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Biophysical Characterization of a Transit Peptide Directing Chloroplast Protein Import†

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Received October 11, 1991; Revised Manuscript Received March 19, 1992

ABSTRACT: We have investigated the biophysical properties of a 35 amino acid peptide representing the entire length of a chloroplastic targeting sequence. The peptide, termed γ-tp, corresponds in sequence to the transit peptide of the γ subunit of the chloroplast ATP synthase from *Chlamydomonas reinhardtii*. We found that γ-tp blocks the import of the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase into isolated pea chloroplasts ($K_I \approx 5$ μM), suggesting that it interacts with higher plant plastids in a physiological manner. We also found the γ-tp to have a high affinity for nonpolar environments, but not to cause a general disruption of membrane integrity. Hydrophobic moment analysis suggests that the γ-tp can adopt an amphipathic β structure. However, circular dichroism measurements indicate that the peptide is largely a random coil, in both the presence and absence of sodium laurylsulfate micelles. In the absence of a recognizable secondary structural targeting motif, we asked whether the presence of a transit peptide on a chloroplast protein increases the protein's overall affinity for nonpolar environments. Phase-partition experiments with Triton X-114 suggest that this is not the case. These results are discussed in relation to the mechanism of protein targeting to chloroplasts.

Chloroplasts and mitochondria are unique among organelles in that they are capable of a certain amount of semiautonomous protein synthesis. Nonetheless, most of their complement of proteins is encoded in the nucleus, synthesized on cytoplasmic ribosomes, and taken up posttranslationally by the organelles. Proteins destined for these organelles are synthesized as high molecular weight precursors and contain an amino-terminal extension of amino acids termed a transit

† This work was supported in part by National Science Foundation Grant DCB88-17373 to S.M.T.
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0006-2960/92/0431-5053$03.00/0

peptide [reviewed by Keegstra et al. (1989)]. Since transit peptides have been shown to direct normally cytosolic proteins into chloroplasts and mitochondria, it is known that they possess all the information required for organelle targeting. The mechanism through which this information is encoded, however, is still unclear, and is the subject of this work.

Transit peptides which direct chloroplastic and mitochondrial protein traffic do not display any significant amino acid sequence homologies with one another. They do, however, share a number of general physical characteristics. Unlike the signal peptides responsible for protein targeting to the endoplasmic reticulum or to the bacterial plasma membrane, transit peptides are not particularly hydrophobic, are generally rich in aromatic and hydroxylated amino acids, and can contain a few basic, but no acidic, residues (Keegstra et al., 1989; Landry & Gierasch, 1991).

The lack of a targeting motif present in the primary sequence of topogenic peptides has led researchers to investigate the possibility that targeting information is encoded in the peptides' secondary structures. This view has led to the recognition that most mitochondrial transit peptides have a theoretical propensity to form amphipathic α-helical structures (von Heijne, 1986; Roise et al., 1986; von Heijne et al., 1989). Experimentally, peptides representing both mitochondrial transit peptides and ER and bacterial exoprotein signal peptides have been found to partition into membrane/water interfaces when injected into a stirred buffer solution overlaid by a lipid monolayer (Roise et al., 1986, 1988; Tamm, 1986, 1991; Demel et al., 1990; Landry & Gierasch, 1991). More significantly, when these peptides are allowed to interact with detergent vesicles dispersed in aqueous solution, mimicking interactions with membrane surfaces, their secondary structures change from random coil to α-helix (Roise et al., 1986, 1988; Endo et al., 1989; Endo & Oya, 1989; McKnight et al., 1989; Tamm & Bartoldus, 1990; Pak & Weiner, 1990; Karslake et al., 1990). These observations have led some investigators to postulate that a membrane-seeking (hydrophobic or amphipathic) α-helix is one structural element that encodes targeting information in secretory and mitochondrial topogenic sequences, with the membrane-seeking properties playing a more dominant role than the helix.

Despite their similarities, transit peptides on chloroplastic precursors differ from their mitochondrial counterparts in several respects. Whereas mitochondrial transit peptides usually consist of approximately 25 amino acids, chloroplastic transit peptides often contain from 60 to greater than 100 residues. In addition, analysis of the hydrophobic moments of chloroplastic transit peptides indicates that they cannot form amphipathic α-helices. However, the same analysis revealed that many of them could adopt an amphipathic β structure, especially toward their extreme carboxyl termini (Cramer et al., 1989; von Heijne et al., 1989). By analogy with the topogenic sequences targeting proteins to the secretory pathway or to mitochondria, this observation suggests that an amphipathic β structure may play a role in targeting polypeptides to chloroplasts.

The relative length of chloroplastic transit peptides has generally precluded their complete synthesis by chemical techniques. Recently it was noted that the transit peptides of chloroplastic precursors in the green algae *Chlamydomonas reinhardtii* are usually shorter than their higher plant counterparts (Franzen et al., 1990). We have previously shown that at least one such precursor, that to the γ subunit of the chloroplast coupling factor from *C. reinhardtii*, is correctly imported in vitro by chloroplasts isolated from higher plants (peas), even though its transit peptide consists of only 35 amino acids (Yu et al., 1988). In this study, we have used a synthetic 35mer that faithfully reproduces the entire *C. reinhardtii* γ-subunit transit peptide (γ-tp) to probe its secondary structure and other biophysical properties. To our knowledge, this is the first time a synthetic peptide corresponding to a full-length chloroplastic transit peptide has been used in experiments aimed at understanding the manner in which targeting information is encoded by these sequences. This takes on new significance in light of recent reports suggesting that different domains of a chloroplastic transit peptide are involved in precursor binding and membrane transport (Perry et al., 1991; van't Hof et al., 1991).

