

Purification of the Fusicoccin-Binding Protein from Oat Root Plasma Membrane by Affinity Chromatography with Biotinylated Fusicoccin¹

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Fusicoccin (FC), a fungal phytotoxin, evokes a number of physiological responses after binding to the FC-binding protein (FCBP). For characterization of this plasma membrane protein and elucidation of the signal transduction pathway, we purified active FCBP from oat (*Avena sativa* L. cv Valiant) root plasma membranes using avidin-biotin affinity chromatography. For the binding of FCBP to immobilized avidin, a bifunctional FC derivative (FC-biotin, FCBio) was synthesized. FCBio retained high binding affinity for the FCBP ($K_D = 70$ nM), it elicited a biological response comparable to FC, and it was bound by avidin. The purification of the FCBP involved three important steps. First, FCBio was bound to the FCBP in purified plasma membrane vesicles. Next, plasma membrane proteins were solubilized in detergent, and part of the solubilized proteins was precipitated by decreasing the detergent concentration below the critical micelle concentration. The FCBP remained in the soluble fraction, and this fraction was loaded on a "low-affinity" avidin column. Proteins, bound through a biotin moiety to the column, were specifically eluted with excess biotin. This resulted in fractions active in [³H]FC binding and two bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis of 31 and 30 kD. The nonhydrophobic behavior of the FCBP was confirmed by means of phase separation with Triton X-114, wherein the FCBP migrated to the hydrophilic phase. Purification of the FCBP in active form using this novel affinity technique opens the possibility to study other features of the FCBP necessary for inducing physiological responses in plant cells.

The physiological effects of FC, a phytotoxin produced by the fungus *Fusicoccum amygdali* Del., on higher plants are widespread and well documented (Marrè, 1979; Weiler et al., 1990). The first action in the signal transduction pathway is the binding of FC to a PM-localized binding protein, FCBP. The FC-activated FCBP stimulates H⁺-ATPase through interference with the C-terminal autoinhibitory domain (Johansson et al., 1993; Rasi-Caldogno et al., 1993), and the activation mechanism might involve an irreversible change in the phosphorylation status mediated by a protein kinase C-type protein (De Boer et al., 1994a).

To understand the nature of the FCBP and to elucidate the pathway evoked after binding of FC to the FCBP, several attempts have been made to isolate and purify the binding

protein. De Boer et al. (1989) purified the FCBP using affinity chromatography, yielding two major polypeptides of 29.7 and 31.0 kD. Feyerabend and Weiler (1989) and Oecking and Weiler (1991) also identified the FCBP as two major polypeptides on SDS-PAGE with molecular masses in the region of 29 to 35 kD, using photoaffinity-labeling techniques and purification through a number of chromatography steps.

Although the FC-agarose matrix successfully isolated the FCBP from all other membrane proteins (De Boer et al., 1989), this method had two disadvantages: (a) solubilization of the FCBP must be done in the absence of ligand and the solubilized, free binding protein is less stable than the ligand-binding protein complex (Feyerabend and Weiler, 1989) and (b) the coupling of the FCBP to FC is tight such that the FCBP could not be eluted specifically from the column with excess FC. Elution was performed with urea or SDS, which resulted in only partly active FCBP.

Isolation of the FCBP in pure and active form is crucial to further elucidate the biochemical characteristics of the protein. Co-reconstitution experiments using a solubilized and crude FCBP fraction and a purified H⁺-ATPase fraction (Marra et al., 1992) were very important because they showed that PM components alone could elicit the stimulatory effect of FC on the H⁺-ATPase. However, only co-reconstitution of pure proteins, or a study of the biochemical properties of the pure FCBP (kinase, phosphatase or protease activity, or protein-protein interaction), can reveal the transduction mechanism of the FCBP. For these reasons we modified our affinity purification technique.

An FC derivative containing a biotin group (FCBio) was synthesized. This bifunctional ligand binds through the FC moiety to the receptor, and the biotinylated protein thus obtained can be purified with an immobilized avidin column. This novel purification technique has a number of potential advantages: (a) the FCBio can be bound to the FCBP prior to solubilization, which protects the FCBP from loss of binding, (b) after specific binding of the ligand to the FCBP, the ligand-binding protein complex can be specifically eluted from the avidin column using excess biotin, and (c) the ligand concentration can be controlled, which enables discrimination

Abbreviations: FAB, fast atom bombardment; FC, fusicoccin; FCBio, biotinylated fusicoccin; FCBP, fusicoccin-binding protein; OG, *n*-octyl β -D-glucopyranoside; PM, plasma membrane.

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between the FCBP in its high-affinity and low-affinity conformations.

In this paper we describe the purification of the FCBP from oat root (*Avena sativa* L.) PMs and introduce the technique of using biotinylated ligands to purify binding proteins in their active form.

MATERIALS AND METHODS

Chemicals and Reagents

FC was either purchased from Italchemia (Segrate, Italy) or was a gift kindly provided by Professor G.S. Muromtsev (Moscow, Russia). [³H]DihydroFC, a gift kindly provided by Professor R.E. Cleland (Seattle, WA), was synthesized by Amersham (United Kingdom, code TRQ4399; specific activity [May 1986], 35.2 Ci/mmol) and stored in ethanol at -20°C. All avidin and biotin derivatives were purchased from Pierce (Rockford, IL). All other chemicals were of the highest quality available.

