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Fusicoccin-Binding Proteins in *Arabidopsis thaliana* (L.) Heynh.[1]

Characterization, Solubilization, and Photoaffinity Labeling

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ABSTRACT

Using the novel radioligand, [^{3}H]-9′-nor-fusicoccin-8′-alcohol, high affinity binding sites for fusicoccin were characterized in preparations from leaves of *Arabidopsis thaliana* (L.) Heynh. The binding site copartitioned with the plasmalemma marker, vanadate-sensitive K^+, Mg^{2+}-ATPase, when microsomal fractions were further purified by aqueous two-phase partitioning in polyethylene glycol-dextran phase systems and sedimented at an equilibrium density of 1.17 grams per cubic centimeter in continuous sucrose density gradients, as did the ATPase marker. The binding of [^{3}H]-9′-nor-fusicoccin-8′-alcohol was saturable and Scatchard analysis revealed a biphasic plot with two apparent dissociation constants (K_D), K_{D1} = 1.5 nanomolar and K_{D2} = 42 nanomolar, for the radioligand. Binding was optimal at pH 6, thermolabile, and was reduced by 70% when the membrane vesicles were pretreated with trypsin. The data are consistent with the presence of one or several binding proteins for fusicoccin at the plasma membrane of *A. thaliana*. Binding of the radioligand was unaffected by pretreatment of the sites with various alkylating and reducing agents, but was reduced by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, diethylpyrocarbonate, chloramine T, and periodate. A number of detergents were tested to find optimum conditions for solubilization. Nonanoyl-*N*-methylglucamide (50 millimolar) solubilized 70% of the radioligand-binding protein complex in undissociated form. Photoaffinity labeling of membrane preparations with a tritiated azido analog of fusicoccin resulted in the labeling of a 34 ± 1 kilodalton polypeptide. Labeling of this polypeptide, presumably the fusicoccin-binding protein, was severely reduced in the presence of unlabeled fusicoccin.

The *Fusicoccum amygdali* Del. toxin, fusicoccin (FC[2]) produces characteristic biochemical responses in a broad variety of higher plants (15). The stimulation of H^+ extrusion across the plasmalemma appears to be the most direct effect of toxin-action so far known, but the mechanism of FC action remains to be elucidated. While FC has been reported to stimulate the activity of the plasmalemma located, vanadate-sensitive H^+-translocating ATPase (7 and refs. cited therein), the effect does not seem to involve direct binding of the toxin to the enzyme (7, 19). Studies on the metabolism of FC in plants have shown a remarkable metabolic stability, especially with respect to cleavage of the ester bonds (3, and B Siebers, J Backhausen, EW Weiler, unpublished data). These data seem to exclude ester hydrolysis and a resulting cytosol acidification as a mechanism of FC-induced stimulation of ATPase activity. Thus, the presence of FC-receptors mediating FC action needs to be considered. Despite several reports on high affinity FC-binding sites (5, 9, 16, 17, 19), isolation of these sites in pure form has not been accomplished to date. Recently, Feyerabend and Weiler (11, 12) reported the synthesis of the highly bioactive radioligand, [^{3}H]FCol, through a chemically reactive aldehyde intermediate, as well as the characteristics of the FCBP from plasma membranes of *Vicia faba* L. For molecular analysis of the FCBP and the ultimate isolation of its gene, *Arabidopsis thaliana* seems to be the plant of choice. However, FC binding in this species has not been characterized so far. We have now characterized the high affinity FCBP in *A. thaliana* (L.) Heynh. leaf tissue. The properties of the FCBP and the conditions for its solubilization are reported here. We have, furthermore, identified the FCBP on SDS-PAGE after photoaffinity labeling, using an azido analog of FC (M Feyerabend, EW Weiler, unpublished data).

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[2] Abbreviations: FC, fusicoccin; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; FCBP, fusicoccin-binding protein; FCol, 9′-nor-fusicoccin-8′-alcohol; K_D, dissociation constant; Mega-9, nonanoyl-*N*-methylglucamide; [^{3}H]ABE-FC, 9′-nor-8′-(4-azido-3,5-[^{3}H]-benzoyl-ethylenediamine) fusicoccin.

MATERIALS AND METHODS

Plant Material

Arabidopsis thaliana (L.) Heynh. (race Landsberg erecta, seeds provided by Dr. M. Koornneef, Wageningen, NL) was grown in standard soil in short days (8 h) at 24°C (day) and 20°C) (night), relative humidity 75 to 90% and 90 W m^{-2} from fluorescent tubes (Philips TLD). Shoots from 6 week old plants were harvested, washed with cold water and used for membrane preparations.

Chemicals and Reagents

[^{3}H]FCol was synthesized as described elsewhere (11, 12) and had a specific activity of 1.33×10^{14} Bq mol^{-1}. Dextran T 500 was from Pharmacia, PEG 3350 was purchased from Union Carbide. FC was purchased from Italchemica, Segrate,

Italy, and Mega-9 from Oxylchemie, Böblingen, FRG. Most other chemicals and enzymes came from Sigma, except for *N*-octyl-β-glucopyranoside (Calbiochem), chymotrypsin A4 (Boehringer), and Polyclar AT (Serva).