Materials and Methods

Synthesis of the γ-tp. A peptide corresponding to the 35 amino acid transit sequence of the γ subunit of the *Chlamydomonas reinhardtii* chloroplast coupling factor was synthesized by the Protein Structure Laboratory at the University of California, Davis. The sequence of the peptide is MAAMLASKQGAFMGRSSFAPAPKGVASRGSLQVVA. The purity of the γ-tp was judged to be >90% on the basis of its amino acid composition and HPLC elution profile; it was used without further purification.

Protein Import into Chloroplasts. Intact chloroplasts were isolated from pea seedlings as described by Theg et al. (1989). The precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (prSS) and the corresponding mature protein (mSS) were synthesized by sequential in vitro transcription of the clones p81/4 (Lubben & Keegstra, 1986) and p59/3 (Friedman & Keegstra, 1989), respectively, and translation of the resulting mRNA with a wheat germ lysate (Anderson et al., 1983). The import of prSS into isolated intact chloroplasts was performed in a buffer containing 0.33 M sorbitol, 50 mM K-Tricine, 5 mM $MgCl_2$, and 3 mM ATP. Reactions were terminated by centrifuging through silicone oil into perchloric acid (Theg et al., 1989), and the precipitated proteins were analyzed by SDS–PAGE and fluorography. Chloroplast-bound and internalized protein was quantitated by scintillation counting of the radioactive bands excised from the gel.

Monolayer Surface Pressure Measurements. Protein- and peptide-induced changes in the surface pressure of lipid monolayers were measured using the Wilhelmy balance described by Morse and Deamer (1972). Briefly, 4 mL of 20 mM K-Tricine at pH 8.0 and 1 mM Na_2EDTA was placed into a small petri dish containing a stir bar, and a flat piece of platinum suspended from a Conn balance was lowered until it just touched the liquid surface. Next, a small volume of an ethanolic lipid solution was spread on the surface of the liquid until the surface pressure increased to approximately 10 mN/m. When the surface pressure had stabilized, the (poly)peptide was injected into the stirred aqueous layer below the monolayer, and their transfer to the surface was recorded as an increase in surface pressure. Experiments in which no lipid was spread over the surface of the dish prior to (poly)peptide injection into the aqueous phase monitored the transfer of the (poly)peptide to the air/water interface.

The change in the surface pressure over time after (poly)peptide injection was monitored for at least 4 min, and kinetic parameters describing the change as the sum of two rising exponentials (ΔP_{max}, k_1, k_2) were determined using a SIMPLEX algorithm to fit the data (Caceci & Cacheris, 1984). The values plotted in Figure 1 represent ΔP_{max}; in only one instance was the ratio of ΔP at 4 min (ΔP_{4min}) to ΔP_{max} less than 80%, and an identical interpretation of the data emerges using the

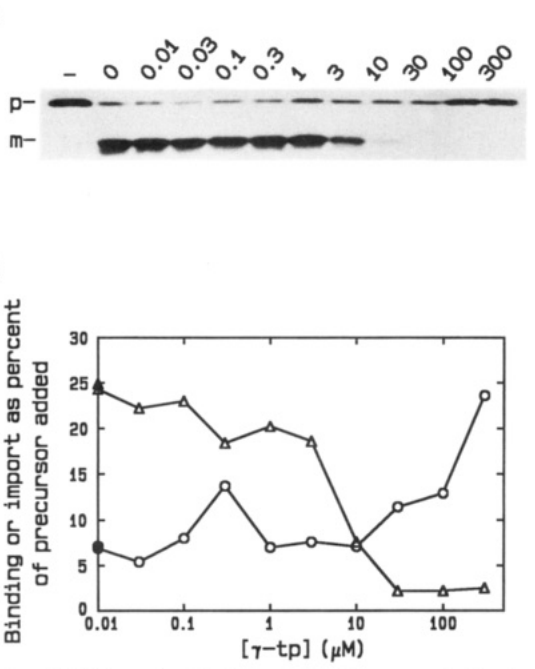

FIGURE 1: Inhibition of prSS import by the γ-tp. Isolated pea chloroplasts, prSS, and the indicated amount of the γ-tp were incubated for 8 min at room temperature as described under Materials and Methods. (A) A fluorograph of the gel is shown. Numbers above the lanes indicate micromolar γ-tp added; the positions of the precursor (p) and mature (m) SS are marked. (B) Quantitation of the fluorograph. The relative amounts of precursor (bound to the chloroplast surface) and mature SS (imported into the chloroplasts) recovered with the chloroplasts are indicated by the open circles and triangles, respectively; the points obtained with no γ-tp addition are indicated by the closed symbols. In control experiments (not shown), it was determined that neither cytochrome *c* at 100 μg/mL nor a 13 amino acid membrane-seeking peptide at 30 μM caused any inhibition of prSS import (see text).

experimentally determined ΔP_{4min} instead of the extrapolated ΔP_{max}.

