Use of aminoglycoside adenyltransferase translational fusions to determine topology of thylakoid membrane proteins

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Abstract We have developed a system to examine the topology of thylakoid membrane proteins using the bacterial *aadA* gene as a reporter. Translational fusions that place the aminoglycoside adenyltransferase domain in the stroma should provide high antibiotic resistance, while those that place it in the thylakoid lumen should give rise to low resistance. Genes encoding chimeric polypeptides consisting of AadA fused to varying lengths of the PsaA polypeptide, whose topology is known, were introduced into the chloroplast genome of *Chlamydomonas reinhardtii*. As expected, chimeras with an even number of α-helices in general resulted in higher resistance. This effect was not due to differences in expression or in catalytic activity. This system should prove useful in analysis of novel proteins predicted to be localized to the thylakoid membrane.

Key words: Topology; Thylakoid; Chloroplast; Photosystem I; Aminoglycoside adenyltransferase

1. Introduction

The inherent asymmetry of biological membranes allows them to perform their various functions, and the topological arrangement of integral membrane proteins is strikingly asymmetric [1]. This topological fidelity is due to the operation of the protein translocation machinery. The Sec translocon in the *Escherichia coli* inner membrane is a channel composed of the SecY and SecE polypeptides through which the extended polypeptide moves, using the ATPase of SecA, with the SecD/F complex coupling the proton electrochemical gradient to polypeptide translocation [2–4]. The signal recognition particle (SRP), and the SRP receptor participate in the eukaryotic translocation machinery at the endoplasmic reticulum [5,6], and portions of this machinery are conserved in eubacteria [7].

Sequence analysis has proved useful in predicting transmembrane (TM) segments and their orientation in the membrane [8]. However, polytopic membrane proteins can have TM α-helices of less hydrophobic character, especially if the polar regions interact together or within an internal channel, leading to uncertainties in assignment. For example, such ambiguity led to speculation that helix 7 of PsaA was membrane-attached rather than membrane-spanning [9]. Thus, the hypotheses formulated by sequence analysis must be tested by other methods. Most biochemical methods rely on specific antibodies to different regions of a polypeptide [10], combined with freeze-fracture and immuno-electron microscopy or protease treatment and immunoblot [11]. The main disadvantages of these techniques are that a certain fraction of purified vesicles have the wrong topology and that it requires generation of a unique antibody for each inter-membrane loop. Manoil and Beckwith [12] devised a genetic approach to analyzing membrane protein topology in *Escherichia coli* by constructing translational fusions of membrane proteins to alkaline phosphatase, which is normally secreted and active in the periplasm. Those fusions that placed the alkaline phosphatase domain in the periplasm had detectable activity on external substrates, allowing elucidation of the topology of the target protein. The important point is not the *inherent activity* of the enzyme domain but its *access* to substrate. Similar methods have been used to study topology and translocation in the yeast *Saccharomyces cerevisiae* [13,14].

Translocation across the thylakoid membrane reflects the eubacterial origin and hybrid nature of the chloroplast. There are four major pathways, three of which (Sec, Tat, and SRP) are shared with eubacteria [15]. In order to construct a genetic system for the chloroplast, we used the bacterial *aadA* gene, encoding aminoglycoside adenyltransferase [16], as a reporter and the chloroplast gene, *psaA*, which encodes the PsaA polypeptide of Photosystem I (PSI), as a test case (see Fig. 1). Expression of *aadA* in the chloroplast results in resistance to both spectinomycin and streptomycin [17], and has been used as a reporter for chloroplast gene expression [18]. PsaA, along with the related PsaB polypeptide, make up the core of PSI. The recent publication of a 2.5-Å crystal structure [19] puts PSI into that rare class of membrane proteins with crystal structures sufficiently resolved to identify individual amino acid residues.

