

# Transit sequence-dependent binding of the chloroplast precursor protein ferredoxin to lipid vesicles and its implications for membrane stability

Ron van 't Hof\*, Ben de Kruijff

Department of Biochemistry of Membranes, Center for Biomembranes and Lipid Enzymology, Institute of Biomembranes, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands

Received 2 January 1995; revised version received 31 January 1995

**Abstract** The binding of the transit peptide (trfd) and precursor of the chloroplast protein ferredoxin (prefd) to large unilamellar lipid vesicles was investigated in relation to the lipid composition of the bilayer. Prefd binds with a dissociation constant of 0.27  $\mu$ M to vesicles with a composition corresponding to the chloroplast envelope outer membrane. Binding is mediated by the transit sequence. From an analysis of binding to vesicles containing the individual lipid components it could be concluded that anionic lipids are mainly responsible for binding, emphasizing the importance of electrostatics for the transit sequence–lipid interaction. Binding is also mediated by the specific chloroplast glycolipid monogalactosyldiacylglycerol. Monolayer experiments revealed that in this case a more extended domain of the transit sequence inserts into the lipid layer. Precursor binding does not result in a loss of vesicle barrier function. However, high concentrations of trfd do cause release of vesicle-enclosed carboxyfluorescein. The results are discussed in the light of the chloroplast protein import process, with special emphasis on the role of monogalactosyldiacylglycerol.

**Key words:** Chloroplast; Protein import; Ferredoxin; Transit peptide; Transit peptide–lipid interaction

## 1. Introduction

Chloroplast biogenesis depends on the import of proteins which are encoded on the nuclear genome and synthesized in the cytosol (for recent review see [1]). These proteins contain an N-terminal extension, the transit sequence, which is necessary and sufficient to direct proteins into the plastid. The

posttranslational import is initiated by binding of precursor proteins to the chloroplast surface. This process depends on the presence of the transit sequence because mature proteins do not bind. Maximal binding requires the utilization of ATP (100  $\mu$ M) in the intermembrane space and the presence of protease sensitive components on the chloroplast surface. Therefore, it is assumed that proteinaceous receptors are involved in protein binding but they remain to be identified. Subsequently, the proteins are translocated across the envelope membranes, which requires the hydrolysis of ATP (1 mM) in the stroma, processed by a stromal protease, routed to their proper localization and finally assembled into holo-enzymes.

Suggestions are accumulating that also precursor protein–lipid interactions can be involved in protein import. Soll et al. [2] showed that treatment of chloroplasts with phospholipase C strongly affects precursor protein binding and import. Precursor proteins bind to chloroplasts in the absence of ATP [3] and after protease pretreatment of chloroplasts [4]. Furthermore, it was shown in model membrane studies that precursor proteins and transit peptides can interact in a specific way with lipids from the target membrane [5,6]. For instance, lipid monolayer studies showed that the precursor protein of ferredoxin (prefd) inserts, transit sequence-dependent, efficiently and specifically in between the lipids extracted from the chloroplast outer envelope membrane [6]. NMR experiments with lipid dispersions revealed that precursor protein–lipid interactions result in transit sequence mediated changes in the lipid organization [7]. The lipid–protein interactions are accompanied by the induction of secondary structure in the otherwise unstructured transit peptide [8].

These observations lead to suggestions on the roles of precursor protein–lipid interactions in protein import. The chloroplast membranes contain lipids like monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG) and sulfoquinovosyl diacylglycerol (SQDG) not found in other cellular membranes [9]. Thus precursor protein–lipid interactions could potentially contribute to organelle specific targeting. Furthermore the changes in transit peptide structure and lipid organization could play roles in the recognition and translocation steps during protein import.

To get more insight into these possibilities we report here studies on the interaction of a chloroplast precursor protein with membranes of large unilamellar vesicles (LUVETs) of varying composition. The extended bilayers of LUVETs serve as a model system for the lipid domains of the chloroplast outer envelope membranes. These membranes have a high lipid-to-protein ratio [10] and the lipids are accessible for proteins because externally added lipid-specific antibodies can interact with chloroplasts [11]. We have chosen to use the precursor

\*Corresponding author. Present address: Center for Protein Technology TNO/WAU, PO Box 8129, 6700 EV Wageningen, The Netherlands. Fax: (31) (8370) 84893. E-mail: ron.vanthof@lmc.lmt.wau.nl

**Abbreviations:** prefd, preferredoxin; trfd, transit peptide of ferredoxin; apofd, apoferridoxin; pCOX IV, presequence of cytochrome c oxidase subunit IV; PC 1–37, peptide corresponding to the 37 N-terminal amino acids of the plastocyanin transit sequence; PC1–43, peptide corresponding to the 43 N-terminal amino acids of the plastocyanin transit sequence; DHFR, dihydrofolate reductase; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipin; BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane; PIPES, piperazine-1,4-bis(2-ethansulphonic acid); CF, 6-carboxyfluorescein; DTT, dithiothreitol; HPLC, high performance liquid chromatography; FPLC, fast protein liquid chromatography; TLC, thin layer chromatography; LUVETs, large unilamellar vesicles.

protein of ferredoxin (prefd) from *Silene pratensis* because its import process is well studied. Prefd uses the general protein import pathway and does not require cytosolic factors for import [12]. The binding of prefd to the vesicles and the consequences for the membrane barrier function were investigated. By comparing the results obtained with prefd with those of the transit peptide (trfd) and the apoprotein (apofd), insight into importance of the various parts of the precursor for the interaction with vesicles was obtained.

