Membrane Biogenesis and Protein Targeting

Editors

WALTER NEUPERT and ROLAND LILL

Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie, Ludwig-Maximilians-Universität München, Goethestraße 33, 8000 München 2, Germany

1992 ELSEVIER
Amsterdam • London • New York • Tokyo
Contents

List of contributors ............................................................... v

Part A  Bacteria

Chapter 1. Where are we in the exploration of Escherichia coli translocation pathways?
Bill Wickner ........................................................................ 3

1. Protein translocation pathways ........................................... 4
2. Open questions ................................................................... 6
References .............................................................................. 7

Chapter 2. Components involved in bacterial protein translocation
Chris Harris and Phang C. Tai ................................................. 9

1. Introduction ........................................................................ 9
2. The minimal length of a prokaryotic signal peptide .............. 10
3. The N(m) element: an export requirement for low basicity as well as low polarity ......................................................... 12
4. Is SecY/PrlA essential for protein translocation? ............... 14
5. In vitro suppression of defective signal peptides ................. 15
6. Roles of SecD and SecF in protein translocation ............... 16
7. An inhibitor of protein translocation ................................. 17
8. Perspective ....................................................................... 18
References .............................................................................. 18

Chapter 3. Molecular characterization of Sec proteins comprising the protein secretory machinery of Escherichia coli
Shoji Mizushima, Hajime Tokuda and Shin-ichi Matsuyama ........ 21

1. Introduction ........................................................................ 21
2. Overproduction of Sec proteins .......................................... 22
3. Purification of Sec proteins .................................................. 23
4. Estimation of the numbers of Sec proteins and of the secretory machinery in one E. coli cell ......................................................... 24
5. Functions of SecA in protein translocation ......................... 25
6. Functions of SecE and SecY .................................................. 27
7. Functions of SecD and SecF .................................................. 28
8. Discussion ....................................................................... 28
References .............................................................................. 30
Chapter 4. Distinct steps in the insertion pathway of bacteriophage coat proteins
Andreas Kuhn and Dorothee Troschel

1. Introduction .......................................................... 33
2. Results and discussion ........................................... 34
   2.1. Pf3 coat protein requires no leader sequence for membrane insertion .... 34
   2.2. Hybrid coat proteins of M13 and Pf3 .................................. 35
   2.3. M13 procoat protein first binds electrostatically to the membrane surface .. 36
   2.4. Both hydrophobic regions are required for the partitioning of the M13 procoat protein into the membrane ................................................. 38
   2.5. Translocation of the negatively charged periplasmic region of the M13 procoat protein is not primarily an electrophoretic event .................. 39
   2.6. M13 procoat protein as a model substrate for leader peptidase ............. 41
   2.7. From membrane to phage ........................................... 42
   2.8. The Sec-independent insertion pathway is limited to simple membrane translocation domains ......................................................... 42
3. Conclusions ......................................................... 45
References ........................................................................ 46

Chapter 5. Steps in the assembly of a cytoplasmic membrane protein: the MalF component of the maltose transport complex
Beth Traxler and Jon Beckwith

1. Introduction .......................................................... 49
   1.1. Issues in the study of membrane protein assembly and structure .......... 49
   1.2. The MalF protein as a model system ...................................... 51
   1.3. The mechanism of insertion into the membrane of MalF .................... 52
2. The nature of topogenic signals in MalF ...................................... 53
4. Assembly of MalF into the quaternary MalF–MalG–MalK structure ............. 57
5. Summary ...................................................................... 59
References ........................................................................ 60

Chapter 6. Structural characteristics of presecretory proteins: their implication as to translocation competency
Shoji Mizushima, Katsuko Tani, Chinami Hikita and Masashi Kato

1. Introduction .......................................................... 64
2. Amino terminal positive charge of the signal peptide .............................. 64
3. Central hydrophobic stretch of the signal peptide .................................... 65
4. Function of the positive charge can be compensated for by a longer hydrophobic stretch ................................................................. 67
5. Carboxyl terminal region of the signal peptide including the cleavage site .. 68
6. Roles of charged amino acid residues in the mature domain in protein translocation .............................................................. 70
7. Chemical structure of the mature domain tolerated by the secretory machinery 72
References ........................................................................ 73
Chapter 7. Sequence determinants of membrane protein topology
Gunnar von Heijne .................................................. 75