Plant Material and PM Preparation

Plant material was harvested, and the PM preparation was carried out as detailed by De Boer et al. (1989). Briefly, oat seeds (*Avena sativa* L. cv Valiant; H. Blonk C.V., Moerkapelle, The Netherlands) were germinated on stainless steel screens over a 1 mM CaSO₄ solution at 25°C in the dark. After 6 d, roots were cut off and ground with buffer A (10 mM Tris-HCl [pH 7.5], 250 mM Suc, and 1 mM EDTA) at 4°C. All further steps were carried out at 4°C. The homogenate was filtered through four layers of cheesecloth, and PMSF was added to a final concentration of 1 mM from a stock solution (200 mM in methanol). The filtrate was centrifuged for 20 min at 10,000g. The supernatant was centrifuged for 30 min at 100,000g to obtain the microsomal pellet, which was resuspended in buffer B (5 mM K-phosphate [pH 7.8], 250 mM Suc, and 4 mM KCl). PM vesicles were obtained, and their purity was determined by further purification of the microsomal fraction by aqueous phase partitioning as described by Sandstrom et al. (1987). For every 100 g fresh weight of roots, a 10-g phase system was used, containing after addition of the microsomal fraction: 6.5% (w/w) dextran T500, 6.5% (w/w) PEG 3350, 250 mM Suc, 5 mM K-phosphate (pH 7.8), and 4 mM KCl. After the phase system was mixed, phase separation was achieved by centrifugation for 10 min at 1000g. The resulting upper phase (U₁) was transferred to a fresh lower phase, and mixing and centrifugation was repeated to obtain U₂; the procedure was repeated again to yield U₃. The lower phases were re-extracted with a fresh upper phase to obtain U'₁, U'₂, and U'₃. The U₃ and U'₃ fractions were pooled and washed by adding 10 volumes of buffer C (10 mM Tris-HCl [pH 7.5] and 250 mM Suc) and centrifuging at 100,000g for 30 min. The resulting pellet was resuspended in buffer C to give a final PM protein concentration of approximately 5 mg/mL. The activity of specific membrane marker enzymes (vanadate-sensitive K⁺-Mg²⁺ ATPase, nitrate-sensitive ATPase, azide-sensitive mitochondrial ATPase, and latent IDPase [Sandstrom et al., 1987]) indicated that the enrichment of PM vesicles during aqueous phase partitioning was more than 10-fold and that contami-

nation with membranes other than those originating from the PM was low. The purified PM vesicles were stored at -80°C.

Synthesis of FCBio

FC (5 mg) was converted to 9'-Nor-FC-8'-aldehyde by OsO₄/NaIO₄ oxidation as described by Feyerabend and Weiler (1987). For biotinylation of the ligand, 0.7 μmol of the FC aldehyde dissolved in methanol was added to 1 mL of 5 mM biotin-long chain-hydrazide in 100 mM sodium acetate plus 0.02% NaN₃, pH 5.5, and stirred for 1 h at room temperature. Thereafter, 50 μL of NaCNBH₃ (10 mg/mL in H₂O) was added and incubation proceeded for 12 h at 4°C. The formation of FCBio could be followed and analyzed on TLC using isopropanol:hydrogen acetate:chloroform (85:10:5, v/v) as the solvent system. The R_F values were 0.80 for FC, 0.76 for FC aldehyde, and 0.34 for FCBio. All FC derivatives on TLC plates could be visualized by spraying the plates with 5% H₂SO₄ in methanol and heating for 5 min at 120°C. The FCBio was purified using HPLC and was stable for several months at 4°C and pH 5.5. The HPLC system consisted of two Gilson model 303 pumps, a Rheodyne injection valve with a 20-μL loop, and a Lichrosorb 10RP8 analytical column (Phenomenex, Torrance, CA) for detection of FCBio or a 2-mL loop with a Hypersil 10C18 250- × 10-mm column (Phenomenex) for preparative purification of FCBio. Both FC derivatives and biotin derivatives were monitored at 205 nm with a Perkin-Elmer LC 235 diode array detector. The mobile phase, degassed by vacuum, was a linear gradient of 60 to 75% aqueous methanol (flow rate, 1.5 mL/min). The FC aldehyde eluted at 73% methanol, the FCBio eluted at 68% methanol, and biotin-long chain-hydrazide and salts eluted at low methanol concentrations. The concentration of FCBio was determined by the method described by Lin and Kirsch (1979), with biotin as a reference. The molecular mass of FCBio (2 μL, 0.5 mM) was determined by FAB MS using a MAT 90 instrument (Finigan MAT, Bremen, Germany) and a saddle field gun (type iinf; Ion Tech, Teddington, UK) with a source temperature of 50°C. FAB was performed at 7 KeV using xenon, ionization current of 0.3 mA, and glycerol as matrix. The measured mass was 1039 [M + H]⁺, which agrees well with the mass calculated from the structural formula of FCBio (C₅₁H₈₄N₅O₁₅S). The interaction between the biotinylated ligand and avidin was tested by loading a 0.4-mL monomeric avidin column, previously equilibrated with solution D (20 mM Tris-HCl [pH 7.5], 5 mM MgSO₄, 1 mM CaCl₂, 2.6 mM DTT, 1 mM PMSF, and 0.2% [w/v] OG), with 6 nmol of FCBio (flow rate, 0.1 mL/min). After incubation (30 min at room temperature), the column was washed with 2 mL of solution D and eluted with 2 mL of 2 mM biotin in solution D (flow rate, 0.1 mL/min). Both the wash fraction and the eluent were subjected to reverse-phase HPLC for detection of FCBio.