2. Materials and methods

2.1. Fusion construction

A portion of the *psaA* cDNA was PCR-amplified from the plasmid pOS191 [20] using the pBluescript SK primer (Stratagene) and a primer complementary to exon 3 (see Table 1 for primers used). The plasmid designated pKR300 was constructed by digesting the PCR product with *Hpa*II and *Nco*I and ligating it with plasmid cg20 [18] previously cut with *Nco*I and *Cla*I. This places the *aadA* gene (out of

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frame) after the promoter and 5′-UTR of exon 1, exon 2, and the beginning of exon 3 (up to the *Nco*I site). Varying lengths of *psaA*-3 sequences were produced by PCR using oligonucleotides complementary to the 5′-UTR of exon 3 and downstream sequences (Table 1). The PCR products were digested with *Nco*I and inserted into pKR300 at the unique *Nco*I site. The anti-sense primers were complementary to regions of psaA encoding inter-helical loops and contained an engineered *Nco*I site, which put the ATG (within CCATGG) in frame with the upstream PsaA sequences. All amplified regions and cloning junctions were confirmed by sequencing. Two independent subclones were chosen and used for transformation.

2.2. Growth and transformation of algae

Chlamydomonas reinhardtii cells were grown at 25°C in TAP liquid medium as described [21]. The recipients for transformation were the *psaA-3Δ* [22] and FUD50 [21] strains. Chloroplast transformation was carried out using a helium-driven particle gun (Bio-Rad). The *psaA-3Δ* recipient was plated on TAP plates supplemented with 75 μg/ml spectinomycin, while the FUD50 strain was plated on minimal media. Cells were bombarded with 1-μm gold particles coated with plasmid DNA. The bombarded *psaA-3Δ* cells were incubated for 2 weeks at 25°C in dim light (0.5 μmol photons m^{-2} s^{-1}) and transformants were propagated on TAP plates supplemented with 100 μg/ml spectinomycin. The bombarded FUD50 cells were incubated for 2 weeks at 25°C in medium light (25–50 μmol photons m^{-2} s^{-1}) and were propagated under the same conditions. Transformants were sub-cloned more than three times before characterization.

For growth tests, cells were grown for 72 h in low light to a concentration of $1–2\times10^6$ cells/ml. 10 μl of culture was spotted on TAP plates supplemented with various concentrations of aminoglycoside antibiotics. The plates were allowed to dry and placed in dim light for 4–5 days before being photographed. At least two independent transformants from each of two plasmid subclones were tested (≥ 5 for each fusion) and the experiment was performed five times to ensure reproducibility.

2.3. Preparation and PCR analysis of genomic DNA

Genomic DNA was prepared by a standard protocol based upon published methods [23], and was used for PCR analysis using the ex2(3′-s) and aadA(5′-as) oligonucleotides as primers. Thus, the amplified portion would correspond to most of the *psaA* portion of the chimera, including the junction through *aadA*. This produced a ladder of PCR products (data not shown), each of which was close to the expected molecular weight ($< 5\%$ difference between calculated and measured molecular weights).

2.4. Biochemical assays

Thylakoid membranes were purified from whole cells as described [24]. Total chlorophyll was calculated as previously described [25]. Immunoblotting of the solubilized proteins of the thylakoid was performed following standard protocols [24]. Rabbit polyclonal antibodies against PsaA [26] and AadA [18] were used to bind the immobilized fusion polypeptides.

The assay of aminoglycoside-3″-adenyltransferase (AAD) enzyme activity was modified from Goldschmidt-Clermont [17]. Purified thylakoid membranes from each strain were pelleted by addition of 50 mM $MgCl_2$ and centrifugation at $20\,000\times g$ for 5 min. The samples were resuspended in AAD-RB (25 mM HEPES–KOH, pH 7.8, 5 mM $MgCl_2$, 100 mM NH_4Cl, 0.5 mM DTT, 20% glycerol) and the chlorophyll concentration was determined. All samples were diluted to the same concentration ($\sim$300 μg Chl/ml), sonicated, and incubated 5 min at 25°C before beginning the assay. The reaction was initiated by mixing one volume of 5× reaction solution (1× AAD-RB, 0.5 mM rATP, 725 μg/ml streptomycin, 1 μCi/ml [^{32}P]α-rATP, 0.5 U/ml pyrophosphatase, 25 mM creatine phosphate, 400 μg/ml creatine kinase) with four volumes of sample. Time points were taken immediately and every 2 min thereafter. At each time point, 16 μl of the mixture was removed and placed into a tube with 4 μl of 500 mM EDTA on ice. After completion, the stopped reactions were centrifuged for 2 min at $10\,000\times g$ to remove the residual membranes. 15 μl of each supernatant was spotted on a 21-mm diameter phosphocellulose paper filter (Whatman P81) and allowed to dry for 5 min. The filters were washed in 75°C ultra-pure water briefly, washed at room temperature three times, dried, placed in scintillation fluid (Packard Ultima Gold-XR) and allowed to equilibrate for 2 h before scintillation counting.