Thylakoid Proton Pump Measurements. Alkalinization of the thylakoid suspension medium was measured with a glass pH electrode essentially as described by Dilley (1971). The medium contained 0.5 mM Na-MES at pH 6.5, 25 mM NaCl, 5 mM $MgCl_2$, 50 μM methylviologen, and thylakoids at 40 μg of chlorophyll/mL. The sample containing nigericin was included as a control showing the trace recorded with membranes which had been made permeable to protons, and shows a slight light-responsiveness of our electrode.

Circular Dichroism Measurements and Analysis. CD spectra of γ-tp were measured in a JASCO J500C spectrophotometer in a solution containing 300 mM NaCl, 10 μM γ-tp, and, when present, 10 mM SDS. The CD spectrum for the mitochondrial cytochrome oxidase subunit IV analyzed in Table I was taken from Figure 2 of Endo et al. (1989).

Spectra were deconvoluted into the relative contributions from α-helix, β-turn, β-sheet, and random-coil secondary structure by comparison to model compound spectra (Johnson, 1988). Molar ellipticity of the γ-tp was matched with the relative sums of the molar ellipticities of the model compounds at 13 points along the spectra using a SIMPLEX algorithm (see above); the resultant values are plotted on top of the experimental data in Figure 3.

Phase-Partitioning with Triton X-114. Phase-partitioning of prSS and mSS between aqueous and detergent environments was accomplished using Triton X-114. Radioactive proteins were produced from cloned genes [p81/4 for prSS and 59/3 for mSS (see above)] and subjected to phase-partitioning essentially as described by Bordier (1981).

RESULTS

γ-tp Inhibits the in Vitro Import of prSS into Chloroplasts. It was shown previously that the precursor to the γ subunit of the *C. reinhardtii* chloroplast coupling factor is taken up by isolated pea chloroplasts (Yu et al., 1988). In that study, it was tacitly assumed, but not demonstrated, that the γ subunit entered the chloroplasts through the normal machinery responsible for protein import into the organelle. This question became more of an issue when we undertook measurements of the biophysical properties of this precursor's transit peptide in isolation. Thus, we sought to determine whether the free γ-tp could interact with the pea chloroplast's import machinery in a physiologically relevant manner. Specifically, we asked whether the γ-tp would compete for chloroplastic import sites with a different pea precursor protein, prSS.

Figure 1 documents that the γ-tp did indeed block prSS import into pea chloroplasts. The apparent K_I for this effect was approximately 5 μM. While this concentration is higher than anticipated by the amount of precursor added to the import experiment, it is in the range of inhibitor constants observed by other investigators for different synthetic targeting peptides which inhibit protein import into mitochondria and chloroplasts (Roise et al., 1986; Buvinger et al., 1989; Glaser & Cumsky, 1990; Pak & Weiner, 1990; Schnell et al., 1991; Perry et al., 1991). Interestingly, an increase in the recovery of prSS bound to the chloroplast surface at high γ-tp concentrations was observed on a number of occasions (Figure 1). A similar trend was also seen when the binding reaction was examined independently of the import reaction by performing the experiment at 0–4 °C (data not shown). Other investigators have similarly found that synthetic peptides corresponding to both mitochondrial and chloroplastic topogenic sequences do not necessarily inhibit the binding of precursors to the surface of the organelles (Buvinger et al., 1989; Pak & Weiner, 1990; Glaser & Cumsky, 1990; Perry et al., 1991). The inhibition of prSS import by the γ-tp was probably due to a physiological interaction of the peptide with the chloroplast protein import machinery (see below for a discussion of controls for nonspecific modes of inhibition). This, in turn, justifies further examination of the γ-tp for insights into the mechanism of protein targeting to chloroplasts.

γ-tp Seeks Air/Water and Lipid/Water Interfaces. Like other chloroplastic and mitochondrial topogenic sequences, but unlike secretory signal sequences, the γ-tp is not exceedingly hydrophobic. We found, for instance, that it is soluble in water up to 2 mg/mL. Nevertheless, investigations with mitochondrial synthetic transit peptides have shown them to have a high affinity for nonaqueous environments [Roise et al., 1986, 1988; Tamm, 1986; reviewed by Tamm (1991)]. We investigated whether the γ-tp has a similar tendency to avoid aqueous environments in the experiment shown in Figure 2. As described under Materials and Methods, proteins and peptides were injected into a stirred aqueous phase below an air/water interface, or below monolayers consisting of either neutral or negatively charged lipids. The affinity of the injected material for the water surface or the water/lipid interface was detected with a Wilhelmy balance as an increase in the surface pressure. The figure demonstrates that the γ-tp partitioned into all interfaces with a strength equal to that of cytochrome *c*, a protein with well-characterized membrane-seeking properties (Morse & Deamer, 1973). It is noteworthy that the γ-tp partitioned into water/lipid interfaces containing both charged and uncharged lipids. This indicates that in addition to ionic forces that might cause the association of the