Functional FC and Biotin Moieties

To assess the functionality of the FC moiety, a bioassay was performed. For the bioassay, the elongation zone of 5-d-old oat coleoptiles, grown on vermiculite at room temper-

ature in the dark, was used. Sections, 15 mm long, starting 10 mm below the tip of the coleoptile were collected. These sections were abraded with silicium carbide and preincubated in 1 mM K-phosphate buffer (pH 6.25) for 30 min (Cleland, 1976). After preincubation, the sections (60 mg fresh weight) were incubated in 2 mL of the K-phosphate buffer with different concentrations of FC or FCBio and gently agitated for 90 min at 28°C in the dark. After the sample was incubated, the pH of the medium was measured.

The formation of the triple complex (FCBP-FCBio-avidin) was tested with fluorescent avidin. A quantity of 25 µg of purified PM vesicles was incubated with either 10⁻⁶ M FCBio or 10⁻⁶ M FC in 10 mL of solution E (20 mM Mes-Tris [pH 6.0], 20% [v/v] glycerol, 5 mM MgSO₄, 1 mM CaCl₂, 2.6 mM DTT, and 1 mM PMSF) for 1 h at 30°C. After incubation the vesicles were pelleted (30 min, 100,000g, 4°C) and washed three times with 5 mL of solution F (20 mM Tris-HCl [pH 7.5], 250 mM Suc, 5 mM MgSO₄, 1 mM CaCl₂, 2.6 mM DTT, and 1 mM EDTA) to remove unbound FCBio or FC. Thereafter, the pellet was resuspended in 1 mL of solution F, and 4 × 10⁻¹⁰ M avidin-fluorescein was added (no addition of avidin-fluorescein for control experiments). After incubation for 5 min at room temperature, the vesicles were pelleted and washed once as described above to remove unbound avidin-fluorescein. The resulting pellet was resuspended in 1 mL of solution F and used for fluorescence measurements. All fluorescence measurements were carried out at room temperature in an SLM Aminco SPF-500 spectrofluorometer with an excitation wavelength of 491 nm and an emission wavelength of 518 nm. Both excitation and emission monochromator were used with a bandwidth of 5 nm. Fluorescence was corrected for interferences caused by vesicles alone.

Preparation and Features of the Monomeric Avidin Affinity Column

Native ("tetrameric") avidin-agarose (50% slurry in 0.02% NaN₃) was packed into a 1-mL fast protein liquid chromatography column (Pharmacia LKB, Uppsala Sweden) with 10 mM K-phosphate buffer (pH 7.0) plus 0.02% NaN₃ at a flow rate of 0.2 mL/min. After packing, the column was stripped to obtain monovalent avidin by using the method described by Kohanski and Lane (1990). Briefly, the column was stripped by treatment of the avidin with 6 M guanidine hydrochloride in 0.2 M KCl (pH 1.5) and 3 M guanidine thiocyanate in 0.2 M KCl (pH 1.5). Thereafter, the nonexchangeable biotin-binding sites were blocked with 2 mM biotin in the K-phosphate buffer (pH 7.0). Finally, the exchangeable sites were regenerated by washing the column with 0.1 M Gly plus 0.2 M KCl (pH 1.5), and the column was equilibrated in solution D for protein purification. Column performance was tested with biotinylated BSA dissolved in solution D. The binding capacity of the monovalent avidin column was estimated at 1 nmol biotinylated BSA/mL avidin-agarose.

Solubilization and Two-Step Purification of the FCBP

PM vesicles (5 mg) were suspended in 5 mL of solution E. After FCBio was added to the solution (final concentration,

10⁻⁶ M), the PM vesicles were incubated for 2 h at 30°C to allow binding of FCBio to the FCBP. Thereafter, the vesicles were pelleted at 100,000g for 30 min at 4°C and resuspended in solution G (20 mM Tris-HCl [pH 7.5], 20% [v/v] glycerol, 5 mM MgSO₄, 1 mM CaCl₂, 150 mM NaCl, 2.6 mM DTT, 1 mM PMSF, and 1.4% [w/v] OG; 1:20 [w/w] detergent/protein ratio). The suspension was vortexed, sonified for 3 min (Branson 2200 bath), incubated for 20 min on ice, and centrifuged for 40 min at 100,000g and 9°C. The supernatant was dialyzed (Visking tube, molecular mass cutoff of 8 kD at 4°C) against solution D plus 0.05% (w/v) BSA but without OG. After equilibrium was reached the solution was replaced by the same solution but with 0.2% OG. The dialysis steps were performed to reduce the detergent concentration to a final concentration of 0.2% (w/v) and to remove the remaining NaCl. The dialysate was centrifuged for 30 min at 100,000g and 4°C to remove precipitated proteins. The supernatant (containing the FCBP-FCBio complex) was used for affinity chromatography with monomeric immobilized avidin preequilibrated in solution D. The proteins were pumped overnight through the column at a flow rate of 0.1 mL/min using a closed loop; the total number of cycles was 15. Although binding between avidin and biotin is normally fast, a long exposure time was chosen because of the reduced affinity between biotin and monomeric avidin. Thereafter, the column was washed with solution D until a stable baseline was obtained. The column was eluted with 2 mM biotin in solution D, and fractions of 0.4 mL were collected just before and during elution. The fractions were analyzed on SDS-PAGE and screened for [³H]dihydroFC-binding activity. All affinity chromatography steps were carried out at 4°C.