## 2. Experimental

### 2.1. Materials

**General.** 6-Carboxyfluorescein (CF) (Eastman Kodak Co., USA) was purified according to [13]. Dithiothreitol (DTT) was obtained from Boehringer (Mannheim, Germany). Ellman's reagent: 5,5'-dithiobis(2-nitrobenzoate), sorbitol, HEPES and Tris were from Sigma (St. Louis, USA). Iodoacetamide was from Fluka (Busch SG, Switzerland). All other chemicals were of the highest quality.

**Peptides.** A 47-mer corresponding to the transit sequence of ferredoxin from *S. pratensis*, with the sequence ASTLSTLSVSASLLPK-QQPMVASSLPTNMGQALFGLKAGSRGRVTAM (Mw = 4780), was synthesized on an Excell Pepsynthesizer by Millipore (Watford, UK). Trfd differs only the sequence deduced from the gene [14] by the absence of the N-terminal methionine which in vivo is removed in the cytosol [15]. A 25-mer peptide resembling the presequence of cytochrome c oxidase subunit IV (pCOX IV) from yeast, with the sequence MLSLRQSIRFFKPATRTLCSRYLL, was synthesized on an Excell Pepsynthesizer (Hubrecht Laboratory, Utrecht, The Netherlands). Both peptides contain an amide group on their C-terminus to avoid a negative charge at this position.

The peptides were purified by reversed phase high performance liquid chromatography (HPLC) as described [6]. The purity of the peptides was estimated to be over 98% as determined by analytical HPLC. The identity of the peptides was confirmed by N-terminal sequencing of 20 amino acids by Edman degradation according to [16], by quantitative amino acid analysis [17] and for the presequence also by mass spectroscopy. The peptides were stored as dry materials under nitrogen at  $-20^{\circ}\text{C}$ . By dissolving the peptides in degassed distilled water, stock solutions with concentrations ranging between 1–1.5 mg/ml were prepared which were stored under nitrogen at  $-20^{\circ}\text{C}$ . The bicinchoninic acid protein assay (Pierce Chemical Co.) with bovine serum albumin (BSA) as reference was used to determine peptide concentrations.

$^{14}\text{C}$ -Labeled trfd was obtained by reductive methylation using  $^{14}\text{C}$ -formaldehyde (New England Nuclear, UK; 59 mCi/mmol) as described [18].  $^{14}\text{C}$ -trfd could be visualized as a single band by tricine-SDS-PAGE [19] followed by fluorography. It was shown that all applied radioactivity was present in the peptide band. Trfd had a specific radioactivity of 49 mCi/mmol and contained 0.8  $^{14}\text{C}$ -methyl group per molecule.  $^{14}\text{C}$ -trfd could be imported into chloroplasts along the same general import pathway as used by prefd and is thus functional active [20].

**Proteins.** Prefd (Mw = 15 kDa) from *S. pratensis* was purified from an *E. coli* strain overexpressing the precursor protein as described by Pilon et al. [16]. The precursor was stored in small aliquots in 25 mM Tris-HCl, pH 7.5, 8 M urea and 0.02% (v/v)  $\beta$ -mercaptoethanol at  $-20^{\circ}\text{C}$  at concentrations ranging between 1–1.5 mg/ml. Apofd was prepared as described [17] and stored in 1 M Tris buffer at  $-20^{\circ}\text{C}$  at a concentration of 1 mg/ml. Protein concentrations were determined according to Bradford [21] with BSA as reference.

