

The secondary structure of the ferredoxin transit sequence is modulated by its interaction with negatively charged lipids

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Import of proteins into chloroplasts depends on an N-terminal transit sequence. Transit sequences contain little primary sequence similarity and therefore recognition of these sequences is thought to involve specific folding. To assess the conformational flexibility of the transit sequence, we studied the transit peptide of preferredoxin (trfd) by circular dichroism. In buffer, trfd is in a random coil conformation. A large increase in α -helix was induced in the presence of micelles or vesicles formed by anionic lipids. Less pronounced changes in secondary structure were induced by zwitterionic detergents but no changes were observed in the presence of neutral detergents or vesicles composed of phosphatidylcholine.

Chloroplast protein import; Transit peptide; Circular dichroism; Peptide conformation; Lipid-protein interactions

1. INTRODUCTION

The chloroplast stroma protein ferredoxin is encoded in the nuclear genome. It is synthesized in the cytosol as a precursor with a typical N-terminal transit sequence [1] which is cleaved off by a stromal protease after the ATP-dependent translocation across the chloroplast envelope (see for review [2]). Deletion and gene fusion experiments have shown that the transit sequence contains the information that is both necessary and sufficient for the uptake of its passenger by the chloroplast [3,4]. Using the purified precursor of ferredoxin, it was shown that chloroplast recognition and import do not require cytosolic factors which means that direct transit sequence–chloroplast envelope interactions are involved [5].

In attempts to identify components of the import machinery, most of the attention has been focussed on envelope proteins [2]. The involvement of an ATPase is inferred from the ATP dependency of binding and

translocation [6]. Furthermore, it has been shown that protease treatment of intact chloroplasts diminishes the import, indicating the involvement of surface exposed proteins [7]. Much less attention has been given to the possible roles of envelope lipids. Recently it has been shown that the ferredoxin precursor inserts into a lipid surface composed of lipids isolated from its biological target, the outer envelope membrane [8]. This insertion depends on the presence of the transit sequence and is mainly mediated by the negatively charged lipid phosphatidylglycerol and sulfolipid (SQDG) and the neutral monogalactosyldiacylglycerol (MGDG). Insertion takes place at surface pressures believed to be present in a biological membrane. This capability of the precursor can help to constitute an efficient import pathway. The chloroplast membrane has a high lipid/protein ratio and therefore lipid domains will be relatively exposed [9]. Furthermore, the outer envelope membrane is the only membrane exposed to the cytosol that contains the galactolipids and sulfolipid favoured by the transit sequence. Therefore, specific insertion into the lipid phase followed by lateral diffusion may increase the chance of productive interactions with the import machinery.

Perhaps in contrast to what is expected if a common and specific mechanism is involved, there is very little sequence similarity between the transit sequences of different precursors [10]. This seemingly low shared information content contrasts with the observed specificity and selectivity of the protein targeting pathways to chloroplasts in plant cells. It is difficult to envisage how a degenerate signal like a transit sequence can undergo

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Abbreviations: trfd, transit peptide of ferredoxin; C₁₂-Pglycol, dodecylphosphoglycol; C₁₄-Pglycol, tetradecylphosphoglycol; C₁₂-PN, dodecylphosphocholine; C₁₂-maltose, dodecyl- β -D-maltose; C₁₀-glucose, decyl- β -D-glucose; lysoC₁₆-PC, 1-palmitoyl-*sn*-glycero-3-phosphocholine; lysoC₁₄-PC, 1-myristoyl-*sn*-glycero-3-phosphocholine; DHPC, 1,2-diheptyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulphoquinovosyldiacylglycerol; CD, circular dichroism; TLC, thin layer chromatography; SUVs, small unilamellar vesicles; CMC, critical micelle concentration.

the specific recognitions involved in the import process if this only involves primary structure motifs. Therefore it is proposed that the information of the transit sequence must be in its secondary or tertiary structural motifs [11]. At present, the physical properties of transit sequences that constitute the specific targeting signal and the mechanism by which the translocation machinery decodes these degenerated signals are not understood [11]. Recently it was shown that the ferredoxin transit sequence is unstructured in an aqueous solution but possesses a certain conformational flexibility depending on the environment [12]. By inducing specific structural changes upon interaction with the envelope lipids, defined secondary and tertiary structural elements might be formed which can serve as recognizable motifs for the import machinery.