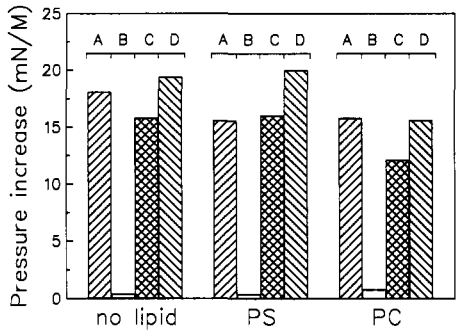

FIGURE 2: Partition of the γ-tp into air/water and lipid/water interfaces. Surface pressure was measured with a Wilhelmy balance as described under Materials and Methods. Bars labeled: (A) 10 μg/mL cytochrome *c*; (B) 10 μg/mL BSA; (C) 1 μM γ-tp; (D) 10 μM γ-tp. Cytochrome *c* and BSA were chosen as well-characterized controls that do or do not interact with lipid monolayers, respectively. Values plotted represent the maximum pressure increase extrapolated as described under Materials and Methods.

peptide with positively charged phosphatidylserine head groups, entropic forces alone were sufficient to drive the peptide into the uncharged water/phosphatidylcholine interface.

Cytochrome *c* is a well-characterized membrane-seeking protein that was used as a positive control for the Wilhelmy balance experiments. This protein can also be used as a control for the experiment shown in Figure 1 to assess whether nonspecific membrane/protein interactions are responsible for the inhibition of prSS import by the γ-tp. We found that a concentration of cytochrome *c* that resulted in monolayer surface pressure increases similar to those observed with 10 μM γ-tp did not inhibit prSS import into chloroplasts, nor did a concentration 10-fold higher (data not shown). Similar experiments revealed that a 10 μM concentration of a synthetic 13 amino acid peptide that did not contain a chloroplast targeting sequence produced an increase in monolayer surface pressure similar to those seen in Figure 2 but did not inhibit prSS import even at 30 μM (data not shown). These controls suggest that the inhibition of prSS import into chloroplasts by the γ-tp shown in Figure 1 was not solely the result of nonspecific adsorption of the peptide to the chloroplast outer envelope membrane.

γ-tp Does Not Increase the Proton Permeability of Thylakoid Membranes. Figure 2 demonstrates that the γ-tp shares a high affinity for nonpolar environments with previously characterized mitochondrial transit peptides. A related property of the latter peptides is their ability to disrupt both native and artificial membrane vesicles (Roise et al., 1986, 1988). To see if this was also a property of the γ-tp, we examined its effect on the permeability of the thylakoid membrane to protons. Figure 3 shows an experiment in which we measured the light-driven thylakoid proton pump in the presence and absence of the γ-tp. We found no significant effect of the peptide either on the amplitude of the medium alkalinization or on the subsequent dark decay rate, both sensitive indicators of proton leakage (Karlish & Avron, 1968). A control trace recorded in the presence of 2 μM nigericin shows the effect we would have observed if the γ-tp had increased the permeability of the thylakoid membrane to protons. From this experiment, we conclude that the chloroplast-active γ-tp does not significantly disrupt this and presumably other membranes. A similar conclusion was reached using different methods by Schnell et al. (1991) and by Perry et al. (1991) in their studies of the interactions of partial transit peptides with chloroplasts.

Secondary Structure of the γ-tp. Analysis of the hydrophobic moment of mitochondrial transit peptides has revealed that they have the potential to form amphipathic α-helices (von Heijne, 1986). It has been found experimentally that an α-helical structure is induced in these peptides when they interact with hydrophobic environments resembling membrane surfaces, i.e., detergent micelles (Roise et al., 1986, 1988; Tamm, 1986; Endo et al., 1989; Pak & Weiner, 1990; Karlaske et al., 1990). This has led to the suggestion that the amphipathic α-helix is a part of the secondary structural motif that allows mitochondria to recognize the precursors of mitochondrial proteins. In contrast, chloroplastic transit peptides do not display any theoretical propensity to form amphipathic α-helices, leading researchers to search for other secondary structural features that might explain their selectivity for chloroplast surfaces (Keegstra et al., 1989). One suggestion that has been recently postulated is that these transit peptides might adopt an amphipathic β conformation at their extreme carboxy termini and that this might be part of the correct targeting motif recognized by chloroplasts (von Heijne et al., 1989; Cramer et al., 1989). A different hypothesis posits that chloroplastic transit peptides are essentially random coils, adopting no particular secondary structure (von Heijne & Nishikawa, 1991). As a test of these competing hypotheses, we undertook an analysis of the secondary structure of the γ-tp by theoretical and experimental means.

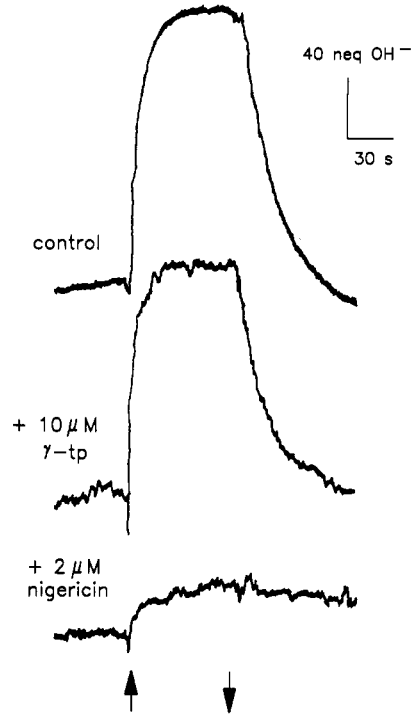

FIGURE 3: Measurement of the light-induced thylakoid proton pump in the presence and absence of the γ-tp. Alkalinization of the thylakoid suspension medium was monitored with a glass pH electrode as described under Materials and Methods. The red light was turned on and off at the upward and downward arrows, respectively, and each sample was individually calibrated after the trace was recorded.