Prefd and apofd were labeled on their cysteine residues with  $^{14}\text{C}$ -iodoacetamide (spec. act. 56 mCi/mmol; Amersham), as described by Pilon et al. [12]. In order to obtain a homogeneous prefd population complete modification of  $-\text{SH}$  groups was obtained by a subsequent incubation with a large excess of iodoacetamide. The specific activity of both prefd and apofd was 162 mCi/mmol corresponding to 2.7  $^{14}\text{C}$ -labeled residues per molecule. Determination of the sulfhydryl groups using the Ellman's reagent [22] according to [12] revealed that for both prefd and apofd all cysteine residues had reacted with iodoacetamide. It was verified by chloroplast import experiments that  $^{14}\text{C}$ -prefd was as efficiently imported as unmodified prefd (data not shown). Monolayer experiments showed that the labeling procedure of

trfd, prefd and apofd did not significantly alter the interaction with lipids. Labeled and unlabeled (poly)peptides caused similar increases in surface pressure in monolayers of lipid extracted from the chloroplast outer envelope membrane and two representative target lipids: MGDG and PG (data not shown). In order to remove the urea which interfered with vesicle pelleting and, due to osmotic effects, with vesicle stability prefd and apofd were immediately before the experiments desalted by means of FPLC using a Fast Desalting column (Pharmacia, Uppsala Sweden) which was eluted with 10 mM Tris, 50 mM NaCl at pH 7.6.

**Lipids.** 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were synthesized according to established methods [23,24]. Phosphatidylinositol (PI) from bovine liver and cardiolipin (CL) from bovine heart were obtained from Sigma (St. Louis, USA). Cholesterol was obtained from Merck (Darmstadt, Germany). MGDG, DGDG and SQDG were isolated out of thylakoid membranes from pea (C.V. Feldham First) according to van 't Hof et al. [6]. A total chloroplast outer envelope membranes lipid extract was obtained according to [6]. The lipid composition of the lipid extract was determined according to [6] and was found to be MGDG 6%, DGDG 30%, SQDG 7%, PC 44%, PG 8% and PI 5% (mol%).

### 2.2. Methods

**Preparation of vesicles.** Large unilamellar vesicles (LUVETs) were made by hydration of lipid films (1  $\mu\text{mol}$  lipid) with indicated composition prepared from chloroform solutions with 1 ml of 10 mM Tris-HCl, pH 7.6, containing either 50 mM NaCl for binding experiments or 50 mM CF for vesicle lysis experiments followed by 10 freeze/thaw cycles and extrusion through two stacked polycarbonate filters (Nucleopore; 0.4  $\mu\text{m}$  pore size) [25]. The vesicle concentration was determined according to Rouser [26]. By TLC [6] it was determined that the lipid composition of the vesicles was not changed during the extrusion procedure.

**Vesicle binding experiments.** LUVETs of in total 200 nmol lipid were incubated with the indicated amounts of radiolabeled (poly)peptides in 300  $\mu\text{l}$  10 mM Tris-HCl, 50 mM NaCl at pH 7.6 for 1 h at room temperature. Vesicles were pelleted by centrifugation for 30 min at 75000 rpm ( $236 \times 10^3 g$ ) at room temperature in a TL 100 ultracentrifuge (Beckmann Instruments Inc., Palo Alto, CA, USA) using a TLA 100.3 rotor. The pellet was resuspended in 200  $\mu\text{l}$  10 mM Tris-HCl, 50 mM NaCl at pH 7.6. Samples of 20  $\mu\text{l}$  were taken to determine the vesicles pelleting efficiency according to Rouser [26] and the amount of bound peptide and protein by means of liquid scintillation counting. The vesicle pelleting efficiency ranged between 70% and 80%. The amount of bound (poly)peptide was corrected for this recovery. We assume that the vesicle population was homogeneous. Control experiments without vesicles revealed that of the added prefd and trfd, respectively 94 $\pm$ 3% and 96 $\pm$ 3% was recovered in the supernatant.

**Vesicle leakage experiments.** LUVETs with indicated lipid composition containing entrapped CF were separated from non-entrapped CF by gel filtration on a Sephadex G75 column (Pharmacia, Uppsala Sweden) ( $0.7 \times 20$  cm), using 10 mM Tris/HCl, 50 mM NaCl at pH 7.6 as eluent. Per measurement, vesicles (36 nmol of lipid) were suspended in 10 mM Tris-HCl, 50 mM NaCl at pH 7.6 to a final volume of 1 ml in a 1 ml fluorescence cuvet and incubated with varying amounts of (poly)peptides at room temperature. The fluorescence was measured on a SPF 500 C spectrofluorimeter (SLM Instruments Inc., Urbana, IL, USA) using an excitation wave length of 430 nm and an emission wavelength of 513 nm. The total amount of enclosed CF was determined by lysing the vesicles with 20  $\mu\text{l}$  10% (v/v) Triton X-100. The CF release is expressed as the percentage of the total amount enclosed.

**Monolayer experiments.** The Wilhelmy plate method was used to measure  $^{14}\text{C}$ -prefd induced changes in the surface pressure of lipid monolayers at constant surface area [27]. A subphase of 20 ml of 10 mM PIPES, 50 mM NaCl, pH 7.6, was placed in a teflon trough with a surface area of 29.6  $\text{cm}^2$  and was continuously stirred. The monomolecular lipid layer was formed by spreading a lipid containing chloroform solution on the air/buffer interface to an initial surface pressure of 20 mN/m. Saturating amounts of  $^{14}\text{C}$ -prefd (15.9  $\mu\text{g}$ ) were added to the subphase through a small hole at the edge of the trough. The surface pressure increase was measured in time until a stable surface pressure was reached. The amount of monolayer associated  $^{14}\text{C}$ -prefd was determined by collecting the monolayer and quantification by liquid scintillation counting. Before collection of the monolayer the subphase was

extensively washed in order to remove the non-associated prefd. Experiments were performed at room temperature.