This latter possibility deserves in our view more attention. Therefore we examined the conformational changes of the transit sequence induced by a lipid surface and the lipid specificity of this process. As a model peptide we have chosen the transit sequence of the well studied ferredoxin precursor. This peptide can be obtained in a state competent for interaction with the import machinery [12].

2. MATERIALS AND METHODS

2.1. Materials

Dodecylphosphoglycol (C_{12} -Pglycol) and tetradecylphosphoglycol (C_{14} -Pglycol) were synthesized as described by De Jongh and De Kruijff [13]. Dodecyl- β -D-maltose (C_{12} -maltose) and decyl- β -D-glucoside (C_{10} -glucose) were a kind gift from Dr. W. de Grip (Utrecht University). Dodecylphosphocholine (C_{12} -PN) was obtained from Cambridge Isotope Laboratories (Cambridge, UK). 1-Myristoyl-*sn*-glycero-3-phosphocholine (lyso- C_{14} -PC) and 1-palmitoyl-*sn*-glycero-3-phosphocholine (lyso- C_{16} -PC) were prepared from, respectively, dimyristoyl-phosphatidylcholine and dipalmitoylphosphatidylcholine by treatment with phospholipase A_2 from bee venom (Sigma) followed by purification by silica column chromatography, essentially as described by Hanahan [14]. Diacyl lipids: 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) were synthesized according to established methods [15,16]. 1,2-diheptyl-*sn*-glycero-3-phosphocholine (DHPC) was a kind gift from R. Dijkman (CBLE, Utrecht, The Netherlands). Monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG) were isolated from pea chloroplasts as described by Van 't Hof et al. [8]. Chemical synthesis and purification of the 47 amino acids long transit peptide (trfd) were performed as described. The purified peptide was dried and stored under N_2 at -20°C . Peptide was quantified as described by Pilon et al. [12].

2.2. Methods

Lipid-phosphorus was assayed by the method of Rouser et al. [17]. The concentrations of SQDG, DGDG and MGDG were determined by quantification of their methylated fatty acids by liquid gas chromatography using C_{17} margaric acid as internal standard.

2.2.1. Sample preparation

For micelle-containing solutions, the appropriate quantities of individual monoacyl lipids and mixtures thereof were prepared from stock solutions in methanol. Solvent was evaporated by reduced pressure for 1 h. Micelles were prepared by the addition of buffer: 10 mM Tris-

HCl, 50 mM NaCl, 1 mM DTT, pH 8.0, followed by vortexing. Peptides (20 μM final concentrations) were added together with the buffer.

To prepare lipid vesicles, the appropriate mixtures of diacyl lipids were prepared by mixing stock solutions in chloroform. Solvent was evaporated as above. The dried lipid film was hydrated in 10 mM Tris-HCl, 50 mM NaCl, 1 mM DTT, pH 8.0. Small unilamellar vesicles were prepared essentially as described by De Kroon et al. [18]. Peptide was added to the vesicles from concentrated stock solutions in 10 mM Tris-HCl, 50 mM NaCl, 1 mM DTT, pH 8.0, to give a final concentration of 20 μM .

2.2.2. Circular dichroism experiments

CD spectra were recorded in a 0.1 mm quartz cuvette at room temperature on a Jasco-600 spectropolarimeter connected to a computer. Typically 30 spectra were accumulated for each sample from 260 to 190 nm, with a resolution of 0.1 nm. The scan speed was 50 nm/min, the time constant 0.125 s, the band width 1 nm and the sensitivity range of the photomultiplier was 20 to -20 mdeg. All data were corrected by subtraction of spectra of protein-free samples. Spectral analysis was performed essentially as described by De Jongh and De Kruijff [13].

