Planta (1989) 178:282–290

Planta

Photoaffinity labeling and partial purification of the putative plant receptor for the fungal wilt-inducing toxin, fusicoccin

Martin Feyerabend and Elmar W. Weiler*

Arbeitsgruppe Pflanzenphysiologie, Fachbereich Biologie/Chemie, Universität Osnabrück, Postfach 4469, D-4500 Osnabrück, Federal Republic of Germany

Abstract. The high-affinity fusicoccin-binding protein (FCBP) was solubilized from plasma-membrane vesicles prepared from leaves of *Vicia faba* L. by aqueous two-phase partitioning. Conditions for the solubilization of intact FCBP-radioligand complexes were worked out. About 60–70% of the complexes can be solubilized with 50–60 mM nonanoyl-N-methylglucamide in the presence of 1 mg·ml^{-1} soybean phosphatidylcholine, type IV S, and 20% (v/v) glycerol at pH 5.5. The slow dissociation of the radioligand, 9′-nor-fusicoccin-8′-alcohol-[^{3}H] from the FCBP at low temperatures permits the purification of FCBP-radioligand complexes at 4–10° C by fast protein liquid chromatography on anion-exchange and gel permeation columns. The FCBP, extracted from plasma membranes with cholate and chromatographed in the presence of this detergent, gave an apparent molecular mass (M_r) of 80 ± 20 kDa on gel permeation columns under the conditions used. By comparison of the elution profiles of the fraction most enriched in FCBP-radioligand complexes with polypeptide patterns obtained on sodium dodecyl sulfate-polyacrylamide gels, a polypeptide with an M_r of approx. 34 kDa co-separated with the radioactivity profile. A second, faint band of approx. 31 kDa was sometimes also observed co-electrophoresing. Photoaffinity labeling of plasma-membrane vesicles with the new compound 9′-nor-8′[(3,5-[^{3}H]-4-azidobenzoyl)ethylenediamine]-fusicoccin ([^{3}H]ABE-FC) and subsequent separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis labeled a single band with an M_r of 35 ± 1 kDa. Labeling in this band was strongly reduced when the membranes were incubated with [^{3}H]ABE-FC in the presence of 0.1–1 μM fusicoccin. From our data, we conclude (i) that the 34–35-kDa polypeptide represents the FCBP and (ii) that in detergent extracts of plasma membranes this polypeptide is probably present as a di- or trimeric structure.

Key words: Fusicoccin – Fusicoccin-binding protein – Photoaffinity labeling – *Vicia*

Introduction

The action of the *Fusicoccum amygdali* Del. toxin, fusicoccin (FC) on cells of higher plants involves a drastic acidification of the apoplastic space concomitant with a strong hyperpolarization of the plasma-membrane potential. As a consequence, ion and metabolite fluxes across the plasma membrane are altered, the osmotic potential of the cell increases and the cell-wall plasticity decreases, leading to increases in growth rate in many plant tissues (for review see Marré 1979). The understanding of the molecular events of FC action will provide insights into such key processes of plant cell function as the regulation of plasma-membrane energetization and ion and metabolite transport across the cell membrane. The mechanism of FC action, however, is still unknown, but might involve the stimulation of H^+-extrusion across the plasma membrane (Rasi-Caldogno et al. 1986) and – or the inhibition of leak currents by the toxin

* *Permanent address:* Lehrstuhl für Pflanzenphysiologie, Ruhr-Universität Bochum, Fakultät für Biologie, Postfach 102148, D-4630 Bochum 1, FRG

Abbreviations: ABE-FC = [(4-azidobenzoyl)-ethylenediamine]-fusicoccin; ABE-NHS = (4-azidobenzoyl)-N-hydroxysuccinimide ester; FC = fusicoccin; FCBP = fusicoccin-binding protein; FCol = 9′-norfusicoccin-8′-alcohol; MAB = monoclonal antibody; Mega-9(10) = nonanoyl(decanoyl)-N-methylglucamide; M_r = apparent molecular mass; PMSF = phenylmethylsulfonyl fluoride; SDS-PAGE = sodium dodecyl sulfate – polyacrylamide gel electrophoresis; TCA = trichloroacetic acid; Tris = 2-amino-2-(hydroxymethyl)-1,3-propanediol

(Blatt 1988). Kinetic studies have revealed that FC binds rapidly and with high affinity to microsomal sites (Dohrmann et al. 1977; Ballio et al. 1980; Stout and Cleland 1980; De Boer et al. 1987; Feyerabend and Weiler 1988). These sites are associated with the plasma membrane (Ballio 1982; Feyerabend and Weiler 1988), with the FC-binding domain facing the apoplastic space (Feyerabend and Weiler 1988). Chemical and enzymatic studies indicate that the binding sites are glycoproteins (Aducci et al. 1984; Feyerabend and Weiler 1988). There is a significant correlation of the biological properties of FC and related structures and their behaviour in cell-free assays of the fusicoccin-binding protein (FCBP) (Ballio et al. 1981a, b; Feyerabend and Weiler 1988). The sum of all available data indicates that the FCBP is the plant's toxin receptor. However, the FCBP has not been identified biochemically to date and methods for its isolation are lacking. In this report, we describe the synthesis, properties and use of a tritium-labeled, biologically active azido-analogue of FC. This compound is used to irreversibly target by FCBP and to identify it on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The data are compared with those obtained from the partial purification of the FCBP (as the reversible FCBP-radioligand complex), using 9′-nor-fusicoccin-8′-alcohol-[^{3}H]([^{3}H]FCol) (Feyerabend and Weiler 1988) as the radioligand. In both cases, a polypeptide with an apparent relative molecular mass (M_r) of 34–35 kDa was identified as the FCBP.