Figure 4 shows an analysis of the hydrophobic moment of the γ-tp. This transit peptide shows some, albeit low, propensity to form an amphipathic α-helical structure (dashed line). It displays a more marked propensity to adopt an amphipathic β structure at its carboxyl terminus (solid line), consistent with the suggestion that this might be a feature that distinguishes chloroplastic transit peptides from those directed to the mitochondria (von Heijne et al., 1989; Cramer et al., 1989). It has recently been pointed out that the transit peptides of *C. reinhardtii* chloroplastic precursors appear to have properties

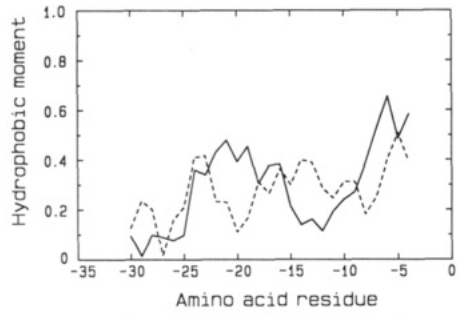

FIGURE 4: Analysis of the hydrophobic moment of the γ-tp. The hydrophobic moment of the γ-tp was analyzed as described in Shiver et al. (1989), with a window size of 11 amino acids. The angle between adjacent residues was set to 100° (dashed line) or 170° (solid line) to represent α-helical or β-sheet conformations, respectively.

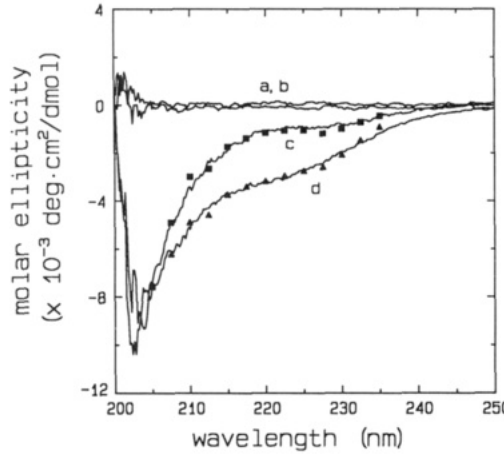

FIGURE 5: Circular dichroism of the γ-tp. Traces a and b, 300 mM NaCl without the γ-tp containing 0 or 10 mM SDS, respectively; traces c and d, 10 μM γ-tp in 300 mM NaCl containing 0 or 10 mM SDS, respectively. Spectra c and d were deconvoluted into the relative contributions of α-helix, β-sheet, β-turn, and random coil as described under Materials and Methods. The conformational contributions (see Table I) were then used to calculate the points overlying traces c and d.

somewhere in between those of normal mitochondrial sequences and normal chloroplastic peptides (Franzen et al., 1990), and the γ-tp appears to adhere well to this pattern.

We next investigated the secondary structure of the γ-tp by circular dichroism. The spectra shown in Figure 5 were recorded in the absence (trace c) and presence (trace d) of SDS at a detergent concentration above the critical micelle concentration. Interactions between this and other detergents with proteins have been used as a model for the interactions between proteins and membranes [cf. Roise et al. (1986) and Endo et al. (1989)]. We noted a slight change in the γ-tp CD spectrum when SDS was added, which we quantitated by comparison to model spectra (Materials and Methods). The results of this quantitation are reported in Table I and reveal that there are in fact no significant changes induced in the secondary structure of the γ-tp by the addition of SDS. Of particular interest is the fact that neither the fraction of β-sheet nor the sum of the β-sheet and β-turn structural components increased significantly when SDS was included in the buffer. This indicates that the γ-tp does not undergo a gross alteration in secondary structure when it encounters a nonpolar environment such as a membrane surface.

Partition of prSS and mSS into Nonpolar Environments. Our CD experiments failed to uncover a particular secondary structural motif that could explain either the selectivity of chloroplasts for chloroplastic precursor proteins or even the fact that γ-tp has a high affinity for membranes. This led us

Table I: Secondary Structures of the γ-tp and the Transit Peptide from Yeast Mitochondrial Cytochrome Oxidase Subunit IV[a]

transit peptide	SDS present	α-helix	β-sheet	β-turn	random coil
γ-tp	no	0.00	0.26	0.14	0.60
	yes	0.04	0.30	0.11	0.54
COX IV	no	0.12	0.17	0.03	0.67
	yes	0.56	0.05	0.00	0.39

[a] Values for the γ-tp were those that produced the best fit to the data in Figure 5. The data for the COX IV transit peptide were taken from Figure 3 of Endo et al. (1989).

