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Lipid–peptide interactions between fragments of the transit peptide of ribulose-1,5-bisphosphate carboxylase/oxygenase and chloroplast membrane lipids

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The interactions of fragments of the transit peptide of ribulose-1,5-bisphosphate carboxylase/oxygenase with lipid monolayers was studied in order to investigate the possible involvement of the membrane lipids in the protein import process The fragments are surface active and have a differential ability to insert in lipid monolayers The fragments have a preference for the chloroplast galacto- and sulpholipids and phosphatidylglycerol and interact with envelope membrane lipid extracts These results suggest that probably transit peptide–lipid interactions are involved in the chloroplast protein import process

Chloroplast protein import, Transit peptide, Peptide–lipid interaction, Galactolipid, Sulpholipid, Small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase

1 INTRODUCTION

Most chloroplast proteins are coded by nuclear genes and are synthesized in the cytoplasm, as precursors with an amino terminal extension, the transit peptide After their synthesis they are post-translationally imported in the chloroplasts in an energy-dependent manner [1] The import is mediated by the transit peptide which contains all the information necessary for import [2,3].

Despite much progress, the molecular mechanism by which a transit peptide is able to direct a precursor protein into a chloroplast is unknown (for review see [4]) Attempts have been made to dissect the transit peptide into functional domains, such as binding domains and those domains participating in processing or translocation [5] At the level of the envelope membranes, proteinaceous receptors involved in precursor binding have been tentatively identified [6–9] However, no components involved in the translocation process have yet been identified. One intriguing possibility that has not received much attention is that the envelope membrane lipids are involved in the import process This hypothesis is particularly attractive because the envelope membranes have a unique lipid composition, containing high amounts of the specific chloroplast galactolipids monogalactosyldiacylglycerol and digalactosyldiacylglycerol as well as the sulpholipid sulphoquinovosyldiacylglycerol

As a first step in evaluating the possible involvement of membrane lipids in the precursor protein import process, we have measured the capacity of peptides mimicking parts of a transit sequence to interact with membrane lipids in monolayers at the air–water interface We made use of three fragments of the transit peptide of the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase from pea, each 20 amino acids long and which correspond to the amino-terminal, the middle and the carboxy-terminal part of the transit peptide It is shown that the peptides have a differential ability to insert into lipid monolayers with a preference for anionic and the typical chloroplast lipids The results are discussed in the light of the results of a recent study [10] in which these peptides were used as inhibitors of the import process

Abbreviations prSS, precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase, MGDG, monogalactosyl diacylglycerol, DGDG, digalactosyldiacylglycerol, SQDG, sulphoquinovosyl diacylglycerol, DOPC, dioleoyl-*sn*-glycero-3-phosphocholine, DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol, PIPES 1,4-piperazindiethane sulfonic acid, HPLC, high performance liquid chromatography, HPTLC, high performance thin layer chromatography

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2. MATERIALS AND METHODS

Fragments of the prSS transit peptide corresponding to the amino-terminal, F_{1-20}, the middle, F_{21-40}, and the carboxy-terminal, F_{41-60}, regions were synthesized by Multiple Peptide Systems [10] The amino

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Table I

Amino acid sequence and charge of the fragments of the transit peptide of SSU used in this study

Fragment	Amino acid sequence	Hydrophobicity
F_{1-20}	1 + + +M A S S I S S S A V T T V S R A S R G Q-amide	-0 8
F_{21-40}	+ 2 + + +S A A V A P F G G L K S A T G F P V K K-amide	4 8
F_{41-60}	− + + +V N T D I T S I T S N G G R V K C M Q V-amide	0 0