3. RESULTS

The main goal of this study is to obtain an insight into the conformational changes in the transit sequence induced by lipid surfaces composed of lipids found in the chloroplast outer envelope membrane. For this purpose, Circular Dichroism is the technique of choice because it provides a very sensitive and quantitative method to analyze the secondary structure of polypeptides in solution. However, lipid vesicles will cause turbidity in the samples which restricts the range of peptide-vesicle systems to be quantitatively analyzed. Moreover, some chloroplast lipids cannot form stable bilayers by themselves. Therefore, we have chosen first to study micellar systems composed of monoacyl lipids with head groups that closely resemble membrane lipids. Neutral detergents with a sugar head group mimic the galactolipids of the chloroplast outer envelope membrane. Anionic detergents were used to mimic

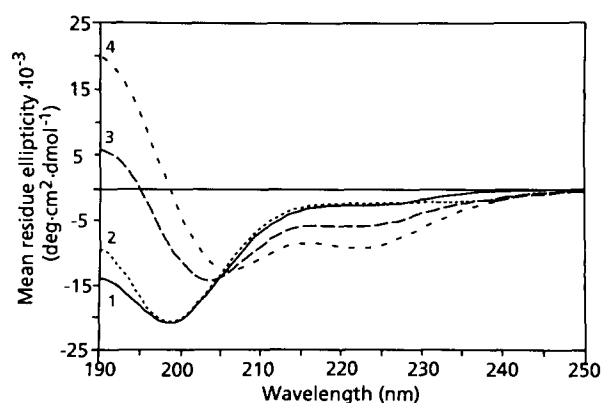


Fig. 1. Influence of micelles consisting of monoacyl lipids on the secondary structure of trfd. CD spectra of trfd (20 μM) were recorded in: (1) buffer or in the presence of micelle forming lipids, (2) 50 mM C_{12} -maltose, (3) 2 mM lyso C_{16} -PC, and (4) 1 mM C_{14} -Pglycol.

phosphatidylglycerol. Zwitterionic detergent and lyso-PC were used to mimic the phosphatidylcholine.

We first investigated the potency of the detergent micelles to induce secondary structure in the transit peptide. CD spectra of trfd in buffer or in the presence of detergents well above their critical micelle concentration (c.m.c.) are shown in Fig. 1. The CD spectrum of trfd in buffer has a characteristic minimum at 198 nm indicative of a polypeptide mostly in random coil conformation. Indeed, deconvolution of the spectrum indicates the presence of only 3% α -helix and 20% β -strand, which is in agreement with published data [12]. The addition of the negatively charged detergent C_{14} -Pglycol causes a significant change in the shape of the CD spectrum, indicating the induction of regular secondary structure. Deconvolution of this spectrum indicates that 52% α -helix and virtually no β -strand is present. The zwitterionic lyso C_{16} -PC also induces significant changes in the secondary structure (32% α -helix, no β -strand). Note that in both these cases the induction of α -helix is accompanied by a loss in β -strand structure. In contrast, the neutral detergent C_{12} -maltose does not significantly affect the secondary structure of trfd.

To assess quantitatively the effect of headgroup, chain length and concentration on α -helix induction, we performed a titration experiment with the different detergents. We recorded CD spectra of the transit sequence in the presence of different detergents over a range of detergent concentrations. The amount of trfd was kept constant at 20 μ M. The CD spectra were deconvoluted. The amount of α -helix as a function of