Triton X-114 Extraction

The temperature-induced phase separation with Triton X-114 (Bordier, 1981) was applied to solubilized PM proteins according to the method described by Hicks et al. (1993). Briefly, 1 mg of PM vesicles was pretreated with 10⁻⁶ M FCBio and solubilized as described in the previous section. The solubilized proteins were diluted seven times with Tris-salt buffer (10 mM Tris-HCl [pH 7.6] and 150 mM NaCl). To 1 mL of the sample, Triton X-114 was added to give a final concentration of 2% (v/v), and the sample was incubated on ice for 5 min. After centrifugation (58,000g for 15 min at 4°C), 500 µL of the supernatant was layered onto a 500-µL Suc cushion buffer (0.25 M Suc plus 0.06% Triton X-114 in Tris-salt buffer), incubated for 5 min at 30°C, and centrifuged at 2500g for 15 min at 25°C. The upper aqueous phase was re-extracted with 1% Triton X-114 and layered onto the previous lower detergent phase after incubation on ice for 5 min. The sample was incubated at 30°C for 5 min and centrifuged as described above. Both the resulting upper aqueous phase and the lower detergent phase were analyzed on SDS-PAGE and/or screened for FC-binding activity.

Tricine SDS-PAGE and Western Blotting

One-dimensional gel electrophoresis and western blotting were carried out on a Bio-Rad Modular Mini Electrophoresis System. PM proteins and purified FCBP were analyzed by

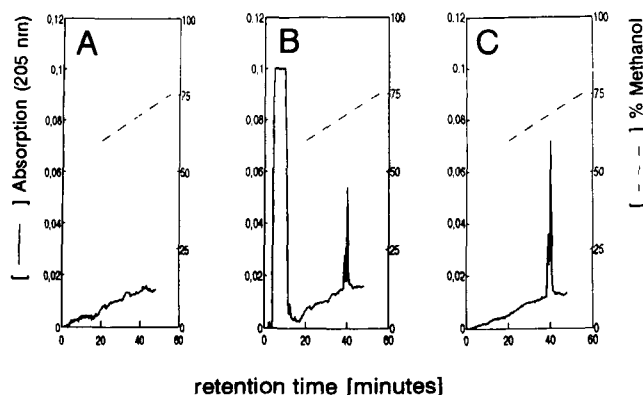


Figure 1. HPLC detection of FCBio in the fractions obtained after affinity chromatography of a sample of FCBio in solution D on a 0.4-mL monomeric avidin column. A, Column fraction; B, eluent fraction; C, control (5 nmol of FCBio in chromatography buffer). The gradient was 60 to 75% methanol in H₂O during 35 min after removal of salts at 5% methanol (see "Materials and Methods"). All chromatograms are corrected for baseline and salt interferences.

Tricine SDS-PAGE under reducing conditions according to the method of Schägger and Von Gadow (1987). A 10% acrylamide running gel with a 4% acrylamide stacking gel was used. Before loading, samples of 25 μ L were heated for 10 min at 80°C in the presence of 60 mM Tris (pH 6.8), 1% SDS, 10% glycerol, 20 mM DTT, and 0.02% bromophenol blue. Electrophoresis conditions were as follows: 1 h, 200 V. Gels were stained with silver (Bio-Rad silver staining kit) or Serva Blue G.

Western blotting was performed on nitrocellulose membranes and 25 mM Tris, 192 mM Gly, 20% (v/v) methanol (pH 8.3) as the transfer buffer. Transfer conditions were as follows: overnight 30 V, 40 mA, room temperature. For detection of biotinylated proteins, the blots were blocked with blocking buffer (Superblock, Pierce) for 30 min, rinsed five times with 20 mM Tris-HCl (pH 7.0), and incubated with horseradish peroxidase-conjugated streptavidin (20 μ g/blot) in 20 mM Tris-HCl (pH 7.0) and 150 mM NaCl for 1 h. Blots were rinsed with 20 mM Tris-HCl (pH 7.0) and stained with 0.224 mM 4-chloronaphthol/3,3'-diaminobenzidine tetrahydrochloride in stable peroxide substrate buffer (Pierce).

Protein and FC-Binding Assay

The protein concentration was determined with bicinchoninic acid protein assay reagent (Pierce) with BSA as the standard. Before protein concentrations were measured, samples were subjected to TCA precipitation according to the method of Brown et al. (1989).

Binding assays were performed with [³H]dihydroFC with the following binding conditions: 1 h, 30°C, 3×10^{-9} M [³H]-dihydroFC in solution E for PM vesicles; 4 h, 30°C, 3×10^{-9} M [³H]dihydroFC in solution D for solubilized proteins; 4 h, 30°C followed by 12 h, 4°C, 10^{-7} M [³H]dihydroFC in solution D for solubilized proteins labeled with FCBio. Radioactivity was measured in a LKB Wallac Rackbeta 1219 scintillation counter using Pharmacia OptiPhase HiSafe 3 scintillation cocktail.