[1]The original methionine in pea prSS is changed for serine
[2]The original methionine in pea prSS is changed for alanine

acid sequence, calculated charge and the mean hydrophobicity, according to the normalized consensus of Eisenberg [11], are listed in Table I The methionine residues at the positions 4 and 33 in the amino acid sequence of pea prSS were changed for serine and alanine, respectively, in order to reduce oxidative damage of the peptides It is unlikely that these changes have a deleterious effect on the transit peptide functions because the serine and alanine are most often found at these positions in prSS of other species [12] The peptides were purified by reversed phase HPLC on a 25×1 cm column packed with S1100 (10 μm) poloyl RP_{18} (Serva) eluted with linear water-acetonitrile gradients containing 0 1% (v/v) trifluoracetic acid The purity of the peptides was more than 95% as was shown by HPLC After lyophilization, the peptides were stored as dry material at −20°C Stock solutions were prepared by dissolving the peptides in distilled water at a concentration of 0 25 mg/ml by weight The actual concentration was determined with identical results according to Bradford [13] and Lowry [14] using bovine serum albumine (BSA) as reference The stock solutions were stored at −20°C

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine and 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol were synthesized according to established methods [15]

Chloroplasts were isolated from 14 days old pea seedlings cv Feltham first as described [16] by using the Percoll-gradient centrifugation method. Outer and inner envelope membrane fractions were isolated according to [17] Lipid extracts were obtained by extraction according to Bligh and Dyer [18] The lipid composition of the outer- and inner membrane lipid extracts was determined by 2-dimensional HPTLC analysis in the solvent systems $CHCl_3$/MeOH/H_2O (65 25 4, v/v) and $CHCl_3$/acetone/MeOH/HAc/H_2O (100 40 20 10, v/v) The amount of MGDG, DGDG, and SQDG was determined according to Roughan et al [19], the amount of phospholipids according to Rouser [20]

MGDG, DGDG, and SQDG were isolated from a Bligh and Dyer extract [18] of thylakoid membranes by chromatography on a carboxymethyl cellulose column (3×40 cm) (CM52, sodium form, Whatman) eluting with chloroform/methanol mixture according to Comfurius and Zwaal [21] MGDG, DGDG, and SQDG were found to elute at 2%, 14% and 25% methanol in chloroform, respectively Remaining traces of pigments were removed by preparative TLC using the eluting solvent $CHCl_3$/acetone/MeOH/Hac/H_2O (100 40 20 20 10) The spots were scraped off and the lipids were eluted from the silicagel by means of hexane extraction It was shown by HPTLC-analysis that the purity of the individual lipids was more than 99%

Monolayer experiments were performed at 25°C in a thermostatically controlled box [22] The subphase buffer consisted of 50 mM sodium chloride, 10 mM PIPES (pH 7 4) The monomolecular layers were formed at the water–air interface in a teflon trough with a diameter of 2 cm and a depth of 0 6 cm The subphase was stirred with a magnetic bar The peptides were added to the subphase through a small hole at the edge of the trough The surface pressure increase was measured after an equilibrium was reached Each experiment was carried out at least 3 times Mean values and standard deviations are shown in the figures Injection of the peptides did not change the subphase pH

3 RESULTS

Peptide–lipid interactions can be determined by the change in surface pressure of a lipid monolayer after the addition of peptide to the aqueous phase The surface pressure changes are interpreted as a result of the insertion of peptides into the lipid layer Fig 1 shows for the three fragments of the prSS transit peptide the surface pressure increase in DOPG (selected as a model anionic lipid) monolayers at different initial pressure. The limiting pressure is defined as the pressure where the peptide can no longer penetrate and consequently the change in the surface pressure is zero. The strongest interaction is found for peptide F_{21-40}, with a limiting pressure of 33 mN/m Peptide F_{1-20} and F_{41-60} show smaller pressure increases with limiting pressures of 31 and 26 mN/m, respectively. For DOPG monolayers at an initial pressure of 20 mN/m, maximal pressure changes of 3.7, 5.4

