FEBS Letters 416 (1997) 190–192 FEBS 19341

The fungal elicitor cryptogein is a sterol carrier protein

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Received 8 August 1997; revised version received 15 September 1997

Abstract Cryptogein is a protein secreted by the phytopathogenic pseudo-fungus, *Phytophthora cryptogea*. It is a basic 10 kDa hydrophilic protein having a hydrophobic pocket and three disulfide bridges. These common features with sterol carrier proteins led us to investigate its possible sterol transfer activity using the fluorescent sterol, dehydroergosterol. The results show that cryptogein has one binding site with strong affinity for dehydroergosterol. Moreover, this protein catalyzes the transfer of sterols between phospholipidic artificial membranes. This is the first evidence for the existence of an extracellular sterol carrier protein and for a molecular activity of cryptogein. This property should contribute to an understanding of the role of cryptogein in plant-microorganism interactions.

Key words: Dehydroergosterol; Elicitin; Fluorescence; *Phytophthora*

1. Introduction

Plants can acquire resistance to pathogens as a result of a previous interaction with non-pathogenic microorganisms. The process is initiated at the site of the inducting interaction which typically involves the formation of a restricted necrotic lesion, such as that occurring in the hypersensitive response of plants against pathogens [1,2].

We previously studied the response of *Nicotiana tabacum* to elicitins, proteinaceous elicitors of defense reactions secreted by *Phytophthora* species. These proteins induce necroses in tobacco plants which then become resistant to pathogens [3,4]. In suspension-cultured tobacco cells treated with cryptogein (the elicitin from *Phytophthora cryptogea*), the earliest events reported are a high-affinity binding of the elicitin to a specific protein on the plasmalemma [5], an alkalization of the extracellular medium and a concomitant electrolyte leakage [6], a fast and large influx of Ca^{2+} [7], and a transient production of active oxygen species [8–10]. These responses are blocked by the protein kinase inhibitor staurosporine [11] indicating that phosphorylation steps are implied in the transduction of the elicitation signal. The plasma membrane is the likely target for the initial interaction between tobacco cells and cryptogein, but nothing is known about the molecular mechanism involved.

Cryptogein is a small hydrophilic holoprotein (pI 8.5) containing 98 amino acids (MW 10 323 Da) and three disulfide bridges [12]. Recently, the crystal structure of this protein showed the presence of a hydrophobic pocket [13]. Cryptogein did not exhibit any protease, β-glucanase or phospholipase activity [14], and no other enzymatic activity has been reported so far. The characteristics of cryptogein reported above seem to be similar to those of lipid transfer proteins [15] or of a sterol carrier protein (SCP2) [16], despite different primary structures. Thus, the aim of this work was to test a possible sterol transfer activity of cryptogein, using the fluorescent sterol $\Delta^{5,7,9(11)22}$-ergostatetraen-3β-ol dehydroergosterol (DHE), which has been previously used to study the interactions between sterols and macromolecules or membranes [16–20].

2. Materials and methods

2.1. Chemicals

All phospholipids, dehydroergosterol and cholesterol were purchased from Sigma. Cryptogein was obtained as described earlier [12], dissolved in the measuring medium and stored at −30°C. The concentration of dehydroergosterol dissolved in ethanol was corrected using the extinction coefficient [19].

2.2. Fluorescence measurements

Fluorescence measurements were performed at 25°C with a Shimadzu RF 5001 PC spectrofluorimeter in a stirred fluorometric cuvette with 2 ml of measuring medium containing 175 mM mannitol, 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4, and 5 mM MES, pH 7.0. The excitation and emission wavelengths were set at 325 nm and 370 nm, respectively. The fluorescence of cryptogein alone was negligible. Fluorescence intensity was expressed in arbitrary units (a.u.).