### 3. Results

The binding of prefd and its subdomains, trfd and apofd to vesicles composed of a total lipid extract of the chloroplast outer envelope membrane is shown in Fig. 1A. Addition of increasing amounts of prefd leads to an increased and saturable binding of the precursor to the vesicles. In contrast, apofd binds only weakly, indicating that the observed prefd-vesicle binding is a specific process depending on the presence of the transit sequence. That it is the transit sequence itself, which is largely responsible for prefd binding, is shown by the efficient vesicle binding of trfd (Fig. 1A). Scatchard analysis of the binding data revealed that the binding of trfd is biphasic (Fig. 1B) with a high affinity binding ( $K_d = 0.27 \pm 0.12 \mu\text{M}$ ) and low affinity binding component ( $K_d = 2.28 \pm 0.43 \mu\text{M}$ ). This two-phase binding was not observed in case of prefd, but the analysis revealed a single binding constant of  $0.175 \pm 0.022 \mu\text{M}$ .

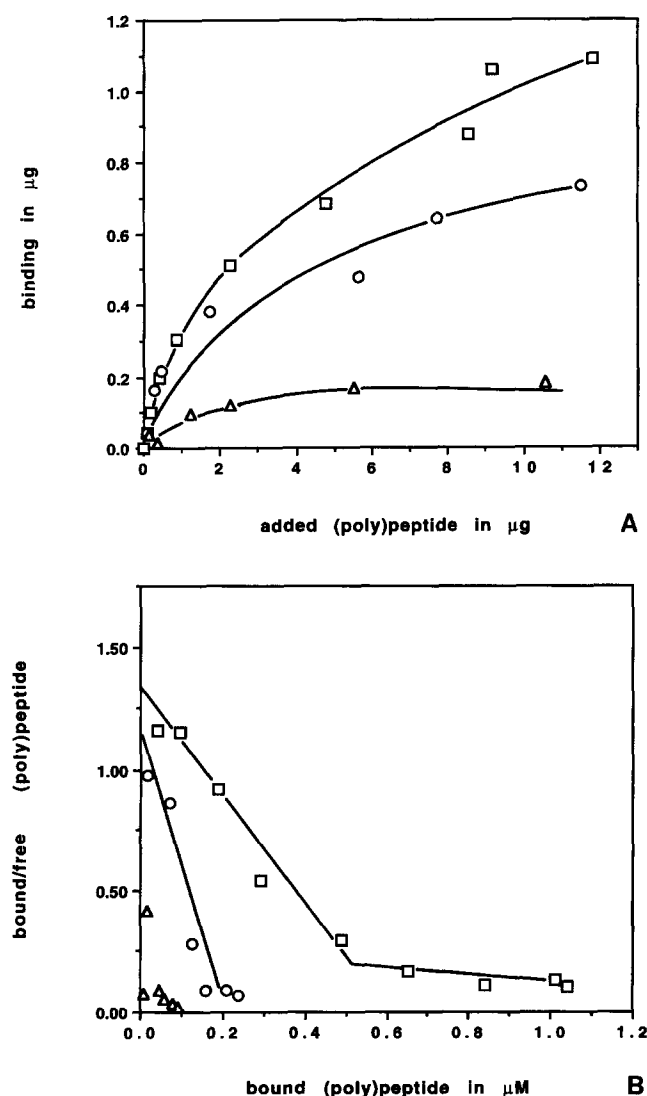


Fig. 1. (A) Binding of prefd ( $\circ$ ), trfd ( $\square$ ) and apofd ( $\triangle$ ) to vesicles composed of a lipid extract of the chloroplast outer envelope membrane. (B) A Scatchard plot of these binding data.