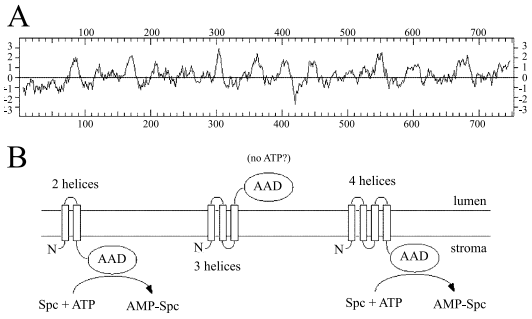

Fig. 1. Construction of chimeric genes. Panel A: Hydropathy plot of PsaA polypeptide (Kyte-Doolittle algorithm). Panel B: Model of the topological arrangement of two-, three-, and four-helix chimeras. Fusion of an odd number of TM helices is expected to localize the AadA domain in the thylakoid lumen, but an even number of TM helices should place it in the stroma.

3. Results

3.1. Chimeric gene construction and introduction

Varying lengths of coding sequence from the *psaA* gene were PCR-amplified and inserted into a plasmid containing the promoter, 5′-UTR and 5′ coding portion of the *psaA* gene, giving rise to a series of plasmids containing from 2 to 8 TM α-helices of PsaA fused to AadA. The sequences flanking the chimeric gene directed homologous recombination to a non-coding sequence between the *atpB* gene and the inverted repeat [18]. The chimeric genes were introduced by ballistic transformation [27] into two different strains. The FUD50 mutant contains a deletion in the *atpB* gene, and recombination with the introduced plasmid restores photosynthetic growth. This enabled us to select for growth on minimal medium rather than antibiotic resistance, eliminating selective pressure for *aadA* expression. The *psa-3Δ* recipient strain was also used to avoid the complication of having long stretches of homologous *psaA* sequences in two places in the genome as well as the presence of immuno-reactive PsaA polypeptide. Because of the lack of PSI in this strain, they were incapable of photosynthetic growth [28], but all of the transformants possessed enough antibiotic resistance to grow on low amounts of antibiotic sufficient to suppress the growth of the recipient strain. PCR on genomic DNA was used to make sure that the intact chimeric genes had been integrated correctly into the chloroplast genome (data not shown; see Section 2.3 for details).

3.2. Immunological detection of fusion polypeptide

The expression of a representative chimeric protein in the thylakoid was verified by immunoblot analysis. Membranes from the *psaAΔ* strain expressing the two-helix fusion were separated by SDS–PAGE and blotted. Antiserum raised against the PsaA N-terminus recognized a polypeptide of the predicted molecular mass (52 kDa) in total membranes (Fig. 2). This polypeptide was strongly enriched in purified thylakoid membranes, but was not detectable in envelope membranes. A comparable polypeptide was not seen in thylakoid membranes from the non-transformed *psaAΔ* strain (Fig. 2). An antibody raised against the AadA polypeptide also recognized a 52-kDa polypeptide in the thylakoid membrane of transformants (data not shown). This indicates that

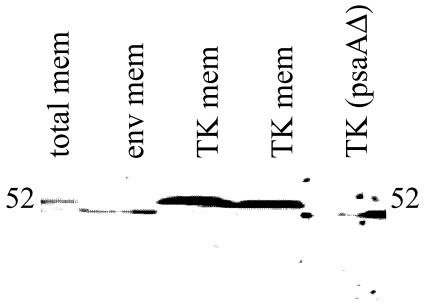

Fig. 2. Immunoblot of PsaA(two-helix)-AadA chimera. The *psaAΔ* strain expressing the PsaA(two-helix)-AadA chimera was subjected to subcellular fractionation. Equal amounts of membrane protein (20 μg; except for the fourth lane, which had 10 μg) were run on 10% SDS–PAGE gels ('total' = total membrane, 'env' = envelope membranes, 'TK' = thylakoid membranes), electroblotted, and probed with the anti-PsaA antibody. The expected size of the chimeric protein (52 kDa) is indicated. A cross-reacting polypeptide of slightly higher mobility was seen in the non-transformed control strain and is not related to the chimera.