RESULTS

Properties of the FCBio

To demonstrate the potential of the avidin-biotin technology for the purification of plant cell receptors, a number of requirements should be met. One important requirement is the ability of the newly synthesized and purified bifunctional ligand, FCBio, to interact with the monomeric avidin. Therefore, FCBio was applied to the monomeric avidin column, washed, and eluted as described in "Materials and Methods." HPLC analysis of the wash and eluted fractions showed that in the wash fraction no peaks could be detected (Fig. 1A), whereas in the eluted fraction (Fig. 1B) a peak was observed with the same pattern and at the same retention time as that in the FCBio (control; Fig. 1C). Peak integration revealed that approximately 70% of the initial amount of FCBio applied to the column was eluted. This shows that FCBio can bind to the avidin column and can be eluted with an excess of biotin.

The ability of FCBio to bind to the FCBP of oat root is a second important requirement, and this was examined in a number of ways. The cellular H⁺ efflux, one of the physiological effects induced by binding of the ligand to the binding protein, was studied *in vivo* using a bioassay. To this end, different concentrations of FCBio and FC were added to abraded coleoptiles (60 mg fresh weight) and the decline of the pH of the medium was measured after incubation. Addition of both FCBio and FC at a concentration greater than 10^{-8} M induced an H⁺ efflux (Fig. 2). Untreated coleoptiles as well as coleoptiles treated with 10^{-5} M biotin acidified the medium only slightly (0.1 pH unit). The presence of a biotin group on the FC molecule had no significant effect on the *in vivo* activity of FC.

In *in vitro* experiments we determined the ability of FCBio to compete with [³H]FC for binding and to form the entire FCBP-FCBio-avidin complex. From ligand competition experiments with [³H]dihydroFC we calculated a K_D of FCBio of 70 nM, which is lower than that of unlabeled FC ($K_D = 3$ nM) (data not shown).

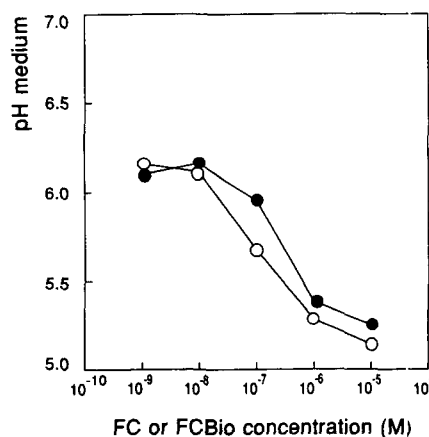


Figure 2. Acidification of 2 mL of incubation medium (initial pH 6.25) caused by H⁺ efflux of 5-d-old abraded coleoptiles (60 mg fresh weight) after addition of either FC (O) or FCBio (●) at different concentrations. The coleoptiles were incubated at 23°C and agitated for 90 min in the dark.

Because the K_D of FCBio is still relatively small we determined the displacement of bound FCBio by labeled FC, which is of relevance to the measurable binding activity in the purification steps. Time-dependent displacement of bound FCBio at 30°C with an excess of [3 H]dihydroFC (10^{-7} M) was slow; in 4 h only 25% displacement had occurred (Fig. 3). Incubation for a longer period (overnight) did not result in a higher degree of displacement, and at 4°C displacement was negligible. This indicates that during our purification procedure (performed at 4°C) FCBio remains tightly bound to the FCBP.

The formation of the FCBP-FCBio-avidin complex was investigated using fluorescein-labeled avidin. Purified PM vesicles were incubated with either FC or FCBio prior to addition of fluorescein-labeled avidin. Figure 4 shows the fluorescence of FC-pretreated vesicles before (Fig. 4A) and after (Fig. 4B) exposure to avidin-fluorescein. Incubation of these vesicles with avidin-fluorescein had no significant effect on the fluorescence. However, with FCBio-pretreated vesicles there was a significant increase in fluorescence after exposure to avidin-fluorescein (Fig. 4, C and D). The increase in the fluorescence of FCBio-pretreated vesicles after addition of avidin-fluorescein compared to FC-pretreated vesicles demonstrates that the FCBP-FCBio-avidin complex is indeed formed *in vitro*.

Solubilization and First Step of the Purification of the FCBP

Typically, 5 mg of purified PM vesicle (protein) were pretreated with FCBio, followed by solubilization as described in "Materials and Methods." Thereafter, the binding activity was measured in the soluble fraction and the pellet. Seventy percent of the initial activity was found in the soluble fraction, and 30% was in the pellet (the same results were obtained with untreated vesicles). The soluble fraction was dialyzed to decrease the detergent (OG) concentration from

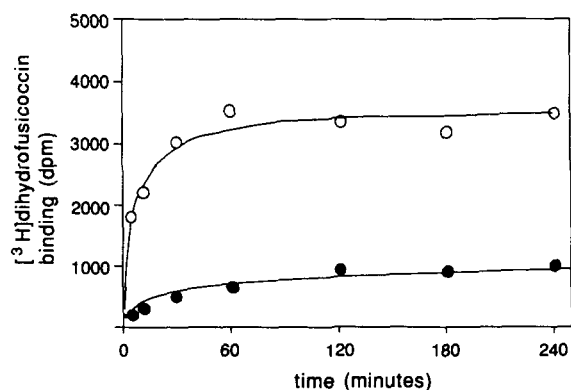


Figure 3. Time course of [3 H]dihydroFC (10^{-7} M) binding to solubilized PM proteins (5 μ g) pretreated for 4 h with FCBio (10^{-6} M) (●) and pretreated in the absence of FCBio (○) at 30°C. Unbound FCBio was removed by washing and pelleting the PM vesicles prior to solubilization. All values are corrected for nonspecific binding. At 30°C, displacement of FCBio is slow (25% after 4 h). At 4°C, no displacement could be observed even after a prolonged period of incubation (2 d).