Preparation of Membranes

Freshly harvested, washed shoots of *A. thaliana* were homogenized in a Braun MX 32 blender for 90 s at 4 to 10°C in 50 mM Hepes-KOH (pH 7.5) containing 0.5 M sucrose, 3 mM EDTA, 0.6% Polyclar AT, and 3mM DTT. The brei was squeezed through gauze and the homogenate centrifuged for 10 min at 11,000*g*. The supernatant was recentrifuged for 30 min at 80,000*g* (in some experiments, 50,000*g* were used) and the microsomal pellet was resuspended in binding buffer (10 mM Tris/Mes [pH 6.0] containing 1 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM KF, 1 mM EDTA, and 2.6 mM DTT [0.4 mg/mL]). These membranes were used for binding studies either directly, or, if necessary, were stored in 60% glycerol (in binding buffer) at −20°C (protein concentration ≥ 0.5 mg mL^{-1}). For the preparation of plasma membrane vesicles, the technique of aqueous two-phase partitioning (13) was used with minor modifications. The microsomal pellets (60–80 mg protein) were resuspended in 9 mL K-PO_4, 5 mM, containing 330 mM sucrose (pH 7.8) and added to 27 g of the phase system. Phase partitioning was performed as described elsewhere (12, 13) at 6.4% (w/w) dextran T 500 and 6.4% (w/w) PEG 3350, 330 mM sucrose, 5 mM K-PO_4, 4 mM KCl (pH 7.8) (all values are final concentrations). The phases U_3 and $U_{3'}$ (13) were combined, diluted with a threefold volume of 5 mM Tris/Mes buffer (pH 6.5) containing 330 mM sucrose (for enzyme assays) or binding buffer (see above, for binding assays) and repelleted for 45 min at 100,000*g* (4°C).

Marker Enzyme Assays

All marker enzymes were assayed as described (7). Protein was determined according to Bradford with the Bio-Rad protein assay kit and BSA as reference standard.

Binding Assays

Microsomal membranes (typically 100 μg protein) or plasma membranes (typically 50 μg protein) were incubated in a total volume of 1 mL binding buffer (see above) in the presence of 10 nM [^{3}H]FCol and optimal additives as specified in the results section. After incubation for 1 h at 25°C, 0.5 mL chilled binding buffer was added and the membranes were pelleted by centrifugation for 30 min at 80,000*g* (4°C). The pellets were resuspended in 100 μL water and added to 5 mL scintillation cocktail for the determination of bound radioligand. To determine unspecific binding, parallel incubations were carried out in the presence of excessive (3 μM) FC. All data were corrected for this unspecific binding. Duplicate determinations of each data point were performed and each experiment repeated two to three times. This binding assay is described in detail elsewhere (12). Modifications of the standard protocol are mentioned where appropriate.

Pretreatments of Membranes Prior to Binding Assays

For the protein modification and protease assays, microsomal membranes (200 μg) were incubated, in a total volume of 1 mL, with the indicated reagent or enzyme in the buffer specified for each treatment (see "Results"). All buffers contained the following additives: 1 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM EDTA, 1 mM KF. Incubations were carried out for 30 min or 60 min (proteases) at 25°C, followed by addition of 0.5 mL of the respective, ice-cold, buffer and centrifugation (30 min, 80,000*g*, 4°C). The membranes were then resuspended in binding buffer and assayed for binding of [^{3}H]FCol under standard conditions. Untreated controls and controls for the subtraction of unspecific binding were always included. All data are based on two separate experiments per treatment.

Solubilization of the FC-Binding Protein

Microsomal membranes (5–7 mg mL^{-1}) were first incubated with 100 nM [^{3}H]FCol under standard conditions. Aliquots of prelabeled membranes (100 μL, 0.5–0.7 mg protein) were then solubilized in 10 mM Tris-HCl (pH 8.4) in the presence of 20% (v/v) glycerol, 1 mg mL^{-1} purified asolectin and the appropriate concentration of detergent (total volume: 0.5 mL). The mixed samples were then sonicated for 5 min in a Sonorex RK 510 S-sonication bath at 22°C, followed by incubation on ice for 15 min. After the addition of 1 mL solubilization buffer, the samples were centrifuged at 4°C for 30 min (120,000*g*). Solubilized ligand-site complexes were determined by the polyethylenimine filter assay (8, 9) and residual pelletable radioactivity by resuspending the pellets in 100 μL water, followed by the addition of 5 mL scintillation cocktail.

Photoaffinity Labeling of the FC-Binding Protein

Plasma membranes (U_3 + $U_{3'}$, see above), equivalent to 1 mg of protein, were incubated as described above, but using 4 nM [^{3}H]ABE-FC (specific activity 1.33×10^{15} Bq mol^{-1}, M Feyerabend, EW Weiler, unpublished data) as radioligand. Parallel samples contained 4×10^{-5} M unlabeled FC. The final incubation volume was 2.5 mL. The labeled membranes were then repelleted for 30 min at 80,000*g* (4°C) and resuspended in 1 mL irradiation buffer (10 mM K-PO_4, 150 mM NaCl, pH 7.4). Samples were irradiated for 10 s in a quartz cuvette kept at 4°C (light source: Osram HBO 500 W high pressure arc lamp with quartz collector and Schott WG 305 long pass filter). Protein was precipitated with a 10-fold excess of methanol at −80°C (30 min) and pelleted for 10 min at 50,000*g* (4°C). The pellet was then dissolved in sample buffer and subjected to SDS-PAGE as described elsewhere (7).