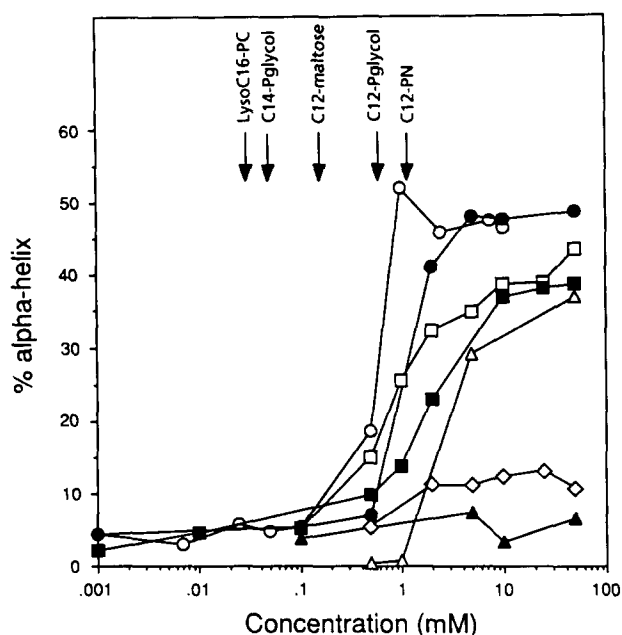


Fig. 2. Dependence of α -helix content in trfd on the concentration of different micelle forming lipids. \circ : C_{14} -Pglycol; \bullet : C_{12} -Pglycol; \square : lyso- C_{16} -PC; \blacksquare : lyso- C_{14} -PC; Δ : C_{12} -PN; \diamond : DHPC; \blacktriangle : C_{12} -maltose. The critical micelle concentrations of the detergents (see [13] and [25]) are indicated in the top of the figure by arrows.

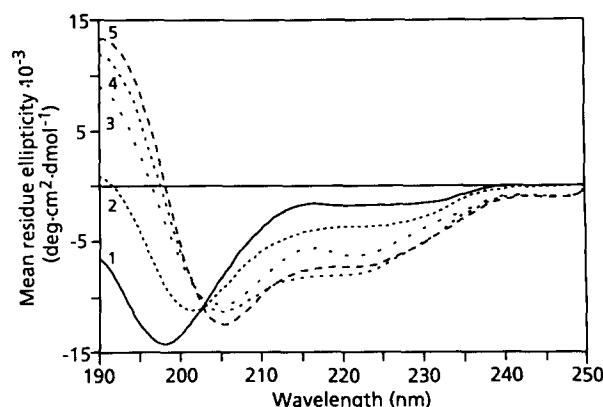


Fig. 3. Influence of charge density in micelles on the secondary structure of trfd. CD spectra of trfd in the presence of mixed micelles consisting of C_{12} -Pglycol and C_{12} -maltose at different molar ratios. The total micelle concentration was kept constant at 10 mM. The mole percentage of C_{12} -Pglycol was 1: 0%; 2: 20%; 3: 40%; 4: 70% and 5: 100%.

detergent concentration is plotted in Fig. 2. The critical micelle concentration values are indicated with arrows in the figure. α -helix induction only occurs above the c.m.c., demonstrating that a lipid aggregate is required. The effect of the c.m.c. is most clearly demonstrated when C_{12} -Pglycol and C_{14} -Pglycol are compared. These detergents only differ in their chain length and consequently in their c.m.c.. The anionic monoacyl lipids are most effective in α -helix induction. Zwitterionic monoacyl lipids are slightly less effective. In contrast, only minor changes are observed in the presence of micelles of the zwitterionic diacyl lipid DHPC and the non-ionic detergent C_{12} -maltose (see Fig. 2) and C_{10} -glucose (not shown).

A loss of signal in the CD spectra was observed in the presence of low concentrations of micelles of anionic detergents, 0.25 mM for C_{14} -Pglycol and 1.0 mM for C_{12} -Pglycol (not shown). A possible explanation for this loss of signal is that the peptide aggregates on the micellar surface, which results in a local high concentration of chromophores. Consequently, the probability for photons to encounter a chromophore is decreased, which results in a deviation from the Beer-Lambert relationship giving a reduced adsorption [19].

α -helix induction was most effective in the presence of micelles consisting of detergents with anionic head groups. However, in the biological target membrane, the chloroplast outer membrane, the amount of negatively charged lipids is approximately 15–20% of the total lipid [8]. We therefore investigated the secondary structure induction in trfd by different mixtures of C_{12} -Pglycol and C_{12} -maltose at a constant total lipid concentration. The CD spectra in the presence of these mixed micelles are shown in Fig. 3. C_{12} -maltose by itself does not induce structural changes in trfd. Already at low C_{12} -Pglycol concentrations, a significant secondary structure change occurs. Interestingly, the spectra in

Fig. 3 exert an isodichroic point at 203 nm indicating that the change in secondary structure in the transit peptide induced by the anionic detergents is a two state process, involving a defined conformational change. Deconvolution of these spectra reveals that the increase in α -helix content is linear with the Pglycol content until a saturation is reached at approximately 50 mol% of C_{12} -Pglycol.