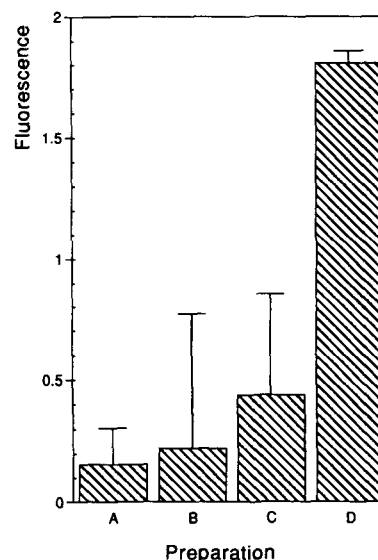


Figure 4. Fluorescence of PM vesicles (means \pm SD) after pretreatment with FC or FCBio, followed by coupling to fluorescein-conjugated avidin. A and B, Fluorescence of FC-pretreated vesicles before (A) and after (B) coupling to avidin-fluorescein; C and D, fluorescence of FCBio-pretreated vesicles before (C) and after (D) coupling to avidin-fluorescein. Unbound avidin-fluorescein was removed by washing and pelleting the PM vesicles.

1.4 to 0.2% (w/v) and to remove the NaCl. Decreasing the OG concentration below its critical micelle concentration resulted in a precipitation of hydrophobic membrane proteins. About 70% of the solubilized proteins were precipitated in this way. An SDS-PAGE gel of the precipitated and soluble membrane proteins is shown in Figure 5A. The pattern of the protein bands of the soluble fraction (lane b) is quite different from the pattern of the precipitated fraction (lane c). This means that the precipitation of proteins during dialysis is not due to partial precipitation of all of the PM proteins but to precipitation of a number of specific proteins with a hydrophobic character. Pretreatment of the PM vesicles with 10^{-6} M FCBio before solubilization did not affect this precipitation: the pattern of protein bands of the soluble and precipitated fractions (lanes b and c) of FCBio-pretreated and solubilized proteins is similar to that of untreated proteins (lanes e and f). A binding assay showed that 77% of the initial binding activity of FCBio-pretreated proteins was found in the soluble fraction after dialysis, and 23% was found in the precipitated fraction (Fig. 5B). When the FC-binding site on the FCBP was not occupied with FC or FCBio before solubilization, only 3% of the initial binding capacity was found in the soluble fraction, and 7% was in the precipitated fraction. This indicates that the binding site is ligand stabilized.

To confirm the "nonhydrophobic" behavior of the FCBP during dialysis in low detergent concentrations, FCBio-pretreated solubilized PM proteins were subjected to temperature-induced phase separation with Triton X-114 according to the method of Bordier (1981). Extraction of these vesicles with Triton X-114 resulted in an aqueous phase containing

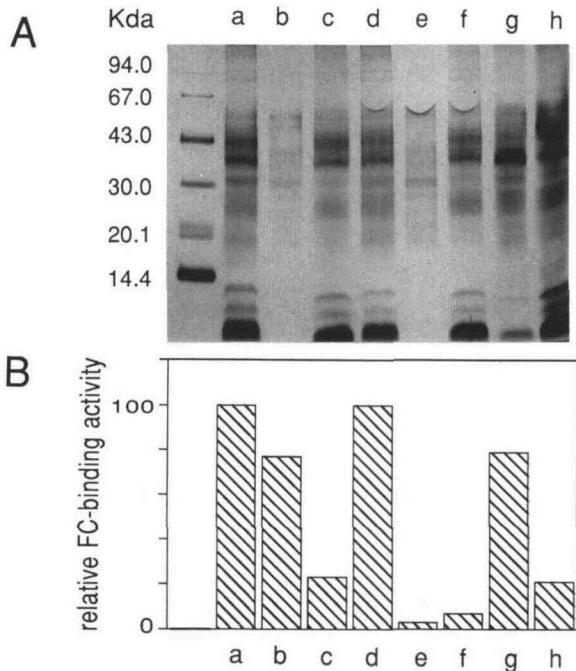


Figure 5. A, SDS-PAGE gel of FCBio-pretreated solubilized PM proteins in the soluble (lane b) and precipitated fraction (lane c) and untreated solubilized PM proteins in the soluble (lane e) and precipitated fraction (lane f) after dialysis. Lanes a and d are solubilized PM proteins before dialysis with (lane a) or without (lane d) pretreatment with FCBio. Triton X-114 phase separation of FCBio-pretreated PM proteins resulted in an aqueous phase (lane g) and a detergent phase (lane h). All lanes are stained with silver. Molecular mass markers are: phosphorylase b (94.0 kD), BSA (67.0 kD), ovalbumin (43.0 kD), carbonic anhydrase (30.0 kD), soybean trypsin inhibitor (20.1 kD), and α -lactalbumin (14.4 kD). B, [3 H]DihydroFC-binding activity of the above-described fractions expressed in percentages of the initial binding activity (before dialysis or Triton X-114 phase separation). The initial binding activities are 1.12×10^6 dpm for FCBio pretreated and 4.16×10^6 dpm for untreated solubilized PM proteins. All values are corrected for nonspecific binding.