RESULTS

Characteristics of Membrane Preparations

The specific activities of several marker enzymes were determined for the microsomal membranes and the U_3 + $U_{3'}$ (plasma membrane) fraction obtained from phase-partitioning (Table I). It can be seen that although the U_3 + $U_{3'}$ fraction apparently still contains a range of other membranes

Table I. *Activities of Some Marker Enzymes in Microsomal Fractions and U_3 + $U_{3'}$ Phases from Leaf Tissue of A. thaliana*

ATPase activities were assayed in the presence of 0.02% Triton X-100 (7, 13).

Enzymes	Specific Activity		Rate
	M[a]	U_3 + $U_{3'}$ phase	U_3 + $U_{3'}$/M
	nkat (mg protein)$^{-1}$		
Vanadate-sensitive K^+, Mg^{2+}-ATPase	6.3 ± 2.7	14.7 ± 3.4	2.32
Nitrate-sensitive ATPase	2.4 ± 1.3	2.1 ± 1.0	0.88
Glucan synthase II	0.007 ± 5 × 10^{-6}	0.018 ± 0.0025	2.75
NADH-Cyt *c*-reductase	0.66 ± 0.06	0.43 ± 0.09	0.65

[a] M = microsomes.

of the endomembrane system as well as mitochondrial membranes, only the plasmalemma markers, vanadate-sensitive K^+, Mg^{2+}-ATPase and glucan synthase II, are enriched in this fraction whereas the specific activities of the other markers are lower in this preparation as compared to total microsomal membranes. The most drastic purification is for chloroplast membranes, as becomes evident from the low level of Chl in the U_3 + $U_{3'}$ phases (typically from 3 to 7 μg/mg protein as compared to 60–80 μg/mg protein in the microsomal preparations). The sidedness of the plasma membrane vesicles was determined from the latency of the vanadate-sensitive K^+, Mg^{2+}-ATPase in the presence and absence of Triton X-100 (13). The preparations contained between 50 and 70% right-side out vesicles.

Conditions of [^{3}H]FCol-Binding

Binding of the radioligand was proportional to the protein added to the standard binding test (10 nM [^{3}H]FCol) in the range up to 750 μg/ml microsomal protein. An aliquot of 100 μg of microsomal protein and 50 μg of plasma membrane protein was used routinely (total assay volume 1 mL). Binding of the radioligand was strongly dependent on pH (Fig. 1). Very little binding was observed at pH <5 and >9 and the pH optimum for binding was 6.0.

Binding was temperature dependent (Fig. 2) and, at an incubation time of 1 h, optimum around 20°C. At higher temperatures, binding of [^{3}H]FCol strongly decreased. Binding was virtually abolished above 55°C. At 25°C standard incubation temperature, the kinetics of [^{3}H]FCol-binding to plasma membranes was rapid (Fig. 3, $t_{1/2}$ = 12 min) and binding was virtually complete after 1 h, the standard incubation time used in all later experiments. Under optimized conditions, unspecific binding (determined as nondisplaced radioligand in the presence of 3 μM FC in parallel incubations) was 10% of the total pelletable radioactivity.

Localization of FC-Binding Sites

The FC-binding sites appear to be located at, or associated with, the plasma membrane. This conclusion is based on two observations: (a) The specific activity of [^{3}H]FCol binding to plasma membrane enriched U_3 + $U_{3'}$ fractions was consistently higher than that obtained for microsomal membranes. Typical values are 1326 ± 400 Bq (mg protein)$^{-1}$ for U_3 + $U_{3'}$ membranes as compared to 432 ± 107 Bq (mg protein)$^{-1}$ for microsomal membranes which is equivalent to 10 pmol (mg protein^{-1}) (U_3 + $U_{3'}$) membranes) and 3.3 pmol (mg protein^{-1}) (microsomes) of bound radioligand. (b) On sucrose density gradients, the FC-binding sites sediment at an equilibrium density of 1.17 g cm^{-3} and peak in the same fraction as the bulk of the K^+, Mg^{2+}-ATPase activity, consistent with a plasma membrane localization of the sites (Fig. 4).

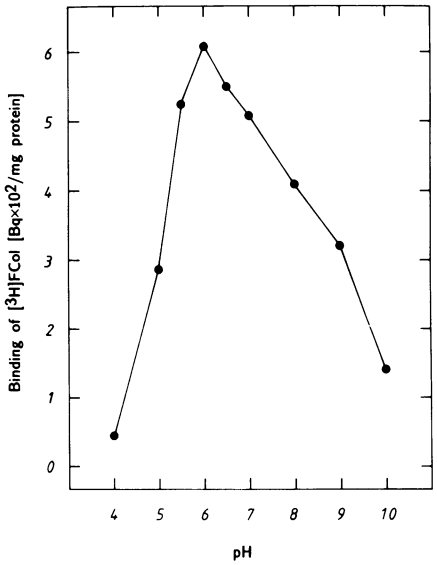

Figure 1. Influence of assay pH on the binding of [^{3}H]FCol (10 nM) to microsomal membranes from leaf tissue of *A. thaliana*.

