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

John Lee Franklin\textsuperscript{a,b,1}, Jianying Zhang\textsuperscript{a}, Kevin Redding\textsuperscript{a,b,}\textsuperscript{*}

\textsuperscript{a} Department of Chemistry, The University of Alabama, 120 Lloyd Hall, 6th Ave., Tuscaloosa, AL 35487-0336, USA
\textsuperscript{b} Department of Biological Sciences, The University of Alabama, 120 Lloyd Hall, 6th Ave., Tuscaloosa, AL 35487-0336, USA

Received 2 December 2002; revised 3 January 2003; accepted 3 January 2003

First published online 17 January 2003

Edited by Ulf-Ingo Flüge

Abstract We have developed a system to examine the topology of thylakoid membrane proteins using the bacterial aad\textsuperscript{A} 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 Aad\textsuperscript{A} fused to varying lengths of the PsaA polypeptide, whose topology is known, were introduced into the chloroplast genome of \textit{Chlamydomonas reinhardtii}. As expected, chimeras with an even number of \(\alpha\)-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.

\textsuperscript{*}Corresponding author. Fax: (1)-205-348 9104.
E-mail addresses: jfranklin@path.ua.edu (J.L. Franklin), zhang914@bama.ua.edu (J. Zhang), kevin.redding@ua.edu (K. Redding).

1 Present address: Department of Pathology, University of Alabama-Birmingham, 1670 University Blvd., VH G031, Birmingham, AL 35294-0019, USA.

© 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

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 \cite{1}. This topological fidelity is due to the operation of the protein translocation machinery. The Sec translocon in the \textit{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 \cite{2-4}. The signal recognition particle (SRP), and the SRP receptor participate in the eukaryotic translocation machinery at the endoplasmic reticulum \cite{5,6}, and portions of this machinery are conserved in eubacteria \cite{7}.

Sequence analysis has proved useful in predicting transmembrane (TM) segments and their orientation in the membrane \cite{8}. However, polytopic membrane proteins can have TM \(\alpha\)-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 \cite{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 \cite{10}, combined with freeze-fracture and immuno-electron microscopy or protease treatment and immunoblot \cite{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 \cite{12} devised a genetic approach to analyzing membrane protein topology in \textit{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 \textit{active} activity of the enzyme domain but its \textit{access} to substrate. Similar methods have been used to study topology and translocation in the yeast \textit{Saccharomyces cerevisiae} \cite{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 \cite{15}. In order to construct a genetic system for the chloroplast, we used the bacterial aad\textsuperscript{A} gene, encoding aminoglycoside adenyltransferase \cite{16}, as a reporter and the chloroplast gene, \textit{psaA}, which encodes the PsaA polypeptide of Photosystem I (PSI), as a test case (see Fig. 1). Expression of aad\textsuperscript{A} in the chloroplast results in resistance to both spectinomycin and streptomycin \cite{17}, and has been used as a reporter for chloroplast gene expression \cite{18}. PsaA, along with the related PsaB polypeptide, make up the core of PSI. The recent publication of a 2.5-Å crystal structure \cite{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 p\textit{saA} cDNA was PCR-amplified from the plasmid pOS191 \cite{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 \textit{HpaII} and \textit{NcoI} and ligating it with plasmid cg20 \cite{18} previously cut with \textit{NcoI} and \textit{CiaI}. This places the \textit{aadA} gene (out of