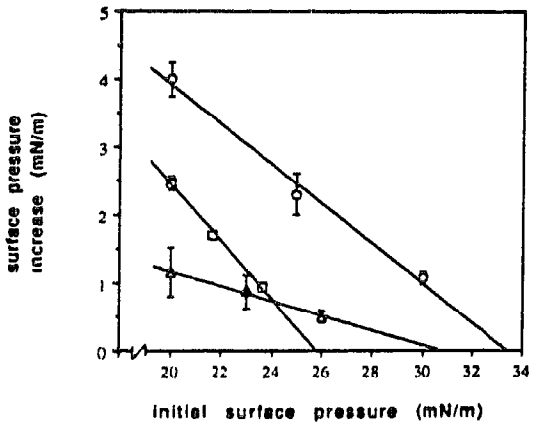

Fig 1. Surface pressure increases after injection of prSS transit peptide fragments F_{1-20} (○), F_{21-40} (▽), and F_{41-60} (□) underneath lipid monolayers of DOPG at different initial pressures The peptide concentration was 1 25 μg/ml

and 4 8 mN/m were measured for F_{1-20}, F_{21-40} and F_{41-60}, respectively at peptide concentrations of 5 μg/ml

The influence of the charge density of the monolayer on the interactions was studied by forming monolayers of DOPG and DOPC mixtures Since DOPG and DOPC occupy practically the same molecular areas, in the mixtures only the charge density is changed and not the molecular packing Fig. 2 shows that, at an initial surface pressure of 20 mN/m, there is no interaction of the peptides with the zwitterionic lipid DOPC and that the pressure increase becomes greater with increasing DOPG concentration. The pressure increase due to the added peptide is nearly maximal at 20 mol% DOPG For all lipid mixtures the surface pressure increase after peptide addition decreased in the following order: $F_{21-40} > F_{41-60} > F_{1-20}$.

To investigate if the peptides are also able to interact with the specific chloroplast lipids, monolayers were formed consisting of MGDG, DGDG, or SQDG at an initial pressure of 20 mN/m. The results are shown in Fig. 3 Most interestingly, fragment F_{41-60} was able to give a relatively large surface pressure increase in MGDG and SQDG monolayers and to a lesser extent in DGDG monolayers. These surface pressure increases are only slightly smaller than the surface pressure increase in DOPG but much larger than that observed for the zwitterionic, but overall neutral DOPC. Fragment F_{21-40} caused a comparable surface pressure increase in monolayers of SQDG as F_{41-60}. The surface pressure increase caused by F_{21-40} in MGDG and DGDG were significantly smaller than the surface pressure increase in SQDG. Fragment F_{1-20} caused only small surface pressure increases in MGDG and DGDG The surface pressure increase with SQDG caused by F_{1-20} was smaller than for F_{21-40} and F_{41-60} but approached the value observed for DOPG.

In order to determine whether the fragments are also capable to interact with monolayers of which lipid composition reveals the lipid composition of the outer and inner envelope membrane, the fragments are injected under monolayers of total lipid extracts of the outer or inner envelope membrane (Fig 4). The measured lipid compositions of the outer (4% MGDG, 32% DGDG, 6% SQDG, 45% PC, 5% PC and 5% PI) and inner envelope membrane (49% MGDG, 30% DGDG, 6% SQDG, 6% PC, 7% PG and 1% PI) are in good agreement with previously published results [23,24] Fragment F_{41-60} causes with lipid extracts from either membrane a considerable surface pressure increase which is comparable to the pressure increase caused by F_{21-40} with the outer envelope membrane lipid extract. The surface pressure increase caused by F_{21-40} with the inner envelope membrane lipid extract was significantly smaller than the surface pressure increase with the outer envelope membrane lipid extract. Fragment F_{1-20} again