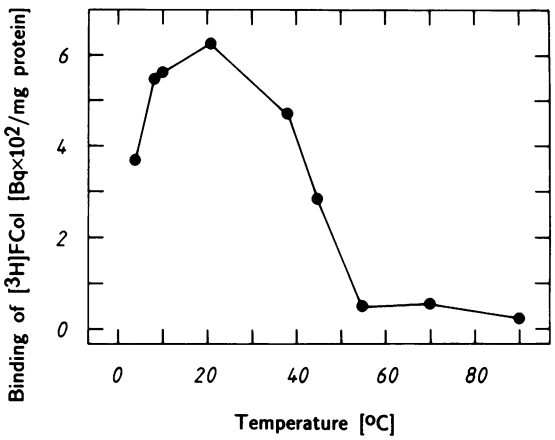

Figure 2. Temperature dependence of [^{3}H]FCol binding to microsomal membranes. Standard assay conditions as detailed in "Materials and Methods", incubation time 60 min.

Saturability and Reversibility of FC-Binding Sites

FC competed effectively with [^{3}H]FCol for the binding sites when added simultaneously (Fig. 5). Nanomolar concentrations were already effective and 1 to 2 μM FC practically reduced the binding of the radioligand to background levels. Fifty percent displacement of radioligand binding to plasma

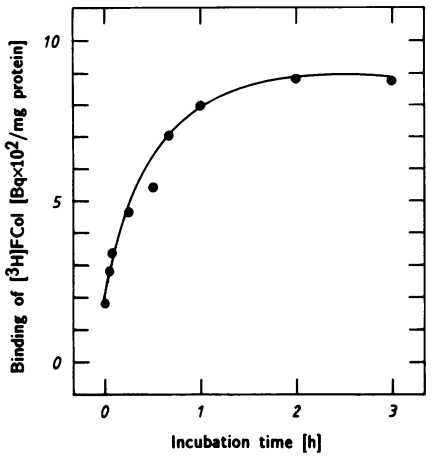

Figure 3. Kinetics of association of [^{3}H]FCol with binding sites at plasma membrane (U_3 + $U_{3'}$ phases).

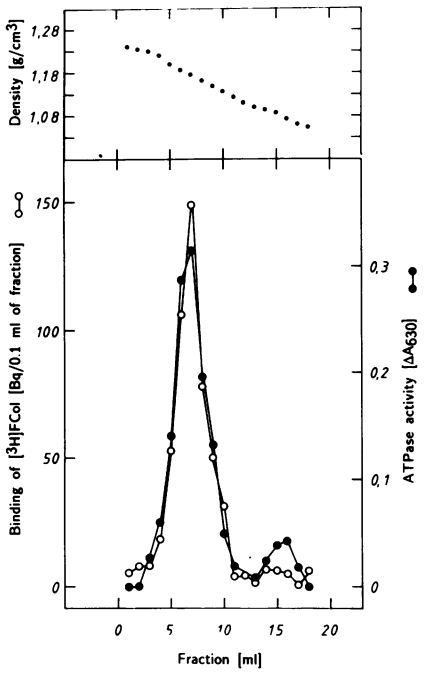

Figure 4. Localization of FC binding and vanadate-sensitive K^+, Mg^{2+}-ATPase on isopycnic sucrose density gradients. Microsomal membranes were loaded on the gradient and centrifuged for 3 h at 4°C and 90,000*g*. FC binding was assayed using the standard protocol with subsequent recentrifugation. ATPase activity was measured as inorganic orthophosphate release in the presence of 0.02% Triton X-100 as described (7).

membrane fractions occurred at 5 nM FC. Excessive FC (3 μM) added to plasma membranes preequilibrated with [^{3}H] FCol also displaced the radioligand, although slowly (Fig. 6, $t_{1/2}$ = 4 h 18 min at 25°C).

As shown in Figure 7A, increasing concentrations of [^{3}H] FCol saturated the binding sites. A Scatchard analysis (18) revealed the presence of two classes of binding sites (Fig. 7B) with apparent K_D values of 1.5 ± 0.85 nM (K_{D1}, n = 3) and 42 ± 21.8 nM (K_{D2}, n = 3) and apparent site concentrations of 5.6 ± 1.8 pmol (mg protein)$^{-1}$ and 13.3 ± 1.8 pmol (mg protein)$^{-1}$, respectively. The data were derived from three

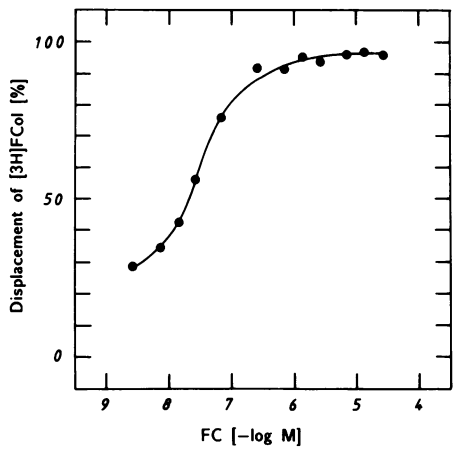

Figure 5. Inhibition of binding of [^{3}H]FCol (10 nM) to U_3 + $U_{3'}$ plasma membranes by simultaneously added, unlabeled FC. All other assay conditions were standard (see "Material and Methods").