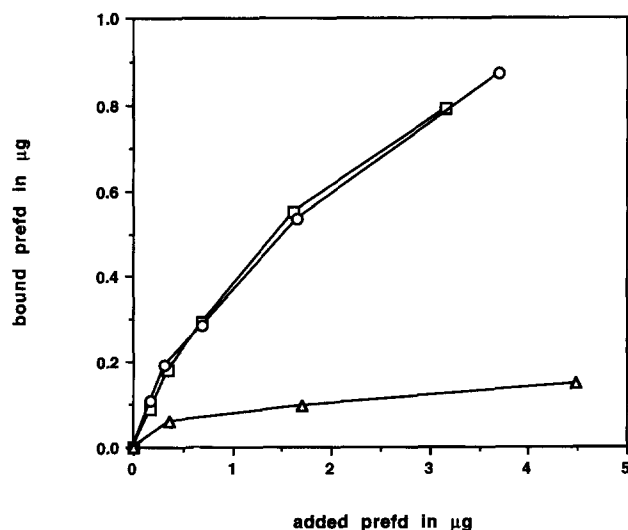


Fig. 2. Lipid specificity of prefd-vesicle binding. The vesicles had the following composition: ( $\square$ ), comparable to the chloroplast outer envelope membrane (MGDG 5%, DGDG 30%, SQDG 10%, DOPG 10%, DOPC 40%, PI 5%); ( $\circ$ ), comparable to the rat liver [38] mitochondrial outer membrane (DOPC 50%, DOPE 30%, DOPS 10%, CL 5%, cholesterol 5%) and ( $\triangle$ ), DOPC.

To get a first insight into the lipid specificity of prefd-vesicle binding, lipid mixtures corresponding to two organellar membranes involved in post-translational protein import were compared. Fig. 2 demonstrates that prefd binds with comparable affinity to vesicles mimicking lipid domains of the chloroplast outer envelope and the mitochondrial outer membrane. PC is the most abundant lipid class in both systems, yet prefd does not bind to vesicles prepared of this lipid (Fig. 2). Similar results were obtained for trfd-vesicle binding (data not shown). It can thus be concluded that specific lipids present within both systems are responsible for binding of the precursor to the lipid bilayers.

To search for these lipids the binding of prefd and trfd was investigated with vesicles composed of DOPC and 20% of either MGDG, DGDG, SQDG, DOPG or DOPE (Fig. 3). Incorporation of DOPE (which does not occur in chloroplasts), MGDG (the most abundant chloroplast lipid), DOPG and SQDG all significantly stimulated the binding of prefd to vesicles with the largest effects for the anionic lipids SQDG and DOPG. This demonstrates that SQDG and DOPG are primarily responsible for the binding of the precursor to the outer envelope membrane lipid extract. In analogy, it can be suggested that PS and CL are primarily involved in the binding of prefd to the lipid extract of the mitochondrial outer membrane.

Insertion of prefd in monolayers of MGDG and DOPG resulted in similar surface pressure increases [6], which appears to contradict the vesicle binding data. To get insight into this controversy, we determined the amount of monolayer-associated [ $^{14}\text{C}$ ]prefd at an initial surface pressure of 20 mN/m (data not shown). This revealed that 14.5% of the added prefd was associated with DOPG monolayers, whereas 5.9% was associated to the MGDG monolayer, in agreement with the preferential binding to DOPG-containing vesicles. This demonstrates that prefd is differently inserted into MGDG and DOPG monolayers. Using the  $\pi$ -A characteristics of DOPG [28] and MGDG [29] it was calculated that an inserted prefd molecule

in a DOPG monolayer occupies  $406 \text{ \AA}^2$  and in a MGDG monolayer  $648 \text{ \AA}^2$ .

Association of (poly)peptides to lipid domains can exert large effects on the lipid organization as is recently shown for the transit sequence of preferredoxin [7]. In order to investigate whether this results in a loss of barrier function vesicle leakage experiments were performed. The carboxyfluorescein (CF) release method [30] was used for this purpose. Polypeptide induced release of CF results in a strong dilution of the probe and an increase of its fluorescence level.

Fig. 4 demonstrates that addition of up to  $10 \mu\text{M}$  prefd to vesicles made of a lipid extract of the chloroplast outer envelope membrane does not cause a release of enclosed CF. The same is true for the apo-protein. It can thus be concluded that the barrier of the vesicles remains intact upon transit sequence mediated binding of the precursor. Addition of trfd to the vesicles does cause a partial release of CF from the vesicles. It should be noted that peptide-lipid ratios required to induce CF release ( $36 \mu\text{M}$  lipid,  $2.5 \mu\text{M}$  peptide) are much higher than those used in the binding experiments ( $670 \mu\text{M}$  lipid,  $0.27\text{--}5.7 \mu\text{M}$  peptide). Compared to the presequence of the mitochondrial precursor protein cytochrome *c* oxidase subunit IV (pCOX IV) trfd is much less potent to release CF (Fig. 4). The strong surface seeking property of pCOX IV [31] and its ability to adopt amphipathic helical structures upon interactions with lipid domains may be responsible for this. The lipid specificity of trfd induced CF release was investigated by the addition of trfd to vesicle suspensions composed of DOPC and 20% of MGDG, DGDG, SQDG, DOPE or DOPG (Fig. 5). It is demonstrated that trfd only mediates CF release if the vesicles contain the anionic lipids DOPG and SQDG which corresponds to the observed specificity of trfd binding.