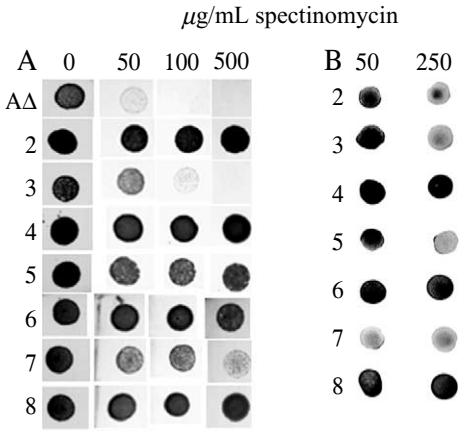

Fig. 3. Assessment of in vivo resistance levels. Cultures of *psaA-3*Δ transformants (A) and FUD50 transformants (B) were spotted on TAP plates supplemented with indicated concentrations (50–500 μg/ml) spectinomycin and grown for 5 days.

the full-length chimeric protein was expressed and present in the thylakoid membrane.

3.3. Antibiotic resistance in vivo

Antibiotic resistance was assessed by examining growth of transformants on agar plates supplemented with aminoglycoside antibiotics (Fig. 3). The *psaA*Δ transformants were generally more resistant than the FUD50 transformants, likely explained by the use of the *psaA* exon 1 promoter in the chimeric genes and the fact that exon 1 is over-expressed in the *psaAΔ-3* mutant (Redding and Rochaix, unpublished results). In general, lower levels of resistance were seen in the strains expressing the chimeras with an odd number of TM α-helices and higher levels with those having an even number of helices (Fig. 3). This is consistent with the idea that an even number of TM helices placed the reporter domain in the stroma, where it could be maximally effective. This observation was also made with the FUD50 transformants (Fig. 3B), where the level of resistance was generally lower; the chimeras having 2, 4, 6, or 8 TM helices grew better in the presence of 250 μg/ml spectinomycin than those having 3, 5, or 7 TM helices. The *psaA*Δ transformants possessed higher levels of resistance, which allowed us to assess their resistance over a greater range of antibiotic concentrations. However, we still observed the same trend – those with chimeras predicted to produce stromal localization of AadA were resistant to higher levels of antibiotic (Fig. 3A). The one exception was the five-helix fusion, which gave variable levels of expression (Fig. 3A; see Section 4 below).

3.4. Enzyme activity in vitro

The lower level of resistance in the odd-numbered chimeras could be explained in several ways unrelated to topology, such as lowered transcription, mRNA stability, or translation. It is perhaps more likely that the odd-numbered chimeric proteins had lower enzymatic activity, and examination of mRNA or polypeptide levels would not test this. Therefore, we developed an in vitro system using thylakoid membranes to assay for AadA activity. Using streptomycin as a substrate, membranes from all of the transformants catalyzed the transfer of radiolabeled adenylate from α-[^{32}P]ATP, while the non-transformed control strain had only low activity (Fig. 4). There was no significant difference in enzyme activity among the various transformants. Thus, the in vivo phenotypes cannot be explained by variations in the expression or intrinsic activity of the chimeric enzymes.