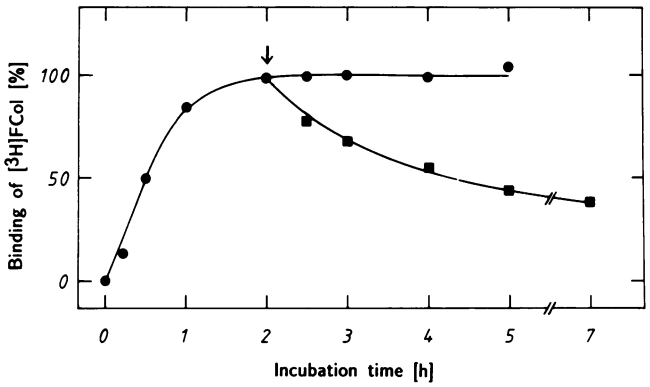

Figure 6. Reversibility of [^{3}H]FCol (10 nM) binding at 25°C by an added excess (3 μM) of unlabeled FC. FC was added after 2 h (arrow). Control = (●), incubated in the presence of FC = (■).

independent experiments. No significant difference was observed for microsomal membranes and U_3 + $U_{3'}$ fractions. At our standard conditions (10 nM radioligand), the higher affinity site should be labeled predominantly, but not exclusively.

Selectivity of the Binding

A range of FC derivatives and related compounds were tested for their ability to compete with the radioligand for the binding sites (Table II). For comparison, data from a recent study of FC-binding sites in *Vicia faba* L., using the same radioligand (11) and data obtained for *Zea mays* L. coleoptiles using [^{3}H]-dihydro-FC as radioligand (6) are also given.

Influence of Proteases and Chemical Modifications

A number of proteases, when preincubated with microsomal membranes, significantly reduced subsequent binding of [^{3}H]FCol (Fig. 8). At low concentrations (< 10 μg mL^{-1}), trypsin was most effective and removed 70% of the binding sites. These findings, together with the thermolability of [^{3}H] FCol binding, suggests that the FC-binding site is a protein. This conclusion is further strengthened by results from the

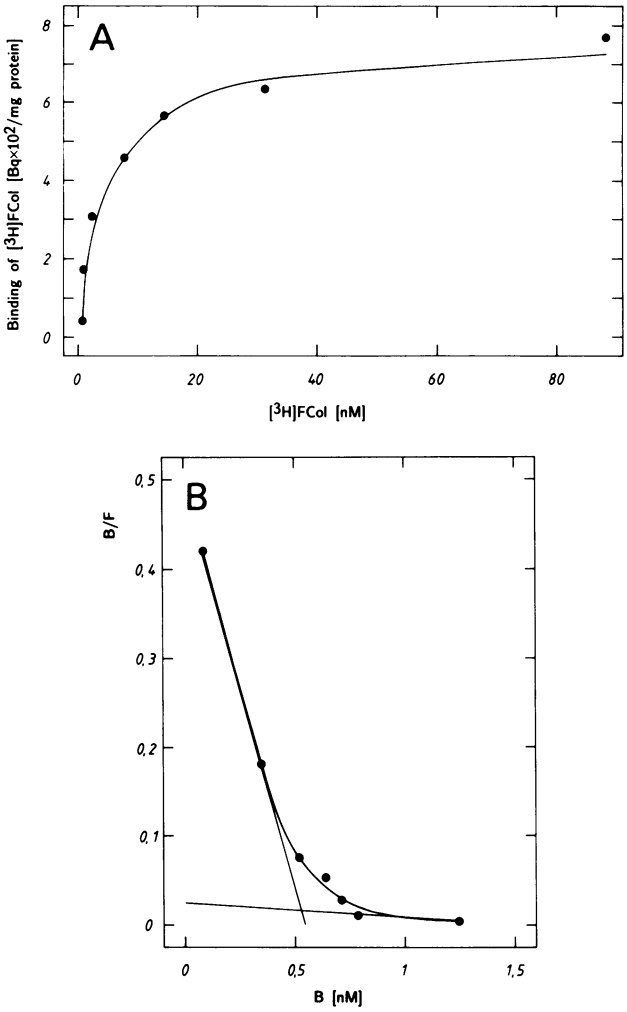

Figure 7. A, Saturation of FC-binding sites with increasing concentrations of [^{3}H]FCol in a typical experiment. For this experiment, microsomal membranes and standard assay conditions except for the radioligand concentration were used. B, Scatchard plot of the same data.

use of various protein-modification reagents (Table III). Three reagents significantly reduced subsequent radioligand binding namely diethylpyrocarbonate (which at the pH used mainly modifies histidine (14), chloramine T (which mainly oxidizes methionine (14) and EDC (a water-soluble carbodiimide which modifies carboxyl groups (14), but may lead to cross-linking through peptide-bond formation). SH-reagents (alkylating, disulfide-bound reducing), NH_2-reagents, *N*-acetylimidazole and dicyclohexylcarbodiimide did not affect subsequent radioligand binding. However, preincubation of the membranes with $NaIO_4$ at mM concentrations nearly completely abolished subsequent binding of [^{3}H]FCol.

