684
16. **Karlin A, Dipaola M, Kao PN, Lobel P** (1987) Functional sites and transient states of the nicotinic acetylcholine receptor. *In* B Hille, DM Fambrough, eds, Proteins of Excitable Membranes, Wiley-Interscience, New York, pp 43–65
17. **Lamed R, Levin Y, Wilchek M** (1973) Covalent coupling of nucleotides to agarose for affinity chromatography. Bioch Bioph Acta **304**: 231–235
18. **Löbler M, Klämbt D** (1985) Auxin-binding protein from coleoptile membranes of corn (*Zea mays* L.). 1. Purification by immunological methods and characterization. J Biol Chem **260**: 9848–9853
19. **Marrè E** (1977) Fusicoccin: a tool in plant physiology. Annu Rev Plant Physiol **30**: 273–288
20. **Marrè MT, Romani G, Bellando M, Marrè E** (1986) Stimulation of weak acid uptake and increase in cell sap pH as evidence for fusicoccin- and K^+-induced cytosol alkalinization. Plant Physiol **82**: 316–323
21. **Pesci P, Tognoli L, Beffagna N, Marrè E** (1979) Solubilisation and partial purification of a fusicoccin-receptor complex from maize microsomes. Plant Sci Lett **15**: 313–322
22. **Sandstrom RP, De Boer AH, Lomax TL, Cleland RE** (1987) Latency of plasma membrane H^+-ATPase in vesicles isolated by aqueous phase partitioning. Plant Physiol **85**: 693–698
23. **Schaller GE, Sussman MR** (1988) Phosphorylation of the plasma-membrane H^+-ATPase of oat roots by a calcium stimulated protein kinase. Planta **173**: 509–518
24. **Shimomura S, Sotobayashi T, Futai M, Fukui T** (1986) Purification and properties of an auxin-binding protein from maize shoot membranes. J Biochem **99**: 1513–1524
25. **Sibley DR, Benovic JL, Caron MG, Lefkowitz RJ** (1987) Regulation of transmembrane signaling by receptor phosphorylation. Cell **48**: 913–922
26. **Stout RG, Cleland RE** (1980) Partial characterization of fusicoccin binding to receptor sites on oat root membranes. Plant Physiol **66**: 353–359
27. **Stryer L, Bourne HR** (1986) G proteins: a family of signal transducers. Annu Rev Cell Biol **2**: 391–419
28. **Tognoli L, Basso B** (1987) The fusicoccin-stimulated phosphorylation of a 33 kDa polypeptide in cells of *Acer pseudoplatanus* as influenced by extracellular and intracellular pH. Plant Cell Environ **10**: 233–239
29. **Wareing PF** (1985) Plant cell responses and the role of growth substances. *In* M Bopp, ed, Plant Growth Substances 1985, Springer-Verlag, Berlin, pp 1–9