0014-5793/03/$22.00 © 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

doi:10.1016/S0014-5793(03)00034-6
frame) after the promoter and 5’-UTR of exon 1, exon 2, and the beginning of exon 3 (up to the NcoI 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 NcoI and inserted into pKR300 at the unique NcoI site. The anti-sense primers were complementary to regions of psaA encoding inter-helical loops and contained an engineered NcoI site, which put the ATG (within CCAATGG 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 FUD50 strain was plated on minimal media. 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 1-2×10⁶ 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(3-5) and aadA antisense oligonucleotides as primers. Thus, the amplified portion would correspond to most of the psaA portion of the chimeras, 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 protein 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’-adenylyltransferase (AAD) enzyme activity was modified from Goldschmidt-Clermont and Montenat [19]. Purified thylakoid membranes from each strain were pelleted by addition of 50 mM MgCl₂ and centrifugation at 20000×g for 5 min. The samples were resuspended in AAD-RB (25 mM HEPES-KOH, pH 7.8, 5 mM MgCl₂, 100 mM NiCl₂, 0.5 mM DTT, 20% glycerol) and the chlorophyll concentration was determined. All samples were diluted to the same concentration (~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 spectinomycin, 1 μg/ml [32P]-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 10000×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 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 foraadA expression. The psaA-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 psaA-3Δ 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.

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 psaAΔ 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 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 psaAΔ 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-32P]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

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequencea</th>
<th>Region of hybridization (orientationb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Khol(326-as)</td>
<td>attccatatataaccaacaggatacc</td>
<td>Middle of psaA exon 3 (a)</td>
</tr>
<tr>
<td>5’-NcoI</td>
<td>ggaagaattttgacgctgtgcagtg</td>
<td>Immediately 5’ of exon 3 (s)</td>
</tr>
<tr>
<td>3’-NcoI-2fsb</td>
<td>tggagttttcccattgctattccaccag</td>
<td>Just after helix 2 of psaA (a)</td>
</tr>
<tr>
<td>3’-NcoI-3fs</td>
<td>tgtgacataccatgagaccttaacccag</td>
<td>Just after helix 3 of psaA (a)</td>
</tr>
<tr>
<td>3’-NcoI-4fs</td>
<td>catatataaccctgacacccctccttg</td>
<td>Just after helix 4 of psaA (a)</td>
</tr>
<tr>
<td>3’-NcoI-5fs</td>
<td>gtaataaatgatcatgcgtcggctaatcag</td>
<td>Just after helix 5 of psaA (a)</td>
</tr>
<tr>
<td>3’-NcoI-6fs</td>
<td>gtctataaatgagagcggagagtagttagg</td>
<td>Just after helix 6 of psaA (a)</td>
</tr>
<tr>
<td>3’-NcoI-7fs</td>
<td>gtactctgagccatgcctgggcttagg</td>
<td>Just after helix 7 of psaA (a)</td>
</tr>
<tr>
<td>3’-NcoI-8fs</td>
<td>tcaacaggggcatgcagcatgattctagc</td>
<td>Just after helix 8 of psaA (a)</td>
</tr>
<tr>
<td>3’-NcoI-10fs</td>
<td>atactctagggcatgcgtgtgtagctcag</td>
<td>Just after helix 10 of psaA (a)</td>
</tr>
<tr>
<td>aadA(5’s-as)</td>
<td>cctctgatgctgtgagcctgatc</td>
<td>5’ end of aadA gene (a)</td>
</tr>
<tr>
<td>ex1-5’-UTR(s)</td>
<td>gcgtgctataaggtgtagaat</td>
<td>5’UTR of psaA exon 1 (a)</td>
</tr>
<tr>
<td>ex2(3’s)</td>
<td>cagtgcacacctttggtcactt</td>
<td>3’ end of psaA exon 2 (s)</td>
</tr>
</tbody>
</table>

aUppercase bases are mutations to introduce NcoI site.
bS = sense, a = anti-sense.
formed psaA
PsaA fused to AadA and are indicated on the bottom.
100

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.

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 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 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 introduction of 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.

Acknowledgements: The following persons contributed to the construction of the translational fusion plasmids: Otello Stampacchia, Gabrielle Gerelle, Bodil Lindved, and Caroline Vandell. K.R. is especially grateful to Dr. Jean-David Roaix, in whose laboratory most of the plasmids were constructed, with the support of Grant 3100-050895.97 from the Swiss National Fund. The work in K.R.’s laboratory was supported by a DuPont Young Professor Award.

References