Conditions for the Solubilization of Ligand-Binding Protein Complexes

Purification of the binding-proteins requires previous solubilization. Because of the slow dissociation of ligand-site complexes at low temperatures, it was determined whether intact ligand-binding site complexes could be solubilized in the cold. As Figure 9 shows, conditions for the effective solubilization of intact complexes were found. About 70% of the radioligand-binding protein complexes were solubilized with 50 mM Mega-9 or octylglucoside. Lysolecithin and Triton X-100 were much less effective. A range of other detergents were excluded because of their ineffectiveness in preliminary experiments. Under optimal conditions, 70% of the radioligand-binding protein complex can be solubilized (Fig. 9) and the purification of this complex is now in progress.

Photoaffinity Labeling of the FCBP

Incubation of plasma membranes (U_3 + $U_{3'}$ phase) in the presence of [^{3}H]ABE-FC in nanomolar concentrations resulted in specific labeling (*i.e.* labeling readily quenched by the simultaneous presence of micromolar levels of unlabeled FC). When 5.5 nM [^{3}H]ABE-FC was used, approximately 1.5 pmol/mg protein (2028 Bq/mg protein) of the azido analog was specifically and reversibly associated with the FCBP. In the presence of an excess of unlabeled FC (30 μM), binding was reduced to 232 Bq/mg protein which is considered due to unspecific binding. Irradiation at $\lambda \geq 305$ nm immobilized approximately 10% of the ligand as judged by the trichloroacetic acid-precipitable radioactivity. When total, photoaffinity labeled, plasma membrane protein was analyzed by SDS-PAGE, a 34 ± 1 kD band (broad) was found to be labeled (Fig. 10). In addition, a significant proportion of radiolabel appeared in the front, presumably representing low molecular mass degradation products or lipid components in the environment of the FCBP.

DISCUSSION

High affinity FC binding to membrane fractions was demonstrated for a number of plants (5, 9, 10, 12, 15–17, 19) and most evidence points to the plasmalemma as the probable location of these sites (9, 10, 12, 19). However, the occurrence and characteristics of FC-binding sites in *A. thaliana*, a plant which lends itself particularly well to molecular genetics and thus is a prime candidate for gene isolation studies, are not known.

The data reported here show that *A. thaliana* does express FC-binding sites, that these sites are proteins (*cf.* Fig. 8) probably located at the plasmalemma (*cf.* Fig. 4) and that their properties are closely similar to those of FC-binding sites reported from other plants (for review, see Ref. 21).

[^{3}H]FCol, a recently introduced radioligand to probe *V. faba* FC-binding sites which has biological activities in stomatal bioassays similar to that of FC (11, 12), proved also suitable to analyze the FC-binding site(s) of *A. thaliana*. Apparent dissociation constants as derived from Scatchard analysis for the highest affinity site detected (1.5 nM) are well within the range (0.3 to 5.7 nM) reported for other tissues (21), and the pH profile, kinetics of association and dissociation and the temperature dependence of radioligand binding were similar to those measured for the corresponding site(s) of *V. faba* L. (12) as well as other systems (5, 9, 16, 21). The similarity of the *A. thaliana* FC-binding site(s) becomes further evident from the structure-activity relationship revealed by testing structurally related compounds for their ability to

Table II. *Relative Activity of FC-Derivatives and Structurally Related Compounds in Inhibiting Binding of Radioligand [^{3}H]FCol, to U_3 + U_3 Plasma Membrane Fractions of A. thaliana*

For comparison, data for *V. faba* (microsomal membranes, radioligand: [^{3}H]FCol [12]) and *Zea mays* (microsomal membranes, radioligand: [^{3}H]-Dihydro-FC (6) are also included.

Derivative	I_{50} *A. thaliana*	Relative activity		
		A. thaliana	*V. faba*	*Z. mays*
	nM		%	
FC	5.0 ± 0.4	100	100	100
Dihydro-FC	5.0 ± 0.1	100	107	100
Monodeacetyl-FC	5.2 ± 0.1	96	98	51
Dideacetyl-FC	36.5 ± 0.7	14	51	60
9′-Nor-FC-8′-alcohol	5.2 ± 0.7	96	92	
de-*t*-Pentenyl-dideacetyl-FC	164.0 ± 24	3.0	4.2	1.4
FC-aglycone	11690.0 ± 1500	0.04	0.2	0.4
Cotylenin A	19.5 ± 1.5	26	37	128
Cotylenin C	70.0 ± 2.8	7	12	13