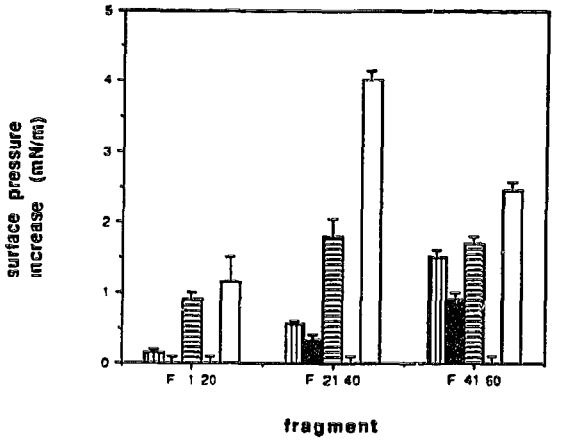

Fig 3 Surface pressure increases after injection of prSS transit peptide fragments F_{1-20}, F_{21-40}, and F_{41-60} underneath monolayers of MGDG (▥), DGDG (■), SQDG (▤), DOPC (▧) and DOPG (□) at an initial pressure of 20 mN/m The fragment concentration in the subphase was 1 25 μg/ml

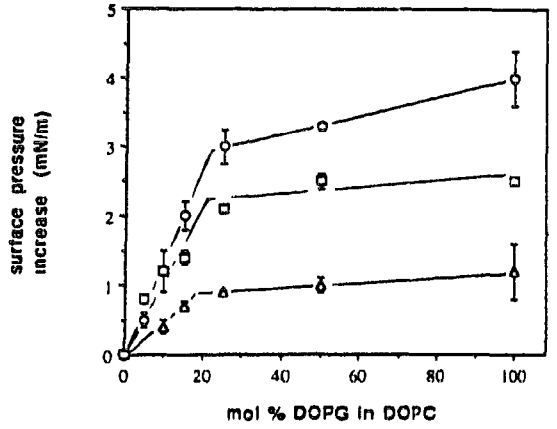

Fig. 2. Surface pressure increases after injection of prSS transit peptide fragments $F_{1\ 20}$ (△), F_{21-40} (○), and F_{41-60} (□) under mixed monolayers. The monolayers contained DOPC with increasing concentration of DOPG at an initial pressure of 20 mN/m The fragment concentration in the subphase was 1–25 μg/ml

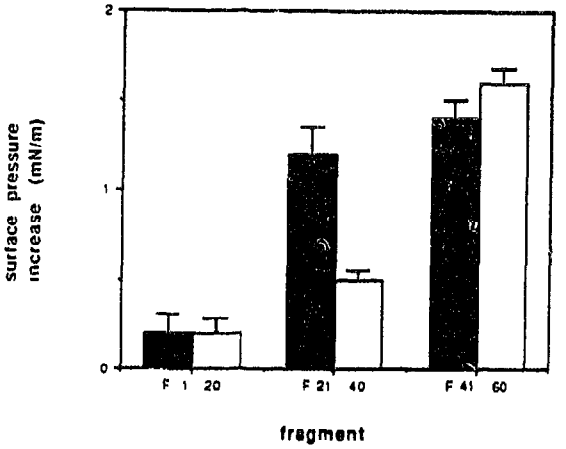

Fig. 4. Surface pressure increases after injection of prSS transit peptide fragments $F_{1\ 20}$, $F_{21\ 40}$, and F_{41-60} underneath monolayers of lipid extracts of the outer (■) and inner envelope membrane (□) at an initial pressure of 20 mN/m The fragment concentration in the subphase was 1.25 μg/ml

caused only small pressure increases with lipid extracts from either membrane.