Table 1
Oligonucleotides used

Name	Sequence[a]	Region of hybridization (orientation[b])
*Xba*I(326-as)	atttcatataaaccaacgtgacc	Middle of *psaA* exon 3 (a)
5′-*Nco*II	gaaagaatttgagccgtgtgcagtg	Immediately 5′ of exon 3 (s)
3′-*Nco*I-2fsb	tggtggtttaCcatGgattcaacgttt	Just after helix 2 of *psaA* (a)
3′-*Nco*I-3fs	gtggaatttcCAtGgAatctacaccag	Just after helix 3 of *psaA* (a)
3′-*Nco*I-4fs	catataaaccCaTgGAaccttcacctg	Just after helix 4 of *psaA* (a)
3′-*Nco*I-5fs	gtaaataatgataCCATGgtaccgtaatcag	Just after helix 5 of *psaA* (a)
3′-*Nco*I-6fs	gtctaataagtCCAtgGagttattagtagg	Just after helix 6 of *psaA* (a)
3′-*Nco*I-7fs	gtatctgagaCcatgGcttgaggacg	Just after helix 7 of *psaA* (a)
3′-*Nco*I-8fs	tcacaagggaCCATgGaacctaagttagc	Just after helix 8 of *psaA* (a)
3′-*Nco*I-10fs	atacttaaagcCATGggttgaattgcag	Just after helix 10 of *psaA* (a)
aadA(5′-as)	cctctgatagttgagtcgatac	5′ end of *aadA* gene (a)
ex1-5′-UTR(s)	gcgttgctaatggtgtaaat	5′UTR of *psaA* exon 1 (s)
ex2(3′-s)	cagtgcacactttggtcaat	3′ end of *psaA* exon 2 (s)

[a]Uppercase bases are mutations to introduce *Nco*I site.
[b]s = sense, a = anti-sense.

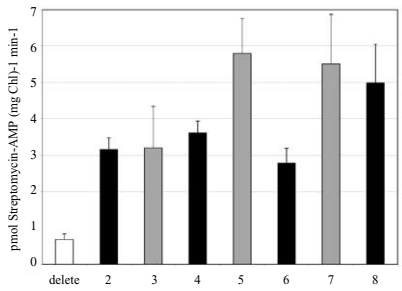

Fig. 4. Aminoglycoside adenyltransferase assays. The rate of streptomycin adenylation was measured in thylakoid membranes. Activity is expressed as pmol streptomycin-AMP min^{-1} (mg Chl)$^{-1}$ along the vertical axis (average of ≥ 3 trials ± S.E.M.). 'delete' = non-transformed *psaA*Δ strain; numbers refer to number of TM helices of PsaA fused to AadA and are indicated on the bottom.

4. Discussion

We have demonstrated that fusions of the AadA domain to integral membrane polypeptides of the chloroplast can be used as a general tool to examine topology. However, we had an anomalous result with the five-helix fusion. Taking the data at face value without further knowledge of PsaA's topology, we would have two possible explanations: (1) hypothetical helix 5 is TM and the resistance is an artifact, or (2) neither hypothetical helices 5 nor 6 are TM (since the six-helix fusion also gave resistance). In this case, we know that helix 5 is TM, and that explanation (1) is the correct one. This is also supported by the results in the FUD50 background, where the five-helix fusion conferred low resistance. We found variable resistance in later *psaA*Δ transformants harboring the five-helix fusion; some had much lower resistance (data not shown). This underscores the importance of analyzing several transformants. It is possible that growth on antibiotic media after transformation selected *psaA*Δ transformants that had higher resistance, although we do not know if or why the five-helix fusion would be more apt to give rise to such transformants. In the FUD50 background, we selected for restoration of photosynthetic growth due to the co-inserted *atpB* gene, and only measured antibiotic resistance later. Use of both genetic backgrounds may thus be useful. In any event, while this method is useful for testing models, it may be dangerous to rely solely upon it for topological assignments.

Over evolutionary time, many genes have been transferred from the chloroplast's ancestor to the nucleus, necessitating the import of thousands of proteins [29]. Adoption of this method for nuclear-encoded thylakoid membrane proteins should be possible. Cerutti and coworkers [30] have developed a dominant selectable marker for nuclear transformation of *C. reinhardtii*, composed of the coding sequence of the eubacterial *aadA* gene (conferring spectinomycin resistance) fused to the 5′ and 3′ untranslated regions, the promoter, and the chloroplast import peptide of the nuclear *RbcS2* gene. Thus, one could simply insert a section of the protein of interest between the import peptide and the *aadA* gene, and introduce the chimeric gene into the nucleus by standard techniques. The chimeric protein should be targeted and transported to the chloroplast, where the TM helices would allow it to be integrated into the membrane in the same way as the protein under examination. In addition, there is every reason to suspect that this technique could be used in higher plants, as the *aadA* gene confers resistance in tobacco [31]. However, it is unlikely to work with chloroplast envelope proteins, as the presence of ATP in the intermembrane space may allow inactivation of antibiotics before entry into the stroma.

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