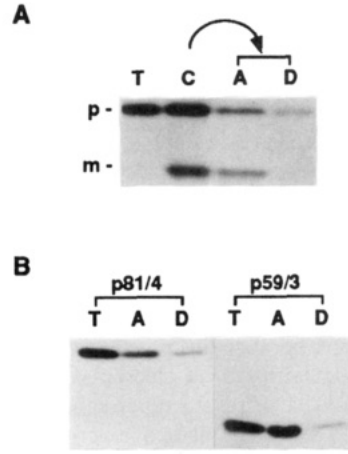

FIGURE 6: Phase-partitioning of prSS and mSS using Triton X-114. (A) prSS and mSS were recovered from a chloroplast protein import experiment. T, prSS translation; C, chloroplasts after an import experiment; A and D, aqueous and detergent phases, respectively, of the sample in C after phase-partitioning. (B) Both prSS and mSS were synthesized by in vitro translation from clone p81/4 and p59/3, respectively. T, translation product; A and D, aqueous and detergent phases, respectively, after phase-partitioning.

to question whether transit peptides, by virtue of their membrane-seeking nature, might simply act to increase the affinities of their associated passenger proteins for cellular membranes. While not necessarily leading to organelle specificity, such an increase in membrane affinity still might be advantageous by confining the precursor to search for a receptor in the two-dimensional plane of a membrane, rather than in three dimensions throughout the cell [cf. Landry and Gierasch (1991)]. We tested this hypothesis by comparing the partition coefficient of a chloroplastic precursor and its mature protein into the polar and nonpolar phases formed when a solution of Triton X-114 is raised from 0 to 30 °C (Bordier, 1981). Figure 6 shows the results of such an experiment performed with prSS and mSS. The experiment shows that a significant portion of the prSS is recovered in the detergent phase formed at 30 °C, suggesting that this protein has a low, but measurable, affinity for nonpolar environments. The results obtained with the mature SS depended on the manner in which the mature form of the protein was generated. When radiolabeled mSS was recovered from the stroma of chloroplasts after import of prSS, essentially all of mSS was found in the aqueous phase (panel A). However, it is known that most, if not all, of the SS entering the stroma in such an experiment is assembled into the Rubisco complex (Lubben et al., 1989), raising the possibility that our experiment examined the partitioning of the assembled Rubisco, rather than mSS. If so, it would not be surprising that the SS associated with this soluble enzyme complex is recovered in the aqueous phase.

In order to avoid the complications arising with Rubisco assembly, mSS was synthesized by in vitro transcription and translation of a clone, p59/3, from which the nucleotides

encoding the transit peptide had been deleted (Friedman & Keegstra, 1989). When this mSS was subjected to phase-partitioning in Triton X-114, it was recovered in both the aqueous and detergent phases, in approximately the same proportions as observed for prSS (panel B). Quantitation of data from eight independent experiments indicated that the amount of protein recovered in the aqueous phase was only slightly less for prSS (72% ± 16%) than for mSS (81% ± 5%) and it is doubtful that this bias is significant. In as much as Triton X-114 is a reliable model for the nonpolar environment of a membrane, these results suggests that the presence of the SS transit peptide does not profoundly increase the overall affinity of the protein for nonpolar (detergent or membranous) environments.

Discussion

In this paper, we have attempted to elucidate the structural features through which chloroplasts recognize chloroplastic precursor proteins in the cytoplasm. To this end, we have examined the biophysical properties of a chloroplastic transit peptide in the absence of its passenger protein. We chose to analyze the transit peptide for the γ subunit of the *C. reinhardtii* chloroplast coupling factor because of its relatively short length, and because this precursor had been previously shown to interact with the pea chloroplast's import machinery (Yu et al., 1988). Our results reported here strongly suggest that the γ-tp, free of the passenger protein, is also able to interact with the protein translocation apparatus in pea chloroplasts. Assuming that the secondary structural motif recognized by chloroplasts is unique, this motif must be present in the γ-tp. We therefore felt justified in using this somewhat unusual peptide (Franzen et al., 1990) as a model for chloroplastic transit sequences.

It is important to note that the γ-tp studied here represents an entire chloroplastic transit peptide. Much useful information has been gained previously through investigations of partial transit peptide sequences (Bruvinger et al., 1989; Schnell et al., 1991; Perry et al., 1991; van't Hof et al., 1991). Interestingly, the transit peptide from prSS has recently been shown to consist of two functional domains: one directing binding of the precursor to the chloroplast surface and the other involved in translocation of the protein into the stroma (Perry et al., 1991). By using a full-length transit peptide which contains both binding and translocation information in our studies, we avoided potential complications in interpretation of biophysical data that might arise if the domains had been separated.

A number of investigations have shown that synthetic peptides representing chloroplastic and mitochondrial transit peptides inhibit the import of proteins without inhibiting their binding to the organelle surfaces (Buvinger et al., 1989; Perry et al., 1991; Pak & Weiner, 1990; Glaser & Cumsky, 1990). We obtained a similar result with the γ-tp (Figure 1 and data not shown). This suggests that the γ-tp's competitive interaction with the chloroplast's import machinery is not at the level of the surface receptors but rather further along in the import pathway. This conclusion was also reached and elaborated more fully by Glaser and Cumsky (1990) and by Pak and Weiner (1990) in their studies of peptide inhibition of mitochondrial protein import.