Our experiments with monoacyl lipids so far have shown that secondary structure is induced by model lipids having anionic head groups and, to a lesser extent, zwitterionic head groups above their c.m.c.. However, these detergents do not occur in natural membranes and therefore it is important to know whether these tendencies are preserved in vesicles composed of membrane lipids. Fig. 4 shows the CD spectra of the transit peptide in the presence of small unilamellar vesicles composed of the zwitterionic DOPC or the anionic DOPG at 10 mM. The CD spectrum in the presence of DOPC is indicative of a peptide mostly in random coil (4% α -helix, 20% β -strand) and very similar to that of the peptide in buffer. In contrast, in the presence of PG vesicles, a considerable induction of α -helix is observed (40% α -helix, 4% β -strand).

To test the influence of vesicle concentration on secondary structure induction, we performed a titration experiment with PG SUVs. In Fig. 5A, the CD signal at 222 nm, which is indicative of the amount of α -helix in trfd, is plotted as a function of the amount of DOPG vesicles. Above 1 mM DOPG clear α -helix induction occurs, at higher DOPG concentrations saturation is seen. α -helix induction already starts at the lowest DOPG concentration measured but between 0.1 and 1.0 mM DOPG, a loss in signal intensity was observed similar to that mentioned for the C_{14} -PG and C_{12} -PG micelles.

To assess whether at a charge density present in the chloroplast outer membrane a transition in the secondary structure of trfd occurs, we performed a titration experiment. CD spectra of the transit peptide were re-

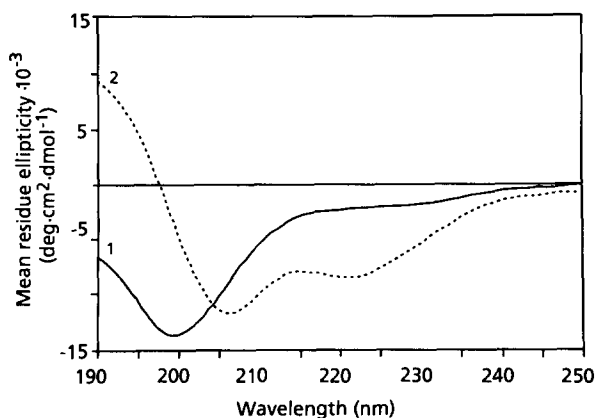


Fig. 4. CD analysis of trfd (20 μ M) in the presence of small unilamellar vesicles. The lipid concentration was 10 mM. 1: DOPC, 2: DOPG.