membrane proteins with a more hydrophilic character (lane g) and a detergent phase containing hydrophobic membrane proteins (lane h). In a binding assay we demonstrated that 79% of the total binding activity was found in the aqueous phase and only 19% was found in the detergent phase (Fig. 5B).

Avidin-Biotin Affinity Chromatography

Proteins remaining in the soluble fraction after dialysis (1.5 mg) were finally subjected to avidin-biotin affinity chromatography. Fractions of 0.4 mL were collected just before and during elution with biotin. These fractions were analyzed on SDS-PAGE and screened for binding activity. Silver staining of the gel revealed a major protein band with an apparent molecular mass of 31.0 kD and a minor band of 30.0 kD in the fractions eluted with biotin (lanes F2–F5, Fig. 6A). This confirms the earlier findings of De Boer et al. (1989). [3 H]FC-binding activity was demonstrated to be present in the

fractions that contained both polypeptides (Fig. 6B). We calculated that the specific [3 H]FC-binding activity of the avidin-biotin-purified FCBP is approximately 400 pmol/mg protein. The specific [3 H]FC-binding activity of solubilized PM proteins pretreated with FCBio is approximately 2.7 pmol/mg protein. This means that purification using this novel affinity technique resulted in a 150-fold enrichment of the FCBP.

Two additional experiments were done to test the specificity of this isolation method and the possibility that native biotinylated polypeptides (Baldet et al., 1991; Wurtele and Nikolau, 1992) were purified. First, solubilized PM proteins without FCBio added were pumped through the avidin column and eluted with biotin. No proteins were eluted and no binding activity was measured in the eluted fractions (data not shown). Second, PM vesicles and solubilized PM proteins were subjected to SDS-PAGE, followed by western blotting. The nitrocellulose blots were stained for detection of biotinylated proteins with horseradish peroxidase-conjugated streptavidin. Despite the high sensitivity of this detection system, no biotinyl groups were detected (data not shown). This excludes the possibility that biotinylated proteins other than the FCBio-FCBP complex co-purified.

DISCUSSION

In the past 15 years, purification schemes for isolation and purification of hormone receptors in animal tissues have been developed, based on the avidin-biotin system using a bifunctional ligand. The use of a monomeric avidin column offers

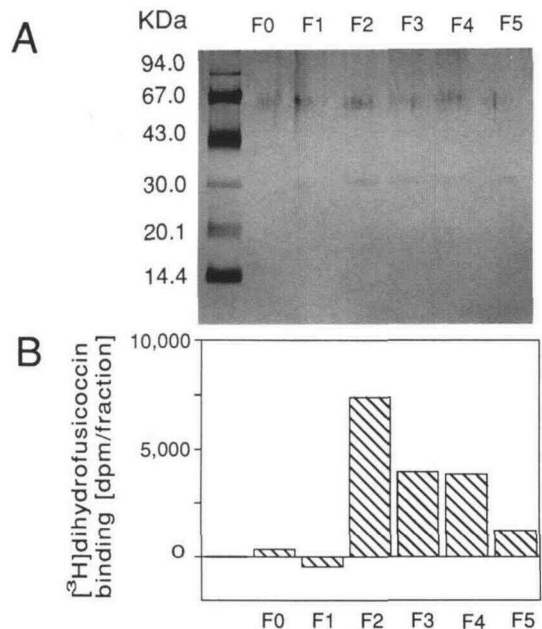


Figure 6. Elution of the FCBP-FCBio complex from a 1-mL monomeric avidin column with 2 mM biotin in solution D. A, SDS-PAGE gel of fractions (0.5-mL fraction size) collected just before (F1 and F0) and during (F1–F5) elution with biotin. The gel was silver stained. B, Total binding activity of the fractions described above, corrected for nonspecific binding.

the advantage of binding the biotinylated ligand-receptor complex with high specificity to the affinity column and eluting the complex under mild conditions in a specific way using an excess of biotin. To apply this technique to the purification of the FCBP from PMs of oat roots using FCBio, a number of demands had to be met. We have demonstrated that the FCBio could act as a bifunctional ligand: (a) it bound to the monomeric avidin column and specifically eluted under mild conditions and (b) the presence of a biotin moiety hardly affected the binding of the ligand to the binding protein in vivo as well as in in vitro experiments. Nevertheless, the most critical step in our purification procedure was the formation of the entire FCBP-FCBio-avidin complex; steric hindrance might have prevented binding of the FCBP-FCBio complex to avidin. The length of the biotinyl group of FCBio, with spacer arm included, is about 24.7 Å. The biotin-binding site of avidin is reported to be 9 Å below the surface of the avidin molecule (Green et al., 1971). The question arises as to whether the length of the spacer arm is sufficiently long to fit both into the binding pocket of the avidin molecule and into the pocket of the FCBP, the depth of which is unknown. Because the biotinyl groups are able to interact with different kinds of avidin derivatives, it was possible to study the formation of the complete FCBP-FCBio-avidin complex using a fluorochrome-conjugated avidin. This fluorometric experiment, shown in Figure 4, demonstrated a significant increase of the fluorescence of FCBio-pretreated PM vesicles after exposure to fluorescein-conjugated avidin as compared to FC-pretreated vesicles. This experiment demonstrates the formation of the FCBP-FCBio-avidin complex on PM vesicles, but because of the lack of quantitative data this experiment does not give information about the efficiency of binding.