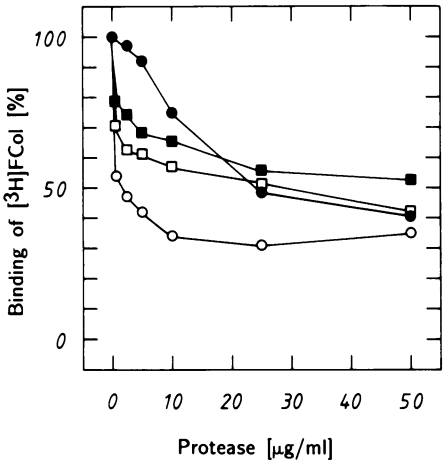

Figure 8. Influence of pretreatment with proteases on subsequent binding of [^{3}H]FCol. Microsomal membranes were preincubated with the enzymes as detailed in materials and methods, then repelleted, resuspended in assay buffer followed by a standard binding assay. ■ = Thermolysin, ● = chymotrypsin A4, □ = papain, ○ = trypsin. Incubation conditions with the proteases were: 10 mM Tris/MES containing 1 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM KF, 1 mM $MgSO_4$ at pH 6.0 (thermolysin), pH 7.8 (chymotrypsin A4), pH 6.0 (papain), and pH 7.8 (trypsin), for 60 min at 25°C.

compete with the radioligand for the binding sites (Table II). Only dideacetyl-FC was somewhat less reactive in *A. thaliana* than in the other systems for which comparable data were reported (2, 12). The ability of the derivatives tested in competing with the radioligand for the binding site parallels the biological activity of the compounds as determined in stomatal opening bioassays (2, 12) whereas some compounds (*e.g.* dideacetyl-FC and the FC-aglycone) are relatively more active in inducing germination of dormant lettuce and radish seeds and stimulating cell enlargement in squash cotyledons (4).

The thermolability of the binding site(s) and the fact that the sites are degraded by several proteases (Fig. 8) indicates that they are protein(s), a conclusion reached also for other systems (9, 12, 19). However, in contrast to recent findings for FC-binding proteins of *V. faba* (12), a significant proportion of the *A. thaliana* binding sites (30–40%) was resistant to proteases. This probably reflects the higher proportion of vesicles with the FC-binding site facing inwards as compared to the *V. faba* plasma membrane vesicles (12). However, it cannot be ruled out at present that two sites or states of one site with different sensitivity to proteases exist in our preparations. Indications for site-heterogeneity were in fact obtained from Scatchard analysis (Fig. 7B). In a recent study (9), trypsinisation of the FC-binding protein and the plasma membrane ATPase has resulted in similar (ca. 30%) degradation of both systems. This would indicate that the ATP and FC-binding domains of the two systems face the same (cytoplasmic) side of the plasma membrane. In contrast to this, over 90% of the FC-binding sites were trypsin labile in predominantly right-side out plasmalemma vesicles prepared from leaves of *V. faba* and FC-protein conjugates strongly inhibited the binding of the radioligand in the same system (12). These data favour an apoplastic localization of the FC-binding domain.

A further feature of the FC-binding site(s) is its susceptibility to periodate in concentrations above 2 mM (Table III). This treatment modifies, by oxidation of vicinal diols, sugar residues, but under the condition employed does not affect aminoacids (20). In accord with the susceptibility of the FC-binding site(s) to periodate treatment is our observation, that preincubation of membranes with commercial cellulase preparations containing mixtures of hydrolases likewise significantly reduces subsequent binding of the radioligand. These findings confirm an earlier report (1) on the inhibition of FC binding from solubilized spinach leaf microsomes following treatment with mannosidase. Taken together, we must conclude that the FC-binding site(s) appear to be glycoprotein(s) and further that the glycosidic part is essential for the functionality of the FC-binding domain(s). Whether this involves conformational stabilization of the binding domain by the glycosidic moiety, a direct participation of it in the binding event or simply protection of the site from proteolytic degradation remains to be elucidated.

A. thaliana expresses an FCBP with properties typical for the high affinity FC-binding sites of other higher plants (5, 9, 10, 12, 15–17, 19). *A. thaliana* consequently should be the

Table III. *Effect of Pretreatment of Microsomal Membranes from the Leaf of A. thaliana with Various Modification Reagents on Subsequent Binding of [^{3}H]FCol*

[^{3}H]FCol-binding was assayed under standard conditions. Preincubation conditions for modification are mentioned for each treatment. After the pretreatment, membranes were repelleted and resuspended in binding buffer. All preincubation buffers contained the following additives: 1 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM KF, 1 mM EDTA. Preincubations were for 30 min at 25°C.

Reagent	Highest Concentration Tested	Preincubation		Residual [^{3}H]FCol Binding at Highest Concentration Tested
		Buffer	pH	
	mM			%
Iodoacetic acid	10	0.1 M Tris-HCl	7.2	120
Iodoacetamide	2	0.1 M K-PO_4	7.0	95
N-Ethylmaleimide	10	0.1 M K-PO_4	7.0	100
DTT	25	0.01 M Tris Mes	6.0	108
KOCN	10	0.05 M Hepes	7.4	98
Pyridoxal phosphate	5	0.2 M Na-borate	8.0	101
Maleic acid anhydride	5	0.05 M Na-borate	9.0	102
Acetic anhydride	5	1 M K-PO_4	5.8	105
Dicyclohexylcarbodiimide	1	0.04 M MOPS	7.4	100
EDC	2	0.1 M Mes/KOH	6.1	75
EDC	20	0.1 M Mes/KOH	6.1	17
$NaBH_4$	10	0.1 M Na-borate	8.0	91
Phenylglyoxal	10	0.025 M Hepes	7.2	111
Chloramine T	10	0.1 M K-PO_4	7.0	27
Diethylpyrocarbonate	20	0.1 M K-PO_4	6.0	51
Fluordinitrobenzol	5	0.5 M K-PO_4	7.0	108
N-Acetylimidazol	10	0.1 M K-PO_4	7.5	96
$NaIO_4$	5	0.1 M K-PO_4	7.5	42
$NaIO_4$	10	0.1 M K-PO_4	7.5	3