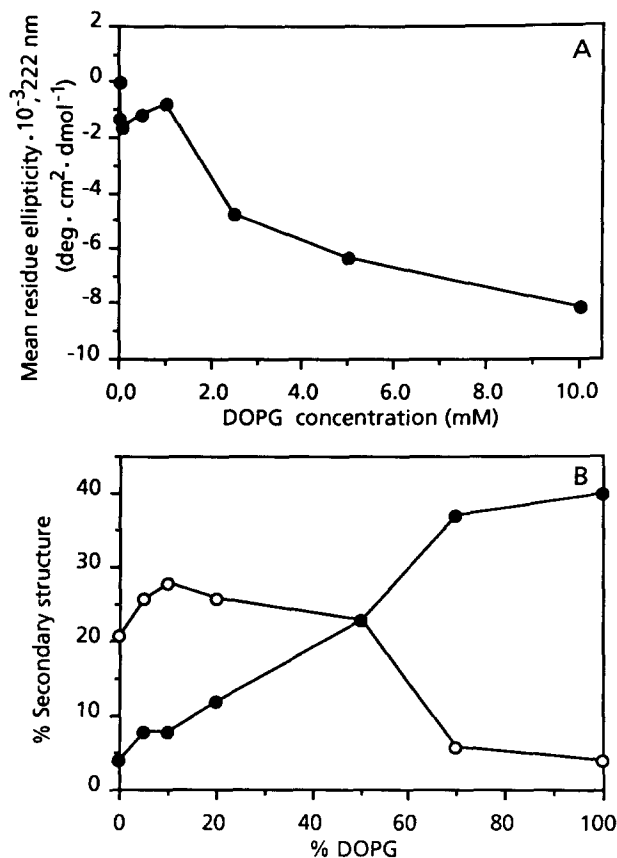


Fig. 5. Influence of the negatively charged phospholipid DOPG on the secondary structure characteristics of trfd (20 μ M) as measured by CD. Panel A: mean residue ellipticity at 222 nm as a function of the DOPG vesicle concentration. Panel B: relative amounts of β -strand (open circles) and α -helix (closed circles) in trfd in the presence of vesicles consisting of DOPC and DOPG at different molar ratios. The total lipid concentration was kept constant at 10 mM.

corded in the presence of 10 mM phospholipid consisting of DOPG and DOPC in different molar ratios. The spectra were deconvoluted to α -helix, β -strand and coil contributions. From the results plotted in Fig. 5B, we see that the decrease in β -strand and increase in α -helix depends on the PG content of the vesicles.

We did not observe a change in secondary structure induced by detergents having a sugar head group. From monolayer experiments, an efficient interaction between transit peptide and glycolipids was observed, suggesting that these lipids form an insertion site in the membrane [8]. Galactolipids do not form stable unilamellar vesicles by themselves. Therefore, their influence on the structure of the transit sequence was assessed by CD in mixed lipid systems. We prepared mixed vesicles consisting of a matrix of 50 mol% DOPC and 20 mol% DOPG to which 30 mol% of either galactolipid, sulfolipid or, as a control, DOPC were added. The calculated amounts of α -helix and β -strand present in the transit peptide in the presence of these lipid vesicles are shown in Fig. 6. The effect of the addition of the anionic

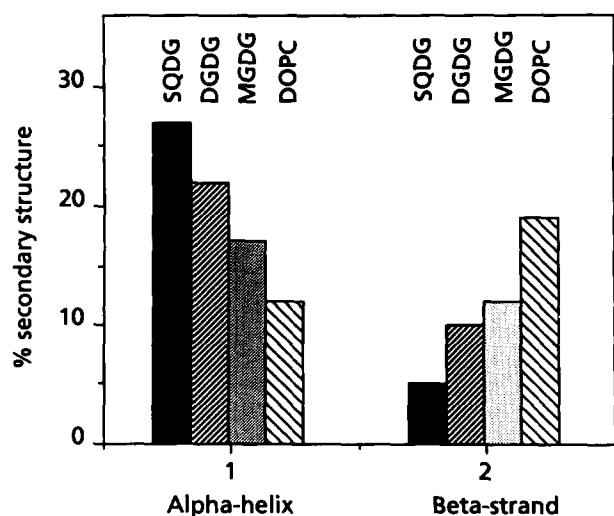


Fig. 6. Influence of the presence of chloroplast lipids in a mixed lipid vesicle on the secondary structure of trfd.

SQDG was similar to that of the vesicles containing 50 mol% PG. Both neutral galactolipids MGDG and DGDG contribute to α -helix induction but to a much lesser extent than PG or SQDG. Note that in all cases, the induction of α -helices is accompanied by a loss in β -strand.

4. DISCUSSION

In this paper we have investigated the possibility of secondary structure induction in a transit sequence by a lipid surface. We have shown that the transit peptide of preferredoxin can undergo specific and defined secondary structural changes upon interaction with lipids. In buffer and in the presence of neutral detergents or vesicles consisting of zwitterionic phospholipids, the transit peptide is mainly unstructured, containing 20% β -strand as regular secondary structure. Upon interaction with negatively charged or zwitterionic detergents or negatively charged lipids, an increase in α -helix occurs which is accompanied by a loss in β -strand structure.

We will first discuss the observed lipid specificity of the secondary structure modulation in trfd and then evaluate this conformational flexibility with regard to its possible role in the import process. The most profound induction of α -helix is seen in micelles composed of anionic monoacyl lipids. Less effective α -helix induction is seen in lyso-PC and C_{12} -PN and small unilamellar vesicles containing negatively charged lipids. Recently, similar characteristics have been reported also for two other chloroplast transit peptides [26,27].