#### 4. Discussion

In this study it is shown that prefd is able to bind to extended lipid bilayers present in large unilamellar vesicles. Prefd-vesicle binding is a specific process depending on the presence of the

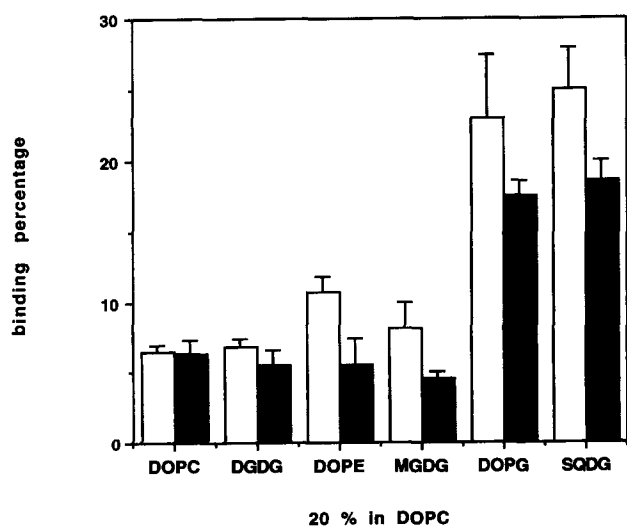


Fig. 3. Lipid specificity of binding of prefd and trfd to vesicles. Binding of prefd (open bars) and trfd (closed bars) is presented as the percentage of the added peptide ( $0.2 \mu\text{g}$  in both cases). Mean values and standard deviations of three individual experiments are shown.

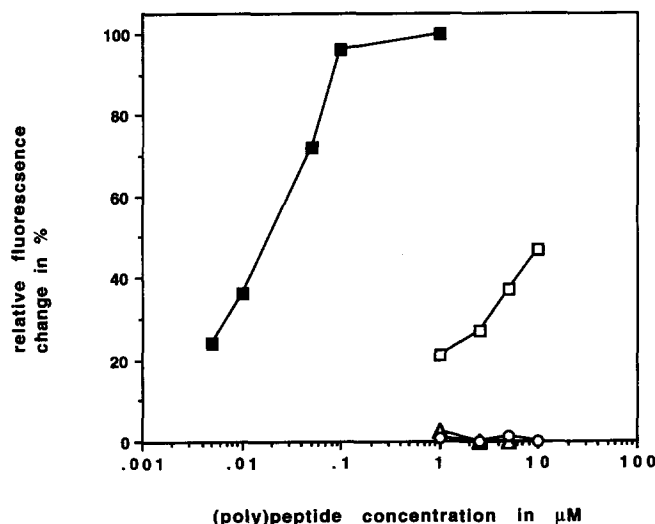


Fig. 4. Effect of trfd ( $\blacksquare$ ), prefd ( $\square$ ), apofd ( $\triangle$ ) and pCOX IV ( $\blacksquare$ ) on the CF-release from vesicles composed of a lipid extract of the chloroplast outer envelope membrane.

transit sequence as was concluded from the strongly reduced vesicle binding of apofd and the efficient vesicle binding of trfd. Analysis of the data of trfd binding to vesicles composed of a lipid extract of the chloroplast outer envelope membrane revealed that this binding was biphasic, consisting of a high and low affinity component. The dissociation constants ( $K_d$ ) of the high affinity binding site for binding of trfd to lipid vesicles is  $270 \text{ nM}$ . This is much larger than the  $K_d$  of  $8.6 \text{ nM}$  reported for binding of the precursor protein of the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (preSSU) to chloroplasts [32]. This difference indicates that the (poly)peptides are bound by different mechanisms to the model and biological membranes, and that other components than lipids are involved in high affinity binding to chloroplasts.

The lipid specificity of prefd-vesicle binding was rather broad. Binding was stimulated by MGDG, SQDG, PG and PE. In contrast, the abundant chloroplast membrane lipids DGDG and PC did not stimulate prefd-vesicle binding. This specificity was also observed for the insertion of prefd into lipid monolayers [6]. The largest prefd-vesicle binding was observed with anionic lipids SQDG and PG. This indicates that these anionic lipids are predominantly involved in binding of prefd to vesicles composed of a lipid extract of the chloroplast outer envelope membrane and that mainly electrostatic interactions are involved in the binding process.

In contrast to the results presented in this article, peptides corresponding to the N-terminal region of the transit sequence of the thylakoid protein plastocyanin do not bind to lipid vesicles under comparable conditions [33]. The reason for this difference is unknown but most likely reflects specific structural features of these different peptides.

Binding of prefd to vesicles made of a chloroplast outer envelope membrane lipid extract does not cause a loss of barrier function despite the ability of transit sequence to reorient lipid membranes [7]. The transit peptide is more lytic and causes partial CF-release in particular due to interactions with the anionic lipids. Apparently, the mature part of the precursor prevents this lytic action.