A number of reports point to the hydrophobic character of the FCBP. De Boer et al. (1989) reported that the FCBP solubilized from oat root interacts with a hydrophobic hexyl agarose matrix. Weiler et al. (1990) concluded from experiments with a lipophilic homobifunctional cross-linker that the FCBP is an integral membrane protein, and the observation that the FCBP is not released from the PM by a low- or high-concentration salt wash (A.H. De Boer, unpublished results) also suggests that the FCBP is an integral membrane protein. However, not all integral membrane proteins are necessarily hydrophobic in nature. This is demonstrated for the FCBP in Figure 5, where 70% of the detergent-solubilized proteins precipitated but only 23% of the FCBP was precipitated when the detergent concentration was reduced below the critical micelle concentration. Also in Triton X-114, a detergent with a temperature-induced phase separation, the FCBP was found predominantly in the aqueous phase. According to Bordier (1981) hydrophobic integral membrane proteins partition into the detergent-rich (Triton X-114) phase, whereas the more hydrophilic peripheral membrane proteins partition into the aqueous phase. We conclude that either the FCBP is an integral membrane protein with relatively few membrane-spanning domains or, due to glycosylation (Aducci et al., 1984; Meyer et al., 1989), the overall character of the FCBP is more hydrophilic, as shown by Pryde and Phillips (1986) for other glycosylated integral membrane proteins.

During dialysis, we found a strong reduction in binding

activity when the binding site was ligand free (Fig. 5B), a reduction that might be due to protease activity. However, we found that the Ser protease inhibitor PMSF (and chymostatin to a lesser extent) was a very good protease inhibitor in oat root PM and the most effective inhibitor in preventing loss of binding activity of the solubilized FCBP of the series of protease inhibitors that we tested (leupeptin, pepstatin, E64, chymostatin, or combinations thereof; P.C.J. van der Hoeven, A.H. De Boer, unpublished results). Possibly during dialysis, a reactive SH group in or near the binding pocket is oxidized (despite the presence of DTT), since we observed that Hg⁺ ions will strongly reduce the binding capacity of ligand-free FCBP but have no effect when a ligand is present.

During avidin-biotin affinity chromatography we eluted a major protein band with a molecular mass of 31.0 kD and a smaller band of approximately 30.0 kD on SDS-PAGE under reducing conditions. The molecular masses of these proteins are comparable to those reported earlier with other purification protocols. De Boer et al. (1989) purified a 31.0- and a 29.7-kD polypeptide from PM of oat roots using affinity chromatography. Oecking and Weiler (1991) reported two polypeptides of apparent molecular masses of 30.5 and 31.6 kD purified from PMs from *Commelina communis*. Photoaffinity labeling with a tritiated azido analog of FC resulted in a 34-kD polypeptide in PMs from *Arabidopsis thaliana* (Meyer et al., 1989) and a 35-kD polypeptide in PMs from *Vicia fabia* (Feyerabend and Weiler, 1989). Aducci et al. (1993) reported that the native molecular mass of the FC receptors (in maize shoots) is 90 kD and suggested that the 31-kD doublet that we and other investigators found is a product of degradation of the 90-kD protein. Furthermore, these authors postulated that the protein is a 31-kD doublet on SDS-PAGE and is not able to bind [³H]FC. However, our fractions obtained after elution with biotin were screened for binding activity with [³H]FC. In all fractions containing the 31-kD polypeptide on SDS-PAGE, binding activity was demonstrated (Fig. 6). The total binding activity is about 4-fold higher because of incomplete displacement of bound FCBio with [³H]FC (Fig. 3). Purification of the FCBP using this protocol allows a 150-fold increase of specific binding activity compared to crude solubilized PM proteins. On the basis of these data and assuming that the protein is pure, we can deduce that approximately 0.7% of the total amount of PM proteins from oat roots is FC-binding sites. This number of FC-binding sites corresponds with the number of FC-binding sites reported by De Boer et al. (1989), 0.4% in oat roots, and by Abramycheva et al. (1991), 0.6% in mesophyll protoplasts of *Vicia faba*. The yield of FCBP is still rather low, and preliminary experiments using fluorescent FC derivatives (P.C.J. van der Hoeven, unpublished results) indicate that this may be due to the deep location of the binding pocket for FC in the FCBP. To test this hypothesis the spacer arm between FC and biotin will be extended.

The purification scheme reported here offers a convenient method for the isolation of highly purified and active FCBP. The versatility of this technique was recently demonstrated with the isolation of a number of GTPase proteins from the PM of oat roots by means of the bifunctional ligand GTP-biotin (De Boer et al., 1994b).

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