2.3. Interaction of cryptogein with dehydroergosterol

To 0.2–3.8 μM DHE in the measuring medium (fluorescence intensity F_0) 2.5 μM cryptogein was added. Fluorescence intensity (F) of the mixture was read after stabilization. The fluorescence titration curve ΔF vs. DHE concentration was made on the basis of the titration ($\Delta F = F - F_0$). Titration of a constant DHE concentration (0.25 μM) with cryptogein, until a constant fluorescence intensity was reached, allowed to determine the fraction of bound ligand. To obtain the dissociation constant and the number of binding sites plots of 1/bound DHE vs. 1/free DHE were made on the basis of the following equation:

$$1/C_b = (K_d/NA)\cdot 1/C_f + 1/NA$$

where C_b and C_f are the concentrations of bound and free DHE, respectively, K_d the dissociation constant, A the concentration of acceptor and N the number of binding sites.

2.4. Preparation of liposomes

Two types of small unilamellar vesicles (SUV) were prepared: donor vesicles contained phosphatidylcholine (PC), phosphatidylserine (PS) and DHE, acceptor vesicles contained PC, PS and cholesterol. Phospholipids and sterols were dissolved in chloroform. For each assay, 4.4 mg PC, 0.8 mg PS and 1.5 mg sterol (cholesterol or DHE) were mixed, chloroform was evaporated under nitrogen and traces of solvent were vacuum evaporated for at least 1 h. Thereafter, 1 ml of exchange buffer containing 10 mM MES (pH 7.0) and 0.02%

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Abbreviations: DHE, dehydroergosterol; SUV, small unilamellar vesicles; PC, phosphatidylcholine; PS, phosphatidylserine; BSA, bovine serum albumin; SCP, sterol carrier protein

PII S0014-5793(97)01193-9

azide was added. The mixture was vortexed under nitrogen and then sonicated for three 5 min periods at 40°C. The suspension of liposomes was centrifuged (150 000×*g*, 1 h) and the supernatant containing SUV was used for measurements. The recovery of SUV estimated on the basis of DHE fluorescence before and after the centrifugation was about 60%.

2.5. Measurements of sterol exchange

Measurements of the steady-state polarization of fluorescence were performed at 25°C with a Kontron SFM-25 spectrofluorimeter equipped with polarizers. In each exchange assay, 280 μg of SUV were used in 2 ml exchange buffer (see Section 2.4) so that the donor:acceptor ratio was 1:9. The intensities of vertically and horizontally oriented components were read during 10 min. The horizontal and vertical components of the fluorescence polarization of liposomes without DHE were subtracted.

3. Results and discussion

3.1. Cryptogein-DHE interaction

The uncorrected emission maxima of DHE excited at 325 nm in the measuring medium were 370, 402 and 424 nm (Fig. 1). The addition of cryptogein to DHE in the measuring buffer resulted in a marked increase in the emission maximum at 370 nm. A similar increase was observed after the interaction of DHE with a rat liver sterol carrier protein [18]. The DHE-cryptogein interaction was time dependent. The relative fluorescence intensity ΔF of bound DHE (0.1–2.0 μM) at 370 nm found after titration of DHE with cryptogein was 10-fold higher than fluorescence intensity F_0 of DHE in the measuring medium. The increase in fluorescence intensity and the change of the shape of the emission spectra upon binding of DHE to the hydrophobic core of cryptogein could be caused by a separation of DHE molecules which were concentrated in micelles. Energy transfer was considered as a possible mechanism of energy dissipation of DHE in micelles [18]. DHE is not very sensitive to solvent polarity [19]. Decreased motion inhibits the transfer of excited state energy and increases fluorescence quantum yield [19].

The interaction of DHE with cryptogein was compared to that obtained with BSA, which is known to adsorb unspecifically many non-polar substances including DHE [17]. Fig. 1 (inset) shows that DHE was rapidly adsorbed on BSA whereas 3–5 min were necessary to reach equilibrium in the case of cryptogein. Moreover, fluorescence intensity of DHE was much lower in the presence of BSA than in that of cryptogein. The slow kinetics of fluorescence increase of DHE bound to cryptogein could be due to a slow penetration of DHE into the hydrophobic pocket of cryptogein.