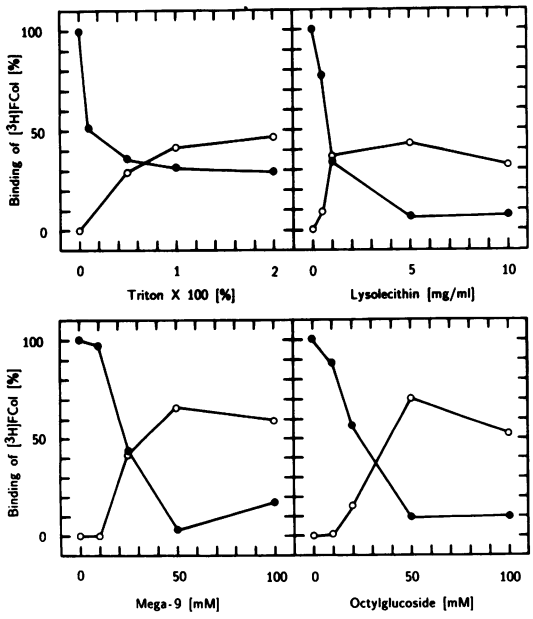

Figure 9. Solubilization of radioligand-binding site complexes from microsomal membranes. ● = residual, nonsolubilized and pelletable radioactivity, ○ = solubilized radioactivity bound to polyethylenimine-coated glass fiber filters. In each case, the nonspecific background was subtracted.

plant of choice for the molecular study of the toxin-binding protein. This involves the isolation of the FCBP in sufficiently pure form. In order to achieve this goal, the solubilization and specific tagging of the FCBP are necessary, both of which have been accomplished in the present study. The optimum conditions for solubilization of the FCBP from U_3 + $U_{3'}$ membranes of *A. thaliana* (see Fig. 9) are quite similar to those reported for the oat root system (9), again stressing the similarity of the FCBP from distantly related species. More important, photoaffinity labeling with the recently introduced azido analog of FC, [^{3}H]ABE-FC (M Feyerabend, EW Weiler, unpublished data) allowed to identify, in U_3 + $U_{3'}$ membranes of *A. thaliana* leaf tissue, a 34 ± 1 kD polypeptide (Fig. 10). The labeling of this band was strongly inhibited in the presence of FC. The electrophoretic front also carried photoaffinity labeled, low molecular mass components, and labeling in this fraction, too, was reduced in the presence of FC. We conclude, that this material represents either proteolytic fragments of the FCBP and/or membrane lipid components in the vicinity of the FCBP which were hit by the photoactivated toxin. No label was found in the 100 to 105 kD region reinforcing the argument of Blum *et al.* (7) that FC does not interact with the plasmamembrane H^+-ATPase directly. Rather, we conclude that the 34 kD polypeptide represents the FCBP, or part of it. The solubilized FCBP migrates with an apparent M_r of approximately 80 kD on gel permeation columns (19). Thus, it is likely, though not yet proven, that the native FCBP is an oligomeric complex including the 34 kD toxin-binding polypeptide.

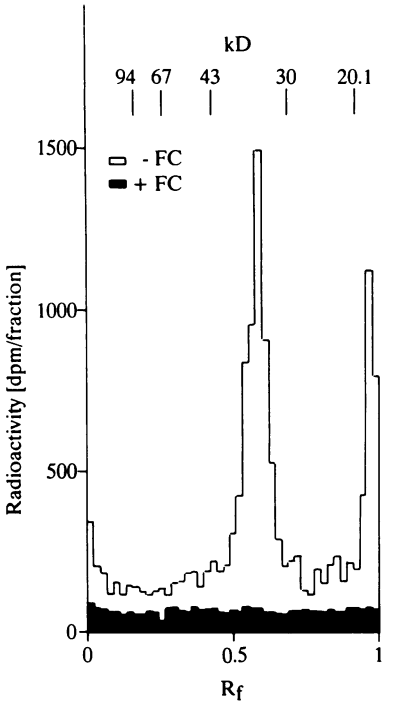

Figure 10. Separation of plasma membrane proteins ($U_3 + U_{3'}$ phase) on SDS-PAGE after photoaffinity labeling with [^{3}H]ABE-FC. The gel (11% acrylamide) was cut into 2 mm strips with a gel cutter, the pieces homogenized and the eluates counted in a scintillation with a gel cutter, the pieces homogenized and the eluates counted in a scintillation counter. Open bars represent the sample incubated with 5.5 nM [^{3}H]ABE-FC alone, closed bars the control incubated additionally in 3 μM FC. The upper panel gives the location and molecular masses of the marker proteins.

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