Material and methods

Chemicals and equipment. Cholate was purchased form Serva, Heidelberg, FRG, dextran T 500 from Pharmacia, Freiburg, FRG, and 4-azidobenzoic acid N-hydroxysuccinimide ester from Pierce, Rockfield, Illinois, USA. Nonanoyl-N-methyl glucamide (Mega-9) was from Oxyl Chemie, Bobingen, FRG and polyethylene glycol 3350 from Union Carbide, Düsseldorf, FRG. The fast protein liquid chromatography (FPLC) equipment consisted of two pumps P 500, gradient programmer GP 250, monitor UV 1, valve MV-7 with Superloop 50 ml and fraction collector FRAC 100 (all from Pharmacia). Fractogel TSK DEAE 650(S) and Fractogel TSK 55-W(S) were obtained from Merck, Darmstadt, FRG. All other gels were from Pharmacia.

Preparation of membrane vesicles. Microsomal membranes were prepared from leaves of three-week-old *Vicia faba* L. as described previously (Blum et al. 1988; Feyerabend and Weiler 1988). Plasma-membrane vesicles were enriched from the microsomal fraction by partitioning between dextran T 500 and polyethyleneglycol 3350 (Larsson et al. 1984; Blum et al. 1988; Feyerabend and Weiler 1988). Membrane protein was determined by the Bradford assay (Bradford 1976) using bovine serum albumin (BSA) as the standard.

Detergent extraction of membrane proteins. Microsomal or plasma-membrane vesicles were, if not stated otherwise, solubilized under the following conditions (final concentrations): protein 0.6–0.8 mg·ml^{-1} in 20% (v/v) glycerol, 1 mg·ml^{-1} soybean phosphatidylcholine, type IV-S (Sigma, Dreieich, FRG; purified as in Cook et al. 1986), 60 mM Mega-9, 1-mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM ethylene diaminetetraacetic acid (EDTA), 1 mM KF, 0.2 mM phenyl methylsulfonyl fluoride (PMSF), 2.6 mM dithiotreitol (DTT), 10 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol(Tris)/2-(N-morpholino)ethane sulfonic acid (Mes), pH 5.5. The samples were sonicated for 1 min in a sonication bath and then centrifuged for 10 min at 4° C and 50000·*g*. The supernatants were then immediately diluted with a 1.5- to 3-fold volume of 20 mM Tris-HCl, 5 mM cholate, pH 8.5.

Prior to solubilization, the membrane vesicles were, if not stated otherwise, incubated in the presence of 10 nM [^{3}H]FCol or 1 nM 9′-nor-8′[(3,5-[^{3}H]-4-azidobenzoyl)ethylene diamine]-fusicoccin([^{3}H]ABE-FC at a protein concentration of 0.1 mg·ml^{-1} as described for [^{3}H]FCol (Feyerabend and Weiler 1988). The vesicles were then pelleted at 4° C (30 min, 50000·*g*) and the pellets resuspended for solubilization. Unspecific binding was always checked by co-incubation of the radiotracers with an excess (0.1–1 μM) of unlabeled FC (Feyerabend and Weiler 1988) and all data, unless otherwise indicated, were corrected for unspecific binding.

Assay of solubilized FCBP. The diluted detergent extracts (protein concentration 1–5 mg in 5–10 ml volume) were loaded at a flow rate of 0.5 ml·min^{-1} on a small column (60 mm long, 9 mm i.d.) filled with 1 ml Fractogel TSK DEAE 650(S) pre-equilibrated in 20 mM Tris-HCl, 5 mM cholate, pH 8.5. After loading, the column was washed with 5 ml of the same buffer. This removed the free, uncharged ligand. The protein was then quantitatively eluted with 2 ml 1 M KCl in 20 mM Tris-HCl, 5 mM cholate, pH 8.5. After the addition of 10 ml scintillation cocktail, the samples were counted in a scintillation spectrometer. All data were corrected for counting efficiency using internal standardization with [^{3}H]toluene.

Purification of the FCBP-[^{3}H]FCol complexes from plasma membranes of Vicia faba. All steps were performed at 0–4° C and as quickly as possible. For the bulk purifications, only one instead of three phase separation steps was carried out (Feyerabend and Weiler 1988). The resulting, almost chlorophyll-free vesicles (approx. 15 mg of protein), were solubilized as described above and, after dilution with 20 mM Tris-HCl, 5 mM cholate, pH 8.5 (=buffer A) to a final volume of 50 ml, loaded at a flow rate of 1 ml·min^{-1} on a Fractogel TSK DEAE 650(S) column (20 cm long, 1.0 cm i.d.; gel bed volume, 15 ml), equilibrated with buffer A. After washing with buffer A until no more protein was detected in the effluent, a linear gradient from 0 to 40% buffer B (20 mM Tris-HCl, 5 mM cholate, 1 M KCl, pH 8.5) was applied over 40 min, followed by a gradient from 40 to 100% buffer B in 10 min. Fractions were collected at 1-min intervals (flow rate=1 ml·min^{-1}). The fractions containing the FCBP-radioligand complexes were pooled, diluted to 15 ml with buffer A and reapplied at a flow rate of 1 ml·min^{-1} to a Pharmacia Mono Q column (HR 5/5) equilibrated in buffer A. The elution profile was the same as in the first elution step (flow rate=1 ml·min^{-1}, fraction size=1 ml). The active fractions were again pooled, diluted to 15 ml and rechromatographed on the same matrix. The active fractions were pooled (1.9 ml) and applied directly onto a Pharmacia Superose 12 (HR 10/30) column. The column was eluted with buffer A (0.5 ml·min^{-1}) and the active fractions were concentrated in a SpeedVac concentrator. The samples were redissolved in sample buffer according to Laemmli, containing 4% SDS and

checked by SDS-PAGE (Laemmli 1970) using 11% gels. Protein was stained with Coomassie Brilliant Blue R-250.