It has been pointed out by many authors that chloroplastic and mitochondrial transit peptides share a number of general features, such as a low hydrophobicity, a preponderance of hydroxylated and aromatic residues, and a lack of acidic amino acids [cf. Keegstra et al. (1989)]. It was not surprising, therefore, that the well-documented affinity of mitochondrial transit peptides for membranes was shared by the γ-tp. Quantitatively similar changes in monolayer surface pressures induced by the mitochondrial cytochrome oxidase subunit IV transit peptide (Roise et al., 1986) and by the γ-tp indicate that the strength of membrane association is similar for the two peptides. Our data do not permit us to assess the type of association between monolayers and the γ-tp, i.e., surface association or insertion, but we are able to rule out electrostatic interactions as the sole cause for this association.

In contrast to an affinity for hydrophobic environments, chloroplast and mitochondrial transit peptides do not share any of the other biophysical properties examined. Specifically, unlike mitochondrial topogenic sequences, the γ-tp does not appear to increase the permeability of membranes to ions, at least not the permeability of thylakoid membranes to protons. Nor did the γ-tp adopt a different secondary structure in the presence of detergent micelles. Thus, while the targeting of proteins to chloroplasts and mitochondria appears to share many mechanistic features, our data suggests that the targeting motif recognized by the respective organelles is different. This is not surprising, given the high fidelity of the selectivity of chloroplasts and plant mitochondria for their own precursors (Smeekens et al., 1986; Boutry et al., 1987; Whelan et al., 1990). [Reports of targeting to both mitochondria and chloroplasts by the same topogenic sequence (Hurt et al., 1986; Pfanner et al., 1989; Huang et al., 1990) may be more a reflection of missorting by heterologous systems than of a single shared targeting motif.]

The relatively small change in γ-tp secondary structure in the presence of SDS micelles merits further consideration. Analysis of its hydrophobic moment indicates that, in principle, the γ-tp could adopt an amphipathic β conformation at its extreme carboxyl terminus. However, adoption of this conformation is unlikely when the peptide is dissolved in water at low concentrations, since the hydrophobic residues in the amphipathic stretch would either be unfavorably exposed to water or shielded from the solvent by other parts of the peptide folded back upon itself. In opposition to this latter notion, Figure 4 does not reveal another region of comparable amphipathicity in the peptide that could potentially shield the carboxyl-terminal region. Thus, if the γ-tp were to adopt an amphipathic configuration, it would be most likely to do so in contact with a membrane surface. The lack of a large change in the CD spectrum of the γ-tp in the presence of SDS argues against this possibility. An alternative explanation would assume that the γ-tp does not interact with the SDS micelles. However, this seems unlikely given the relatively high affinity of the γ-tp for nonpolar environments documented in Figure 2.

A potential complication in the assessment of the secondary structure of the γ-tp arises through the deconvolution of the CD spectra into individual structural elements (Johnson, 1988). While a number of more rigorous deconvolution routines have been published, we chose the relatively simple method of comparison to model spectra (Johnson, 1988) because we were interested in gross, rather than detailed, structural features. The fidelity of our deconvolution routine was assessed by two means. First, points that were calculated using the parameters listed in Table I are plotted on top of the observed spectra in Figure 5, and show a good fit to the experimental data. Second, we used our deconvolution routine to assess the changes in secondary structure of a mitochondrial transit peptide that is known to take on an α-helical conformation in the presence of detergent (Roise et al., 1986; Endo et al., 1989). As seen in Table I, we successfully predicted a large

increase in the α-helical content of this peptide upon exposure to detergent, and our numbers agree well with the amount of α-helix determined by NMR techniques (Endo et al., 1989).

The results of our CD analysis of the structure of the γ-tp suggest that it does not adopt an amphipathic β structure when it encounters a nonpolar surface. Instead, our data would tend to favor the hypothesis put forth by von Heijne and Nishikawa (1991) that the γ-tp and, by extension, other chloroplastic transit peptides are largely devoid of secondary structure. Whether this lack of specific secondary structure could possibly be responsible for the specificity of chloroplast protein targeting reactions remains to be tested.

A number of investigators have suggested that the purpose of the high affinity of topogenic sequences for membranes might be to confine their diffusion to the plane of the target membrane, thereby increasing the chances for interaction with membrane-bound receptor proteins. This model implicitly suggests that the affinity of transit peptides for nonpolar environments might be higher for specific target membranes with particular lipid compositions. However, a number of observations are at odds with this prediction (but see below). Whelan et al. (1990) reported that chloroplastic precursor proteins bind loosely to the surface of isolated mitochondria, a binding which presumably is not productive and which probably takes place through nonspecific interactions. Furthermore, if the transit peptide were responsible for association of the precursor with its proper target membrane, then it would be expected to promote increased association of a passenger protein with nonpolar environments. However, we found that a transit peptide-bearing SS has little or no more affinity for the detergent phase in experiments with Triton X-114 than does the mature SS (Figure 6).

We are finally left with the question of the meaning of the high affinity of chloroplastic and mitochondrial transit peptides for membranes. It could be completely fortuitous, having no relation to the mechanism of protein targeting to these organelles. Arguing against this notion is the fact that the affinity of topogenic peptides for hydrophobic environments has been correlated with their biological activities (Roise et al., 1986, 188; McKnight et al., 1989), suggesting a direct role for those affinities in the targeting mechanism. In addition, we have noticed in unrelated experiments that a low amount of precursor often becomes associated with chloroplast surfaces in a nonproductive manner [Ettinger and Theg, unpublished results; see also Friedman and Keegstra (1989)]. This nonproductive, presumably nonspecific, interaction appears to require the presence of a transit peptide [cf. Figure 2 of Freidman and Keegstra (1989)].