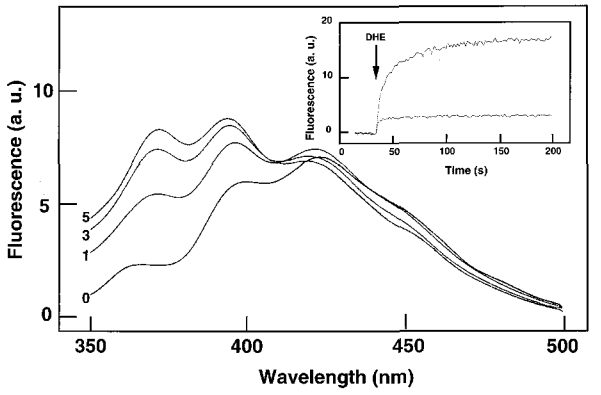

Fig. 1. Time-dependent emission spectra of the mixture dehydroergosterol-cryptogein. The excitation wavelength was set at 325 nm. From the bottom to the top: 2.5 μM DHE, DHE with 2.5 μM cryptogein 1, 3, 5 min after mixing. Inset: Kinetics of the interaction of DHE (1.3 μM), with cryptogein (2.5 μM) (upper curve) and BSA (2.5 μM) (lower curve).

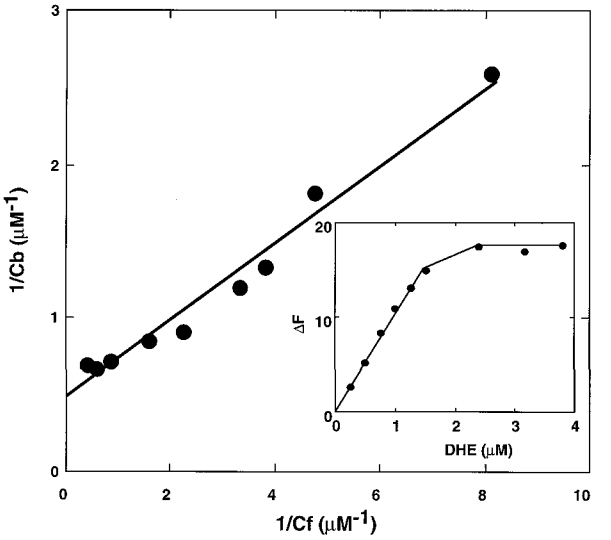

Fig. 2. Binding plots of the titration of cryptogein by DHE. C_b and C_f are the concentrations of bound and free DHE, respectively. Correlation coefficient of the straight line was 0.95. The results are mean values of two experiments. Inset: Fluorescence titration curve of cryptogein (2.5 μM) by DHE.

3.2. Sterol binding activity of cryptogein

The fluorescence titration curve of cryptogein with DHE is shown in Fig. 2 (inset). The plot of 1/bound DHE vs. 1/free DHE of the titration gave 0.90 ± 0.10 binding site per cryptogein molecule and a dissociation constant of 0.56 ± 0.04 μM (Fig. 2). This result indicates that cryptogein has only one binding site with a relatively strong affinity for DHE.

Other proteins are also able to bind sterols. The sterol carrier protein-2 SCP2 from rat liver binds dehydroergosterol with an apparent dissociation constant of 1.2–1.5 μM and a stoichiometry of 0.8–1.0 sterol per protein molecule [17]. Another structurally different sterol carrier protein, SCP from rat liver, binds dehydroergosterol with a K_d of 0.88 μM for DHE and a similar stoichiometry [18]. A rat liver fatty acid binding protein has a K_d of 0.29 μM and exhibits 0.9 binding sites per protein molecule. On the other hand, BSA has a substantially lower affinity for DHE (K_d=2.9 μM) and possesses 6 to 7 binding sites per protein molecule [17]. These results show that cryptogein has a similar affinity and number of binding sites for DHE as previously described sterol binding proteins from mammals.