Electrostatic interactions between the negative charges on the lipids and the positive charges of the transit sequence are most likely involved in the induction of secondary structure. The importance of electro-

static forces are also demonstrated by increasing the salt concentration to 150 mM NaCl. In this case, α -helix induction of trfd by DOPG vesicles was less effective (not shown). Besides charge interactions, a penetration of the peptide into the lipid surface also seems to contribute. Above the c.m.c. lyso-PC greatly enhances α -helix formation, the micelle forming diacyl lipid DHPC is less effective in α -helix formation, while DOPC SUVs are not effective in this respect, despite the identical headgroups. As compared to the DOPC SUVs, the acyl chain density is much lower in lyso-PC and C_{12} -PC micelles, enabling the transit peptide to penetrate more efficiently into the latter two micellar systems. The observed intermediate efficiency of α -helix induction by DHPC can be understood by the presence of the two acyl chains giving a higher chain density in the micelle.

Neutral detergents containing a glucose or maltose head group do not promote secondary structure induction in trfd. It has been concluded from monolayer experiments that diacyl lipids having a sugar headgroup like monogalactosyldiacylglycerol are efficient targets for the transit peptide to insert into. It has been proposed that the transit sequence which is rich in hydroxylated amino acids and does not contain extensive hydrophobic patches likes to reside in the interface of the apolar alkyl chains and the polar head groups. The transit sequence might participate in hydrogen bonding in this region [8]. If the results from monolayer experiments can be extended to the bilayer situation we will expect trfd to insert more efficiently into vesicles containing negatively charged lipids and vesicles containing MGDG. Interestingly, MGDG and DGDG do increase the α -helix induction when present in mixed membranes containing DOPG (Fig. 6). It seems that both penetration of the head group region and charge interactions are required for the specific α -helix induction in trfd.

How could the observed properties of trfd contribute to targeting? In buffer, trfd is unstructured. Its hydrophobic nature and position at the N-terminus of the protein will help to ensure exposure of this domain. The random coil conformation is ideally suited to adopt to the surface distribution of lipid charges [20]. Trfd can insert into a lipid surface with a preference for chloroplast lipids. We have shown here that this insertion can lead to a specific change in secondary structure. Efficient targeting thus might be accomplished by a two step recognition process, in which the lipid affinity of trfd determines the insertion in the chloroplast outer membrane after which an induction of secondary structure takes place. We propose that the induced secondary structure exposes motifs, which can be recognized by the ATP consuming import machinery. The persistence of random coil conformation of trfd (always at least 45%) might be functionally important. Random coil domains might serve as connectors of secondary structure domains which together form a recognition motif. This view can explain the difficulty in aligning different

transit sequences. Based on a theoretical analysis it was calculated that the region between residues 20 and 38 of trfd has the highest probability for α -helix formation. This region also contains two of the more hydrophobic regions present in trfd and is flanked by positively charged residues [12]. Interestingly, the induction of α -helix content up to 40% measured in the presence of anionic lipids matches to this predicted α -helix. However, the exact location of secondary structure elements within trfd needs further investigation.

Conformational flexibility induced by lipids is also a property of secretory signal sequences [21] and mitochondrial presequences [22]. The motifs formed by these latter two topogenic sequences can be rationalized in an evolutionary context. Signal sequences, which are probably in evolutionary terms the oldest cleavable topogenic sequences, might have evolved from transmembrane helices. Indeed, signal sequences contain a long hydrophobic stretch that can form an α -helix. The membrane potential across the mitochondrial inner membrane is used to translocate the presequence of a mitochondrial precursor across the inner membrane [23]. It is possible that presequences have evolved to give optimal response to this membrane potential. Indeed, the positively charged and amphiphilic nature of presequences allows them to respond to an electrochemical gradient which can translocate the presequence across a lipid bilayer [24].

Chloroplast import signals escape the secretion and mitochondrial translocation route and therefore must possess a different functional design. We propose that transit sequences are designed to function in a two-step recognition, in which first insertion into the extended exposed lipid domains of the chloroplast outer membrane takes place, followed by a conformational change in the transit sequence which exposes motifs for further recognition in an ATP-dependent step.

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