While this paper was undergoing review, a paper by van't Hof et al. (1991) appeared in which the membrane-seeking properties of peptides corresponding to different domains of the prSS transit peptide were examined. They found that these peptides interacted with lipid monolayers, leading to the general conclusion, supported by our experiments, that chloroplastic transit peptides have membrane-seeking properties. Interestingly, the effects they observed of the peptides on monolayers formed from different lipids suggested that some of the interactions were mediated by specific properties of chloroplastic lipids. It remains to be tested whether such interactions can be responsible for the specificity of protein targeting to chloroplasts.

In summary, we have shown that a 35 amino acid synthetic peptide corresponding to a chloroplastic transit sequence has a high affinity for membranes but no distinct secondary structure. We have apparently ruled out the suggestion that an amphipathic β structure at the carboxyl terminus of transit peptides targets proteins to chloroplasts. The nature of the structural motif that encodes the targeting information for chloroplast proteins continues to be elusive, and its elucidation remains a goal with implications beyond chloroplast protein trafficking.

Acknowledgments

We thank Dr. David Deamer for the use of his Wilhelmy balance for the monolayer experiments, Dr. Dieter Gruenwedel for the use of his CD spectrophotometer, and both for their advice on the respective techniques.

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DNA Sequence Determinants for Binding of Transformed Ah Receptor to a Dioxin-Responsive Enhancer†

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Received December 24, 1991; Revised Manuscript Received March 19, 1992

ABSTRACT: We have utilized gel retardation analysis and DNA mutagenesis to examine the specific interaction of transformed guinea pig hepatic cytosolic TCDD·AhR complex with a dioxin-responsive element (DRE). Sequence alignment of the mouse CYPIA1 upstream DREs has identified a common invariant "core" consensus sequence of TNGCGTG flanked by several variable nucleotides. Competitive gel retardation analysis using a series of DRE oligonucleotides containing single or multiple base substitutions has allowed identification of those nucleotides important for TCDD·AhR·DRE complex formation. A putative TCDD·AhR DNA-binding consensus sequence of GCGTGNNA/TNNNC/G has been derived. The four core nucleotides, CGTG, appear to be critical for TCDD-inducible protein–DNA complex formation since their substitution decreased AhR binding affinity by 100–800-fold; the remaining conserved bases are also important, albeit to a lesser degree (3–5-fold). The 5′-ward thymine, present in the invariant core sequence of all the DREs identified to date, appears not to be involved in DNA binding of the AhR. The results obtained here indicate that although the primary interaction of the TCDD·AhR complex with the DRE occurs with the conserved "core" sequence, nucleotides flanking the core also contribute to the specificity of DRE binding.

Exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin, TCDD[1]), the most potent member of a large group of halogenated aromatic hydrocarbons (HAHs), results in numerous species- and tissue-specific toxic and biological effects, including tumor promotion, immunotoxicity, hepatotoxicity, teratogenesis, and enzyme induction (Poland & Knutson, 1982; Safe, 1986). The mechanism of induction of cytochrome P450IA1[2] and its associated monooxygenase activity, the most widely studied response to TCDD, is in many ways similar to that described for steroid hormone receptors and steroid-responsive genes (Poland & Knutson, 1982; Yamamoto, 1985; Safe, 1986; Whitlock, 1987, 1990). Induction by TCDD and other related HAHs is mediated by a soluble intracellular protein, the Ah (*a*romatic *h*ydrocarbon) receptor (AhR), which binds TCDD saturably and with high affinity (Poland & Knutson, 1982; Poland et al., 1986; Safe, 1986; Whitlock, 1990). Following ligand (TCDD) binding, the AhR, like steroid hormone receptors, undergoes a poorly defined process of transformation,[3] during which hsp90 (a 90-kDa heat shock protein (Denis et al., 1988; Perdew, 1988)) dissociates from the TCDD·AhR complex and the AhR acquires the ability to bind to DNA with high affinity (Whitlock & Galeazzi, 1984; Henry et al., 1989; Denison & Yao, 1991). Biochemical and genetic studies (Denison et al., 1988a,b; Whitlock, 1987, 1990) have indicated that transcriptional activation of the cytochrome P450IA1 (CYPIA1)[2] gene is stimulated by the binding of transformed TCDD·AhR complexes to *cis*-acting dioxin-re-

† This work was supported by the American Cancer Society (CN-4), the Air Force Office of Sponsored Research (AFOSR 90-0354), and the Michigan Agricultural Experiment Section.

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[1] Abbreviations: AhR, aromatic hydrocarbon receptor; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide. DRE, dioxin-responsive element; DTT, dithiothreitol. HEDG, 25 mM Hepes, pH 7.5, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

[2] Refer to Nebert et al. (1991) for a complete discussion of cytochrome P-450 enzyme and gene nomenclature.

[3] In this report, we have defined transformation as the process by which the TCDD·AhR complex changes to a form which can bind to DNA with a high affinity.

0006-2960/92/0431-5060$03.00/0