Synthesis of 9'-nor-8'-[(3,5-[^{3}H]-4-azidobenzoyl)ethylenediamine]-fusicoccin. The 9'-nor-8'-aldehyde intermediate of FC was prepared and checked for purity as described previously (Feyerabend and Weiler 1987, 1988). The aldehyde (4 µmol, 2.7 mg), dissolved in 0.05 ml methanol was mixed with 0.02 ml of a saturated, aqueous solution of ethylenediamine, adjusted to pH 6.5, followed by the addition of 0.02 ml H_2O and approx. 2 mg $NaCNBH_3$. After 12 h at 4° C, the volume of the reaction mixture was adjusted to 2 ml with water and the solution was passed through a methanol-washed, then water-equilibrated SepPak C_{18} column (Millipore, Eschborn, FRG). The column was washed with 3 ml H_2O, then eluted with 1–2 ml methanol. The derivative chromatographed in *iso*propanol:acetic acid: chloroform = 85: 10:5 (by vol.) at an R_f of 0.1 (FC-aldehyde: $R_f = 0.7$, ethylenediamine: $R_f = 0$) and gave the characteristic FC-specific fluorescence after spraying with dilute methanolic sulphuric acid and heating (Feyerabend and Weiler 1988) as well as a positive reaction with ninhydrin, indicating the presence of a free amino group. The reaction product (abbreviated FC-EDA thereafter) is stable only for a few days when kept at −18° C or lower temperature in the dry state.

The FC-EDA (0.12 µmol, 0.1 mg), dissolved in 0.01 ml methanol was added to 3,5-[^{3}H]-4-azidobenzoic acid-N-hydroxisuccinimide ester (2.78 nmol, $1.35 \cdot 10^{15}$ Bq·mol^{-1}, Amersham, UK), dissolved in 0.02 ml dioxane. Finally, 0.1 ml of 25 mM potassium phosphate-buffer, pH 6.5, was added and the reaction allowed to proceed for 24–30 h at 4° C. The reaction product, 9'-nor-8'-[(3,5-[^{3}H]-4-azidobenzoyl)ethylenediamine]-FC, ([^{3}H]ABE-FC) was isolated by thin-layer chromatography (TLC) on Silica gel in the solvent $CHCl_3$: *iso*propanol = 6:4 (v/v) (R_f, ABE-FC = 0.31, R_f, FC-EDA = 0, R_f, ABE-NHS = 0.86) and eluted with methanol (yield 10–15%, by radioactivity). The structure of ABE-FC was confirmed by fast atom bombardment (FAB+) mass spectroscopy using a VICHY ZAB-E instrument and glycerol/dimethylsulfoxide as matrix: m/z 873 $[M+H]^+$, calculated for $C_{44}O_{13}N_5H_{65} = 872$.

Photoaffinity labeling. Microsomal or plasma-membrane vesicles were incubated as described by Feyerabend and Weiler (1988), but using 1 nM[^{3}H]ABE-FC and 0.1 mg·ml^{-1} of protein (total volume = 1 ml, total radioactivity = 1332 Bq). Unspecific binding was determined from samples incubated additionally with 1 µM unlabeled FC. After 1 h of incubation at 25° C, the membranes were pelleted at 80000·*g* (30 min, 4° C) and the pellets resuspended in 1 ml of ice-cold 10 mM potassium phosphate buffer, 150 mM NaCl, pH 7.4 (phosphate-buffered saline), followed by irradiation, in quartz cuvettes, with a HBO 500-W high-pressure arc lamp (Osram, München, FRG), quartz collector and Schott WG 305, 1-mm-thick, long-pass filter. Samples were placed at a distance of 15 cm from the collector's front lens. Protein was then precipitated with trichloroacetic acid (TCA) (10% w/v final concentration) at 0° C and collected on Schleicher & Schuell, Dassel, FRG GF6-glass-fibre filters presoaked in 5% TCA. The dried filters were immersed in scintillation cocktail and counted after equilibration.

For SDS-PAGE, 1 mg of irradiated membrane protein (or an equivalent of 0.1 ml ascites fluid of monoclonal antibody (MAB) 19-VI-A5 (Feyerabend and Weiler 1987) was precipitated with a five fold volume of chilled acetone at −80° C for 30 min. The washed pellets were redissolved in sample buffer containing 4% SDS and subjected to SDS-PAGE (Laemmli 1970).

Bioassays. The biological activity of FC or the FC-derivatives synthesized was determined as the stimulation of stomatal opening in the dark in isolated epidermal strips of *Vicia faba* (Blum et al. 1988).

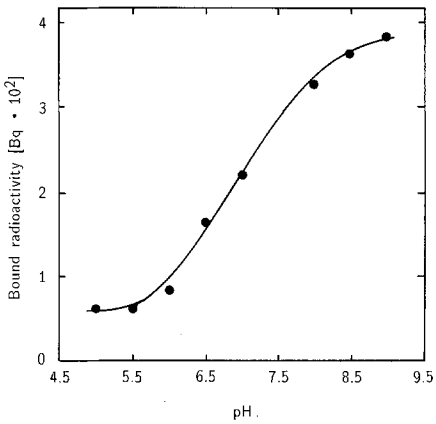

Fig. 1. The influence of pH on the binding of solubilized FCBP of *Vicia faba* to the DEAE TSK 650(S) anion exchanger. Microsomal protein (0.5 mg) was solubilized with Mega-9 as described, after incubation in the presence of 10 nM [^{3}H]FCol, then diluted 10-fold with 0.1 M Mes-Citrate (pH 5–6), Tris-Mes (pH 6.5–8) or Tris-HCl (pH 8.5–9). Further processing was as described in *Material and methods*. Unspecific binding was subtracted

Results

Solubilization and purification of the FCBP-[^{3}H]FCol complex from membrane preparations.