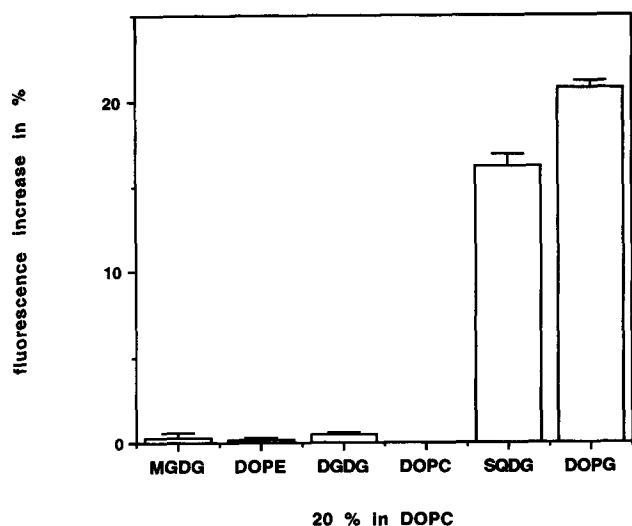


Fig. 5. Lipid specificity of trfd induced CF leakage. The fluorescence increase was measured 7 minutes after the addition of trfd (2.5  $\mu$ M final concentration). The fluorescence increase was constant after 6 min. Mean values and standard deviation of three independent measurements are shown.

Monolayer experiments revealed that prefd induces a significantly larger surface pressure increase into monolayers with a lipid composition comparable to that of the chloroplast outer envelope as compared to monolayers with a lipid composition comparable to that of the mitochondrial outer membrane [6]. This indicates that prefd inserts to a larger extent in its target lipid layer. In this respect, MGDG is of special interest because MGDG in mixtures with PC can serve as insertion site for prefd [6], possibly due to its special surface packing arrangement [7]. Monolayer analysis of the size of the lipid inserted domain of prefd has shown that in DOPG and MGDG monolayers the inserted prefd occupies 406 and 648  $\text{\AA}^2$ , respectively, thus MGDG facilitates specific insertion of a larger domain of the precursor into the lipid phase. In a recent deletion analysis of the functional domains of the ferredoxin transit sequence it was observed that the N-terminal region is involved in the initial chloroplast recognition process and also interacts specifically with MGDG [34]. Because the N-terminal region is enriched in amino acids-containing hydroxylated side chains [35] it could undergo specific interactions via hydrogen bonding to lipids. The inserted N-terminal region of the transit sequence could serve to specifically anchor the precursor into the lipid phase of the chloroplast envelope membrane, as was proposed by Pilon et al. [34]. In addition, the lipid inserted precursor could diffuse in a 2-dimensional way more efficiently to the import machinery than via 3-dimensional diffusion through the aqueous phase. Furthermore, the inserted N-terminus could act as recognition motif for the import machinery and can be responsible for the induced changes in lipid organization [7], which can directly be involved in protein import [36] or be required for the activation of the import machinery.

Finally, we want to discuss our findings in the light of specific targeting of precursor proteins to chloroplasts. This study demonstrates that prefd binds with equal efficiency to vesicles with lipid compositions comparable to the lipid compositions of the chloroplast and mitochondrial outer membrane. Therefore, if

transit sequence-mediated binding of precursor proteins to organelles is facilitated by binding to lipid domains, precursor proteins will bind both to chloroplasts and mitochondria. This was indeed observed for the precursor of the stromal 33 kDa protein subunit of the water-splitting enzyme [37]. The precursor was imported into chloroplasts and bound only loosely to mitochondria. This could indicate an initial and reversible binding of the precursor to the lipid part of membranes. Further steps in import must be responsible for organelle specificity. For instance, subsequent interactions of precursor proteins with proteinaceous components of the import machinery mediated via specific lipid components of the chloroplasts.

**Acknowledgments:** We thank Karin Brouwer for preparing the manuscript, Dr. R.A. Demel for his advice on the monolayer experiments, Dr. M. Pilon for advice in purification and labeling of prefd and apofd, and G. de Korte-Kool for assistance in trfd purification. This work was supported by The Netherlands Foundation for Biological Research (BION) and The Netherlands Foundation for Biophysics with financial aid from The Netherlands Organization for Scientific Research (N.W.O.).