3.3. Sterol transfer activity of cryptogein

Transfer of sterols catalyzed by cryptogein is shown in Fig. 3. The sterol exchange between donor SUV containing DHE and acceptor SUV containing cholesterol was measured on the basis of fluorescence polarization of DHE. In donor SUV, DHE molecules interact to self-quench. This interaction, resulting in radiationless energy transfer, will also decrease fluorescence polarization. As shown by Schroeder et al. [16], the molecular transfer of DHE can be visualized as the increase in steady-state polarization of DHE in the donor-acceptor mixture.

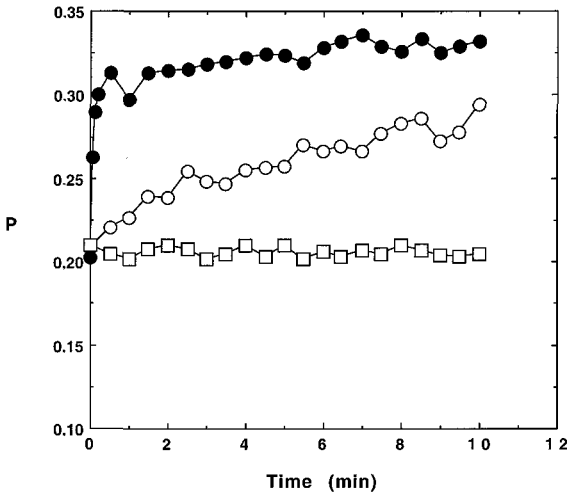

Fig. 3. Exchange of sterols in SUV catalyzed by cryptogein. Donor vesicles contained PC, PS and DHE, acceptor vesicles contained PC, PS and cholesterol. □: control, donor SUV (140 μg/ml)+1 μM cryptogein, ○: spontaneous exchange, donor-acceptor SUV mixture (140 μg/ml), ●: stimulated exchange, donor-acceptor SUV mixture+1 μM cryptogein.

The value of fluorescence polarization (P) of 1.9 μM DHE in exchange buffer was 0.16. After 1 min following the addition of cryptogein (1 μM), the value of fluorescence polarization of the mixture increased up to 0.41. Thus, binding of DHE to cryptogein restricts the degree of DHE mobility, compared to DHE mobility in micelles, as was also shown in the case of the sterol carrier protein SCP2 [16,18]. The value of polarization of DHE in donor SUV was 0.15. Fluorescence intensity of the complex cryptogein-DHE (2 arbitrary units) was negligible compared with that of DHE in SUV (40 arbitrary units) so that it is unlikely that the DHE-cryptogein complex contributed noticeably to the change in the value of polarization. Spontaneous exchange between DHE and cholesterol after the addition of acceptor SUV to donor SUV is shown in Fig. 3 (middle trace). The addition of 1 μM cryptogein to the donor-acceptor mixture stimulated the exchange between DHE and cholesterol (Fig. 3, top trace). As a control, the addition of cryptogein to excess of donor SUV alone did not cause any change in fluorescence polarization during 10 min (Fig. 3, bottom trace).

3.4. Conclusion

Cryptogein is able to bind to either sterols in solution or sterols inserted in liposomal membranes. Therefore, it is a sterol binding protein which is also able to transfer sterols between artificial membranes as observed for the well characterized sterol carrier protein SCP2 from rat liver. This is the first evidence for the existence of an extracellular sterol carrier protein and for a molecular activity of cryptogein. This property should contribute to the understanding of the role of cryptogein in plant-microorganism interactions. Experiments are in progress to verify if the sterol transfer activity is a general property of elicitins. In that case, original functions of elicitins in the *Phytophthora* infection process in plants, could be the primary transport of sterols from plant plasma membranes to *Phytophthora* since these microorganisms are unable to synthesize sterols necessary for their growth and their reproduction [21].

Acknowledgements: This work was supported by a grant from the Conseil Régional de Bourgogne and the MENESR. We thank Pr. N. Latruffe for his help in fluorescence polarization studies performed in his laboratory and S. Brunie for helpful discussions.

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