In contrast to the rapid binding of FC and [^{3}H]FCol to the membrane-embedded FCBP, the dissociation of the complexes, especially at low temperatures, is a slow process ($t_{1/2}$ at 4° C ≤ 6 h; Feyerabend and Weiler 1988). The formation of the complex of FCBP and FC or FC-derivatives has a pH optimum of 5.5–6 (Feyerabend and Weiler 1988). In this pH range, the solubilized complex is rather unstable whereas, at neutral to alkaline pH (7–9), the stability is much better (data not shown). This allowed a facile assay of the solubilized complexes by binding to anion exchangers to remove the unbound ligand. The pH dependence of the binding of FCBP-radioligand complexes to the matrix, Fractogel TSK DEAE 650(S), is shown in Fig. 1. At pH 8.5, 72% of the complex present in the detergent extract is bound to the exchanger and can be recovered quantitatively by elution with high salt concentrations. The relative effectiveness of a range of detergents to solubilize the FCBP-radioligand complexes was compared (data not shown). Approximately 60–70% of the FCBP-[^{3}H]FCol complexes were solubilized intact in the presence of 60 mM Mega-9 or octyl glucoside. Phospholipid (soybean phosphatidylcholine, type IV-S), 0.1% (w/v), and glycerol (20% v/v) sta-

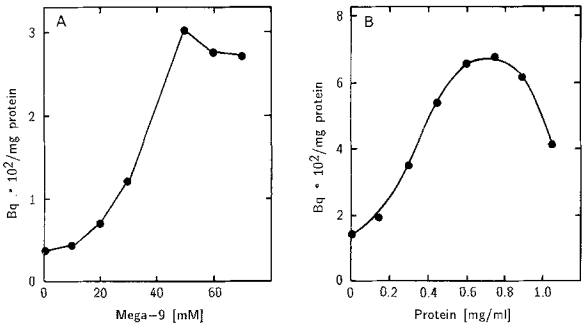

Fig. 2A, B. Solubilization of FCBP-[^{3}H]FCol complexes from microsomal membranes of *Vicia faba*. Membranes (1 mg·ml^{-1} of protein) preincubated in the presence of 10 nM [^{3}H]FCol for 60 min at 25° C were solubilized as described with varying concentrations of Mega-9 (**A**). The efficiency of solubilization of the FCBP-radioligand complexes as a function of the protein concentration and a constant concentration of Mega-9 (60 mM) is shown in **B**

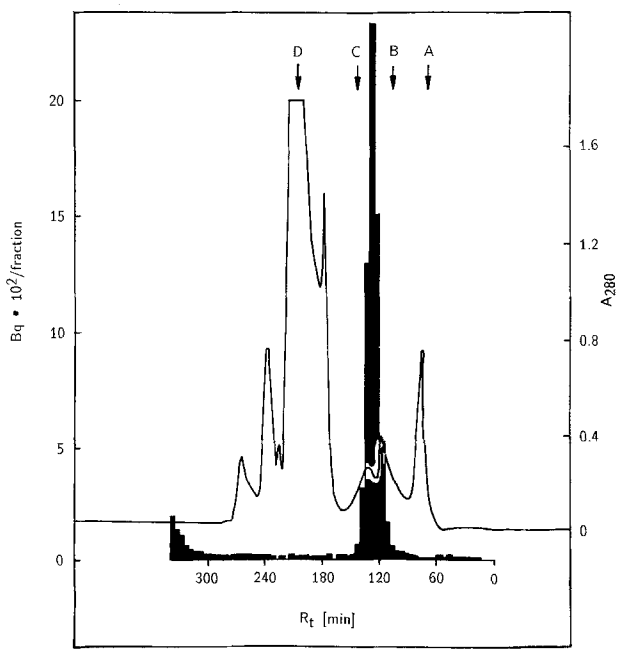

Fig. 3. Gel permeation chromatography of a cholate (23 mM) extract of microsomal membranes of *Vicia faba*. Column: Fractogel TSK 55-W (S) (1.6 cm i.d., 50 cm long; 100 ml gel bed), using 10 mM citrate-NaOH, 5 mM $MgSO_4$, 5 mM EDTA, 1.5 mM cholate, pH 5.5, as mobile phase. Flow rate = 1 ml·min^{-1}, T = 4° C. Unbound [^{3}H]FCol elutes at t ≥ 450 min. *A* = thyroglobulin (M_r = 670 kDa); *B* = Phosphoglucoseisomerase (M_r = 140 kDa); *C* = bovine serum albumin (M_r = 67 kDa); *D* = cytochrome *c* (M_r = 12 kDa)

bilized the solubilized complex. The optimum ratio for Mega-9 was protein:detergent:lipid = 0.7:20:1 (by wt.; Fig. 2). All other detergents tested were less effective and-or dissociated more of the FCBP-[^{3}H]FCol complexes. Zwitterionic or cationic detergents gave an intermediate yield while anionic detergents gave lowest yields of solubilized com-