## References

- [1] De Boer, A.D. and Weisbeek, P.J. (1991) *Biochim. Biophys. Acta* 1071, 221–253.
- [2] Kerber, B. and Soll, J. (1992) *FEBS Lett.* 306, 71–74.
- [3] Flüge, U.I. (1990) *J. Bioenerg. Biomembr.* 22, 769–787.
- [4] Cline, K. (1985) *J. Biol. Chem.* 260, 3691–3696.
- [5] Van 't Hof, R., Demel, R.A., Keegstra, K. and De Kruijff, B. (1991) *FEBS Lett.* 291, 350–354.
- [6] Van 't Hof, R., Van Klompenburg, W., Pilon, M., Kozubek, A., De Korte-Kool, G., Demel, R.A., Weisbeek, P.J. and De Kruijff, B. (1993) *J. Biol. Chem.* 268, 4037–4042.
- [7] Chupin, V., Van 't Hof, R. and De Kruijff, B. (1994) *FEBS Lett.* 350, 104–108.
- [8] Horniak, L., Pilon, M., Van 't Hof, R. and De Kruijff, B. (1993) *FEBS Lett.* 334, 241–246.
- [9] Douce, R. and Joyard, J. (1990) *Annu. Rev. Cell Biol.* 6, 173–216.
- [10] Block, M.A., Dorne, A.-J., Joyard, J. and Douce, R. (1983) *J. Biol. Chem.* 258, 13273–13280.
- [11] Billecocq, (1974) *Biochem. Biophys. Acta* 352, 245–251.
- [12] Pilon, M., De Kruijff, B. and Weisbeek, P.J. (1992) *J. Biol. Chem.* 267, 2548–2556.
- [13] Fabrie, Ch.H.J.P., De Kruijff, B. and De Gier, J. (1990) *Biochim. Biophys. Acta* 1024, 380–384.
- [14] Smeekens, S., Van Binsbergen, J. and Weisbeek, P.J. (1985) *Nucleic Acids Res.* 13, 3179–3194.
- [15] Von Heijne, G., Steppuhn, J., and Herrmann, R.G. (1989) *Eur. J. Biochem.* 180, 535–545.
- [16] Pilon, M., De Boer, A.D., Knols, S.L., Koppelman, M.H.G.M., Van der Graaf, R.M., De Kruijff, B. and Weisbeek, P.J. (1990) *J. Biol. Chem.* 265, 3358–3361.
- [17] Pilon, M., Rietveld, A.G., Weisbeek, P.J. and De Kruijff, B. (1992) *J. Biol. Chem.* 267, 19907–19913.
- [18] Jentoft, N. and Dearborn, D.G. (1979) *J. Biol. Chem.* 254, 4359–4365.
- [19] Schagger, H. and Von Jagow, G. (1987) *Anal. Biochem.* 166, 368–379.
- [20] Van 't Hof, R. and De Kruijff, B. (1994) submitted.
- [21] Bradford, M.M. (1976) *Anal. Biochem.* 72, 248–254.
- [22] Ellman, G.L. (1959) *Arch. Biochem. Biophys.* 82, 70–77.
- [23] Van Deenen, L.L.M., and De Haas, G.H. (1964) *Adv. Lipid. Res.* 2, 168–229.
- [24] Comfurius, P., and Zwaal, R.F.A. (1977) *Biochim. Biophys. Acta* 488, 380–384.
- [25] Mayer, L.D., Hope, M.J., and Cullis, P.R. (1986) *Biochim. Biophys. Acta* 858, 161–168.
- [26] Rouser, G., Fleisher, S. and Jamamoto, A. (1970) *Lipids* 5, 494–496.

- [27] Demel, R.A. (1982) in: *Membranes and Transport*, Vol. 1 (Martonosi, A.N. ed.) pp. 159–164, Plenum Press, New York.
- [28] Smaal, E., Mandersloot, J., Demel, R.A., De Kruijff, B. and De Gier, J. (1987) *Biochim. Biophys. Acta* 897, 180–190.
- [29] Demel, R.A., De Swaaf, M.E., Van 't Hof, R., Mannock, D.A. and De Kruijff, B. (1994) *Mol. Membr. Biol.* (submitted).
- [30] Chen, R.F. (1977) *Anal. Lett.* 10, 787–795.
- [31] Endo, T. and Schatz, G. (1988) *EMBO J.* 7, 1153–1158.
- [32] Friedman, A.L. and Keegstra, K. (1989) *Plant Physiol.* 89, 993–999.
- [33] Endo, T., Kawamura, M. and Nakai, M. (1992) *Eur. J. Biochem.* 207, 671–675.
- [34] Pilon, M., Wienk, H., Sips, W., De Swaaf, M.E., Talboom, I., Van 't Hof, R., De Korte-Kool, G., Weisbeek, P.J. and De Kruijff, B. (1994) *J. Biol. Chem.* (submitted).
- [35] Keegstra, K., Olsen, L.J. and Theg, S.M. (1989) *Annu. Rev. Physiol. Plant Mol. Biol.* 40, 471–502.
- [36] Killian, J.A., De Jong, A.M.Ph., Bijveldt, J., Verkleij, A.J. and De Kruijff, B. (1990) *EMBO J.* 9, 815–819.
- [37] Whelan, J., Knorpp, C., Harmey, M.A. and Glaser, E. (1991) *Plant. Mol. Biol.* 16, 283–292.
- [38] Daum, G. (1985) *Biochim. Biophys. Acta* 822, 1–42.