In an effort to evaluate the involvement of electrostatic interactions, the ionic strength of the subphase solution was varied With monolayers of DOPG and SQDG, both negatively charged lipids, the pressure increases caused by the fragments were decreased by 95% and 20%, respectively, at a sodium chloride concentration of 400 mM (data not shown). The surface pressure increases with monolayers of MGDG and DGDG were not affected by the addition of sodium chloride at concentrations up to 400 mM From these results, we conclude that electrostatic interactions are important in the associations between the peptides and the anionic lipids. Interestingly, the interaction of the peptides wth the lipid extracts from the outer and inner envelope membranes showed only a very small decrease in surface pressure increase, even at a sodium chloride concentration of 400 mM

4 DISCUSSION

From the results presented here, we conclude that peptides corresponding to regions of the prSS transit peptide are surface active and insert into lipid monolayers and that the interactions between the peptides and the lipid monolayers are peptide- and lipid-specific

The extent of the interactions of the peptides with monolayers of the negatively charged DOPG are smaller when compared to the interactions of the mitochondrial presequence of subunit IV of yeast cytochrome *c* oxidase [25] This might be explained by the higher charge (+6), the conformation and the lipid specificity of the presequence

A very interesting result shown in this paper is the ability of the peptides, especially $F_{41\ 60}$, to interact with the uncharged galactolipids while there is no interaction with the zwitterionic and overall neutral DOPC Preliminary results revealed that these interactions are even more pronounced for the full length precursor protein of ferredoxin (R van 't Hof, unpublished results) The interactions with the galactolipids are not of electrostatic nature but are probably mediated by hydrogen bond formation between the hydroxy groups of hydroxylated amino acids of the peptides and the sugar moieties of the lipids. This is probably also the case for the interaction of the peptides with SQDG which is only to a small extent electrostatic in nature The fact that $F_{1\ 20}$, which contains the highest amount of hydroxylated amino acids shows the weakest interaction with the galacto- and sulpholipids suggests that other factors are also involved like the peptide hydrophobicity and conformation The fragments interact with the negatively charged DOPG and to a lesser extent with SQDG, by electrostatic interactions The higher positive charge and probably the higher hydrophobicity of $F_{21\text{-}40}$ (Table I) might explain the stronger interaction of this peptide with those lipids. The electrostatic interaction with DOPG depends on the charge density of the monolayer and is almost maximum at 20 mol% DOPG Interestingly, this value corresponds roughly to the amount of negatively charged lipids in the outer envelope membrane.

Interestingly, the peptides are also able to interact with the chloroplast envelope membrane lipid extracts These interactions are insensitive to high ionic strength and are probably dominated by interactions with the galactolipids It is not possible to quantitatively relate the interactions with the lipid extracts directly to interactions with MGDG and DGDG Therefore it is likely that the interactions are the result of the specific properties of the envelope membrane lipid extracts

In a recent study by Perry et al [10], the same peptides of the prSS transit peptide were used in competition experiments on chloroplast precursor protein binding and translocation It was shown that $F_{1\text{-}20}$ had a slight effect on prSS translocation but lyses the chloroplasts at high concentration From the results it was suggested that $F_{1\ 20}$ is involved in interactions with the lipids of the envelope membrane However, the results shown in this paper make this interpretation very unlikely because $F_{1\text{-}20}$ very weakly interacts with the individual lipids and envelope membrane lipid extracts Neither this paper, nor the paper of Perry et al. makes clear the role of the N-terminus of the prSS transit peptide but possibly it is involved in a step between the initial binding and translocation step Peptide $F_{21\text{-}40}$ had a strong effect on prSS binding and Perry et al concluded that this fragment is involved in the precursor binding process, probably by interacting with a receptor The results which are shown in this paper suggest that the negatively charged lipids SQDG and DGDG might also be involved in this step The C-terminus of the transit peptide, $F_{41\ 60}$, had a strong effect on precursor translocation probably by interacting with some parts of the transport machinery beyond the binding step. Interestingly, this fragment interacts strongly with the specific chloroplast galacto- and sulpholipids Especially the interactions with MGDG are of great interest because MGDG is able to form Hexagonale H_{II} membrane structures [26], which are thought to be involved in protein import processes [27] Therefore, the interactions with MGDG may directly be involved in the translocation step of precursor proteins

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