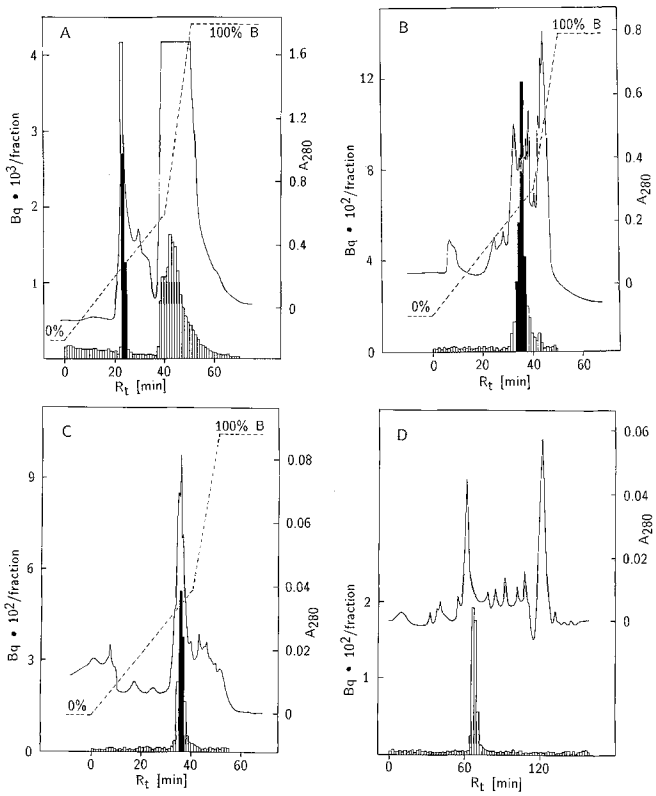

Fig. 4A–D. Partial purification of the FCBP from plasma membranes of *Vicia faba*.
A = Fractogel TSK DEAE 650(S);
B = Pharmacia Mono Q;
C = Pharmacia Mono Q rechromatography;
D = Pharmacia Superose 12. In step **A**, the Mega-9 extract from 16 mg of plasma-membrane protein was applied. Further processing as detailed in *Material and methods*. Free [^{3}H]FCol eluted in the void volume (step **A, B, C**) and at R_t ≥ 180 min (step **D**)

plexes. We observed that anion-exchange chromatography gave better separation of the FCBP-radioligand complexes from other proteins when cholate was used in the eluting buffers instead of Mega-9. The FCBP-[^{3}H]FCol eluted from calibrated Fractogel TSK 55-W(S) columns in the presence of 1.5 mM cholate at pH 5.5 at a M_r of 80 ± 20 kDa in several experiments (Fig. 3). On the basis of these experiments, a sequence of purification steps was established involving (i) the use of plasma-membrane vesicles enriched from microsomal membranes by aqueous two-phase partitioning (this already results in a three- to sixfold enrichment of the FCBP; Feyerabend and Weiler 1988); (ii) solubilization with Mega-9 followed by anion-exchange chromatography first on Fractogel TSK DEAE 650(S), then on the stronger exchanger, Mono-Q (two sequential runs); (iii) subjection of the active fraction to gel permeation chromatography (GPC) using Superose 12. A representative

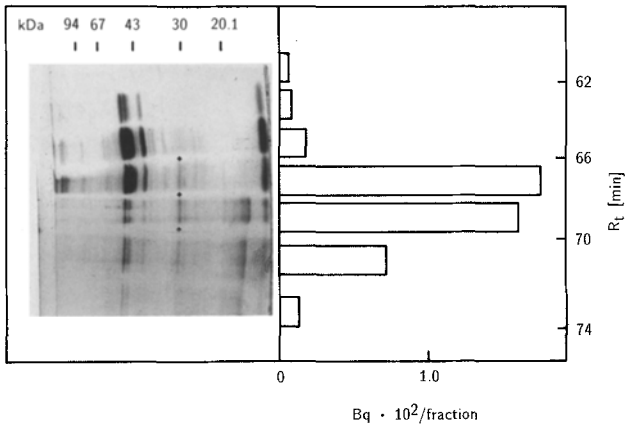

Fig. 5. Protein profiles of partially purified FCBP on SDS-PAGE. A Mega-9 extract of plasma-membrane vesicles (corresponding to 16 mg protein) of *Vicia faba* was purified by DEAE-ion exchange chromatography followed by two passes through Mono-Q and, finally, GPC on Superose 12. Active fractions at this final stage were collected and analyzed individually by SDS-PAGE

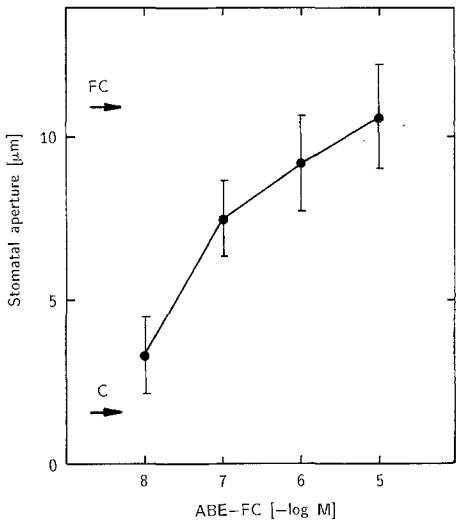

Fig. 6. Biological activity of ABE-FC synthesised as described in *Material and methods*. Increasing amounts of ABE-FC were tested for their ability to induce stomatal opening in the dark. The *arrows* indicate the aperture of buffer controls (*C*) and the aperture of stomata incubated in 10 µM *FC*. For each datum point, n = 150 stomata from three different epidermal sections were evaluated. The *bars* represent mean ± SD

FCBP-purification experiment is shown in Fig. 4A–D. Figure 5 shows an analysis, by SDS-PAGE, of a representive sample after the final GPC run. In the radioactive fractions, several polypeptides were still identified (see Fig. 5). However, only one major band co-eluted with the radioactivity. This band had a M_r of approx. 34 kDa. In some experiments, a second, faint, band at approx. 31 kDa was observed which also co-eluted with the radioactivity.

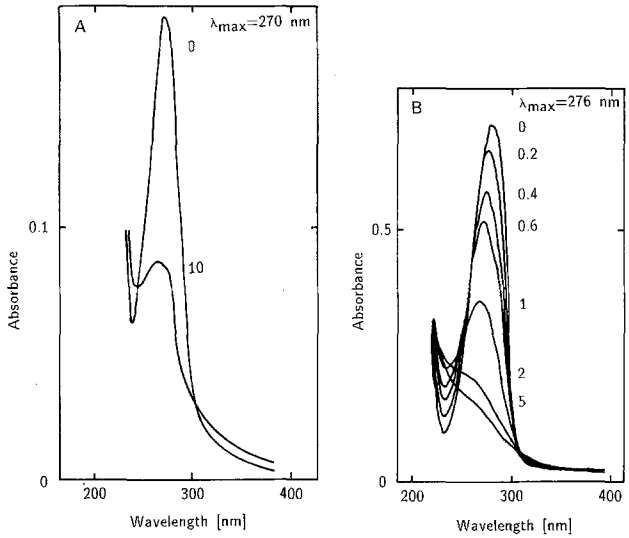

Fig. 7A, B. Photolysis spectra of ABE-FC and ABE-NHS. The UV-spectra of ABE-FC (**A**) and ABE-NHS (**B**) were recorded after illumination with high-intensity UV light ($\lambda \geq 305$ nm). Solvent: methanol. The numbers on the graphs indicate the time of radiation in seconds. ABE-NHS: $\lambda_{max} = 276$ nm, ABE-FC: $\lambda_{max} = 270$ nm

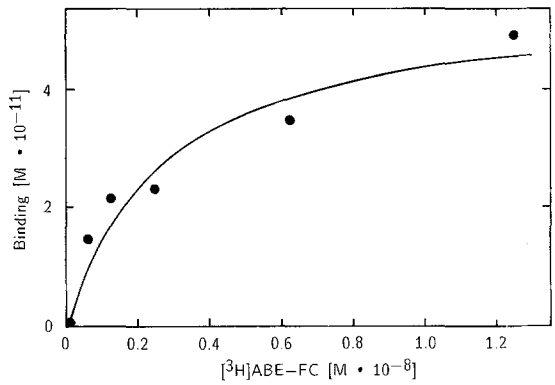

Fig. 8. Saturation of the FCBP with [^{3}H]ABE-FC. Plasma-membrane vesicles of *Vicia faba* corresponding to 120 µg of protein, were incubated with 1 nM [^{3}H]ABE-FC and pelleted. The membranes were then irradiated for 10 s at $\lambda \geq 305$ nm and the protein was precipitated with TCA (10% final concentration). Unspecific binding was subtracted

Photoaffinity-labeling of the FCBP. From earlier studies we know that modification of C-8′ and C-9′ of FC representing the enyl-structure of the *t*-pentenyl substituent yields derivatives which retain to a large extent their biological activity (Ballio et al. 1981a, b; Feyerabend and Weiler 1988). This allowed us to synthesize, in quantitative yield, via the 9′-nor-8′-aldehyde of FC, the corresponding ethylenediamine adduct by reductive amination in the presence of $NaCNBH_3$. The amino-derivative was then reacted with 3,5-[^{3}H]-4-azidobenzoyl-N-hydroxysuccinimide ester ([^{3}H]ABE-NHS) to give the [^{3}H]ABE-FC in 10–15% yield. Unlabeled

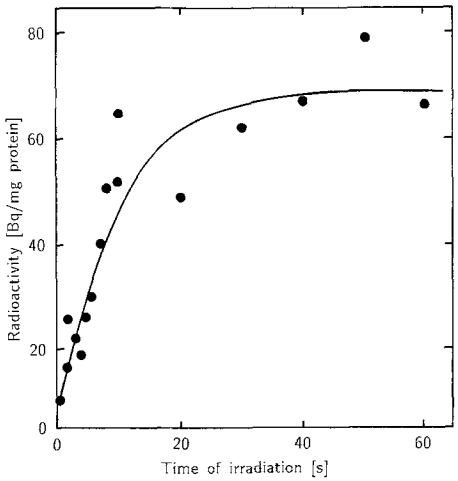

Fig. 9. Time course of photoaffinity labeling of plasma membranes of *Vicia faba* with [^{3}H]ABE-FC. Plasma-membrane protein (100 µg in 1 ml buffer) was irradiated for the times indicated, after labeling with [^{3}H]ABE-FC, pelleting of the membranes and resuspension in PBS, pH 7.4. Unspecific binding was subtracted

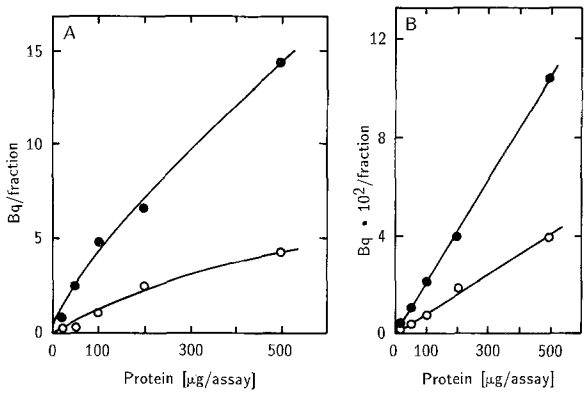

Fig. 10 A, B. Labeling of plasma membranes of *Vicia faba* with [^{3}H]ABE-FC as a function of protein quantity. Increasing amounts of protein were incubated in a final volume of 1 ml with 1.5 nM [^{3}H]ABE-FC alone (●—●) or together with 10 µM unlabeled FC (○—○) under otherwise standard conditions. Membranes were either pelleted and their radioactivities determined directly (**A**) or they were resuspended in PBS, pH 7.4, irradiated for 5 s and the TCA-precipitable radioactivity was determined (**B**)

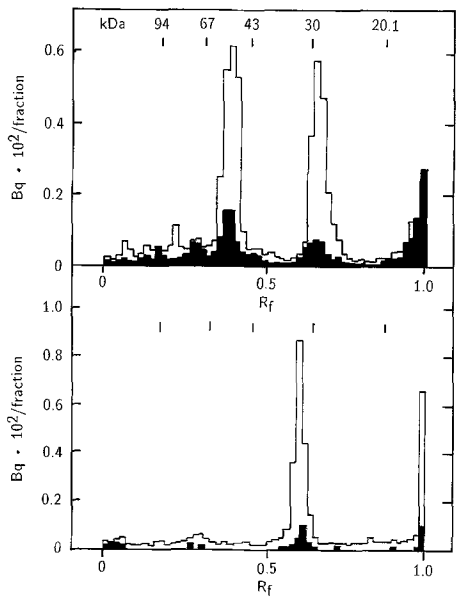

Fig. 11. Analysis by SDS-PAGE of photoaffinity-labeled plasma-membrane proteins of *Vicia faba* (*lower half*) and MAB 19-VI-A5 as a control (*upper half*). The *open bars* represent the incubation in 1 nM [^{3}H]ABE-FC, the *closed bars* give the residual binding in the presence of 1 µM unlabeled FC. The numbers give the M_rs of the marker proteins

ABE-FC was synthesized to determine its biological activity in inducing stomatal opening in the dark (Fig. 6). The compound has, compared to FC, 89% relative activity. The ABE-FC was quantitated spectroscopically in H_2SO_4, a reaction specific for the FC-moiety ($\lambda_{max} = 386$ nm, $\varepsilon_{386} = 8.2 \cdot 10^6$ cm$^2 \cdot$mol^{-1}). Its UV spectrum and that of ABE-NHS are shown under conditions of photolysis in Fig. 7. The saturation of the FCBP with [^{3}H]-ABE-FC is shown in Fig. 8. From this plot, an apparent K_a for [^{3}H]-ABE-FC of $0.9 \cdot 10^9$ M^{-1} was derived (Scatchard 1949). The conditions for photoaffinity labeling of membrane vesicles were optimized using a high-pressure mercury arc lamp with quartz collector. The emitted light passed through a WG $\geq$305-nm long-pass filter. As Fig. 9 shows, irradiation for 15–20 s is sufficient to give maximum photoaffinity labeling. After this time, the photolabile group is completely photolysed (compare Fig. 7). Protein-degradation under these conditions was checked by pre-irradiating membrane vesicles under standard conditions (10 s) followed by incubation with [^{3}H]FCol. No decrease in binding activity was obtained (data not shown). Figure 10 shows the total and unspecific binding of [^{3}H]ABE-FC to plasma-membrane vesicles as a function of protein concentration. Both, the reversible binding (Fig. 10A) and the ligand irreversibly bound after irradiation (Fig. 10B), are shown. Approximately 7–10% of the total reversibly bound ligand appears in the TCA-precipitable protein under standard conditions (1 nM radioligand, 0.1 mg$\cdot$ml^{-1} protein). In addition to plasma membranes of *Vicia faba* L., one further biological system was analyzed which was used as a control: MAB 19-VI-A5 (Feyerabend and Weiler 1987) which recognizes FC and its derivatives in a manner similar to the FCBP of *V. faba*. Both, the heavy and light chain of MAB 19-VI-A5 were found to carry the label. The specificity of the labeling was shown by its reduction by micromolar concentrations of unlabeled FC, when present during incu-

bation (see Fig. 11). Two areas of radioactivity were identified on SDS-gels after separation of the photoaffinity-labeled plasma-membrane proteins of *V. faba* (Fig. 11). The major band migrated at an M_r of 35 ± 1 kDa (average from six determinations) while the migration front also carried label (see Fig. 11). In both areas, the radiolabel was strongly reduced when incubations were performed in the presence of an excess of unlabeled FC.

Discussion

The dramatic alterations in a broad variety of transport processes across the plasma membranes of higher plants, brought about by very low levels of FC without a noticeable lag-phase, supports the notion that the toxin rather directly influences one or several key processes involved in plant plasma-membrane transport and-or its regulation (Marré 1979). Various hypotheses have been put forward to explain the action of FC, most of them involving stimulation of the H^+-translocating, vanadate-sensitive, plasma-membrane ATPase by the toxin (Marré 1979; Rasi-Caldogno et al. 1986). More recently, an approx. twofold inhibition, by FC, of the total leak current into the guard cells of *Vicia faba*, but only a slight stimulation of the pump current were observed (Blatt 1988). Thus, FC might inhibit components contributing to the leak current rather than activate the H^+-ATPase which is considered the dominant enzyme generating pump current in these cells. The mechanism of action of FC, however, is unknown and despite several reports on high-affinity binding of the toxin to plant plasma membranes (Dohrmann et al. 1977; Ballio et al. 1980; Stout and Cleland 1980; De Boer et al. 1987; Feyerabend and Weiler 1988) the binding-protein remained unidentified. We now report it to be a 34–35-kDa polypeptide, based on co-purification studies after solubilization of intact FCBP-FC radioligand complexes and on photoaffinity labeling experiments using a novel, highly bioactive, azido-analogue of the toxin, [^{3}H]ABE-FC, whose synthesis is reported here for the first time. Solubilized FCBP-radioligand complexes obtained from oat root (Stout and Cleland 1980) and broad-bean leaf (Feyerabend and Weiler 1988) membranes under different conditions of extraction exhibit M_rs of approx. 80 kDa on gel permeation columns, indicating that the FCBP occurs as part of an oligomeric complex in the membrane.

From our findings, it can now be ruled out that the toxin interacts directly with the H^+-ATPase. This enzyme is composed of a single polypeptide of 100–105 kDa which is involved in both, H^+ translocation and ATPase hydrolysis (Malpartida and Serrano 1981), and thus is clearly different from the FCBP. The subunit composition of the ATPase from the plasma membrane of higher plants has not been rigorously studied. The enzyme belongs to the class of E1-E2-type ATPases which all appear to be homologous and consist of a 70–100-kDa α-peptide (Pedersen and Carafoli 1987). Only the Na^+/K^+-ATPase contains another (β)-peptide of unknown function and a M_r of 55 kDa (for reviews, see Bowman and Bowman 1986; Pedersen and Carafoli 1987). Thus, the possibility exists that a more complex subunit structure may exist for other E1-E2-type ATPases as well. However, in the purest preparations of plant plasma-membrane H^+-ATPases obtained from oat (Serrano 1984), tomato (Anthon and Spanswick 1986) and maize (Nagao et al. 1987) roots, no evidence for additional subunits other than the 100-kDa polypeptide has been obtained so far. Cross-linking experiments should help to clarify a potential association of the FCBP with the ATPase.

Proteolytic digestions of fungal and yeast plasma-membrane ATPases produce peptides in the range of 30–35 kDa (Vai et al. 1986). Thus, the fact that the FCBP represents a proteolytic fragment of the H^+-ATPase has to be considered. However, this possibility, while not fully excluded, seems highly unlikely for the following reasons: samples prepared in the presence of a mixture of protease inhibitors (leupeptin, *o*-phenanthroline, PMSF, caprylate, benzamidine and aminocaproic acid) when compared to samples prepared under standard conditions (leupeptin and PMSF alone) or in the total absence of protease inhibitors, all, revealed the 35-kDa polypeptide (data not shown). Occasionally (compare Fig. 5), weak labeling of a 67-kDa band was observed, but never any label in the 100–105-kDa region. Yet, vanadate-sensitive K^+, Mg^{2+}-ATPase was readily detectable in our vesicle preparations and also in the detergent extracts prepared from these vesicles (Blum et al. 1988).

The nature of the radiolabeled material seen in the migrating front on SDS gels of both, MAB preparations and plasma-membrane proteins (see Fig. 11) is not yet clear. Radioactivity in this area was not reduced by unlabeled FC in the antibody preparation while it was clearly reduced in the plant membrane preparation. While it seems clear that in the antibody preparation this second band reflects unspecific interactions of the azido-FC analogue with low-M_r contaminants present in the ascites fluid used for the experiments, in the plant membrane preparations this is unlikely. Rather,

proteolytic fragments of the FCBP or membrane components in the vicinity of the FCBP have to be considered. We favour the latter possibility because when the photoaffinity-labeled FCBP is isolated from SDS gels in the absence of protease inhibitors and electrophoresed a second time, the migrating front carries no label.

The long distance between the photoactivated nitrene group and the FC moiety should result in some attachment of the reactive group to residues in the vicinity of the FCBP toxin-binding domain. That this might be a possibility becomes obvious from the MAB experiment (compare Fig. 11). From cross-reaction analyses, we know that the distant portion (C-8′ and C-9′) of the *t*-pentenyl moiety of FC does not contribute significantly to the total binding force to MAB 19-VI-A5 (Feyerabend and Weiler 1987). This moiety can thus be considered protruding from the antibody's antigen-binding area. Thus, the azidobenzoyl moiety which is attached to the pentenyl residue via the ethylenediamine spacer should remain relatively unhindered and freely mobile in the antibody-antigen complex. That this is the case can be proven by the fact that both the heavy and the light chain of MAB 19-VI-A5 are photoaffinity labeled by the azido-FC derivative (see Fig. 11). Antibody 19-VI-A5 binds FC in very much the same way as the FCBP. A similar situation with respect to the steric arrangement of the photoactivated toxin analogue to the antibody and the FCBP can be thus expected. We therefore have to consider the possibility that the 35-kDa protein identified by photoaffinity labelling is not identical with the FCBP but rather a component in the vicinity of the toxin-binding protein. This possibility can be excluded, however, because the partial purification of the reversible FCBP-radioligand complex (see Figs. 4, 5) also yields the 34–35 kDa polypeptide.

With the identification of the FCBP and the possibility of tagging it by photoaffinity labeling, a molecular study of this plant plasma-membrane protein has now become feasible. The results should help to clarify the mechanism(s) underlying the regulation of cell membrane transport in higher plants.

The expert technical assistance of G. Henrichs is gratefully acknowledged. This work was supported by grant SFB171/C10 of the Deutsche Forschungsgemeinschaft, Bonn, FRG and by the Fonds der Chemischen Industrie, Frankfurt, FRG (literature provision).

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Received 6 October 1988; accepted 13 January 1989