1. Introduction ..................................................... 75
2. Results .......................................................... 76
   2.1. Signals and topologies .................................... 76
   2.2. The positive inside-rule ................................... 78
   2.3. Positively charged residues control membrane protein topology .... 78
   2.4. A membrane protein with pH-dependent topology .......... 80
   2.5. Position-specific charge-pairing can affect the topology .......... 80
   2.6. Sec-dependent versus sec-independent assembly ............. 81
3. Discussion ...................................................... 82
References ........................................................ 83

Chapter 8. Lipid involvement in protein translocation

1. Introduction ..................................................... 85
2. Results and discussion ........................................ 86
   2.1. Prokaryotic protein secretion ................................ 86
   2.2. Mitochondrial protein import ................................ 91
   2.3. Chloroplast protein import .................................. 97
3. Concluding remarks ............................................ 99
References ........................................................ 99

Part B  Endoplasmic reticulum

Chapter 9. Membrane protein insertion into the endoplasmic reticulum: signals, machinery and mechanisms
Stephen High and Bernhard Dobberstein ................................ 105

1. Introduction ..................................................... 105
2. Types of membrane proteins and their topological signals .......... 105
   2.1. Proteins with uncleaved signal sequences ................... 106
   2.2. Proteins with cleavable signal sequences .................... 107
   2.3. The loop model for protein insertion into the membrane .... 108
   2.4. Biosynthesis of multiple spanning membrane proteins ....... 109
3. Components involved in the insertion of proteins into the ER membrane . 111
   3.1. Targeting .................................................. 111
   3.2. Membrane insertion ........................................ 111
   3.3. GTP requirement .......................................... 114
4. Discussion ...................................................... 115
References ........................................................ 117
Chapter 10. Translocation of proteins through the endoplasmic reticulum membrane: investigation of their molecular environment by cross-linking
Enno Hartmann and Tom A. Rapoport

1. Introduction .................................................. 119
2. Results ..................................................... 120
   2.1. Experimental strategies ............................... 120
   2.2. The SSR-complex .................................. 121
   2.3. The TRAM protein .................................. 122
   2.4. Other glycoproteins ................................. 123
   2.5. Unglycosylated proteins ............................ 123
   2.6. The Sec proteins of yeast microsomes ............ 124
3. Discussion ................................................. 124
References .................................................. 126

Chapter 11. The role of GTP in protein targeting to the endoplasmic reticulum
Stephen C. Ogg, Jodi M. Nunnari, Joshua D. Miller and Peter Walter

References .................................................. 135

Chapter 12. Consecutive steps of nucleoside triphosphate hydrolysis are driving transport of precursor proteins into the endoplasmic reticulum
Peter Klappa, Günter Müller, Gabriel Schlenstedt, Hans Wiech and Richard Zimmermann

1. Introduction .................................................. 137
2. Results ..................................................... 138
   2.1. Ribonucleoparticles versus molecular chaperones 139
   2.2. Translocase .......................................... 141
3. Discussion ................................................. 143
   3.1. Components involved in protein transport into yeast endoplasmic reticulum 143
   3.2. Model for ribonucleoparticle-independent transport ............... 143
   3.3. Open questions ...................................... 144
References .................................................. 145

Part C Vacuoles

Chapter 13. Mechanism and regulation of import and degradation of cytosolic proteins in the lysosome/vacuole
Hui-Ling Chiang and Randy Schekman

1. Introduction .................................................. 150
2. Intracellular protein degradation in mammalian lysosomes ............... 151
   2.1. Microautophagy ..................................... 151
   2.2. Macroautophagy ..................................... 152
   2.3. Lysosomal protein degradation in cultured fibroblasts ............ 153
3. Protein degradation in the yeast vacuole ................................ 156
3.1. Catabolite inactivation ........................................ 156
3.2. Vacuolar degradation of FBPase ............................. 158
3.3. Mechanism of FBPase degradation ............................ 159
3.4. Covalent modifications and FBPase degradation ............ 160
  3.4.1. Phosphorylation ........................................... 160
  3.4.2. Ubiquitination ............................................ 161
References .......................................................... 162

Chapter 14. The sorting of soluble and integral membrane proteins to the yeast vacuole
Christopher K. Raymond, Carol A. Vater, Steven Nothwehr, Christopher J. Roberts and Tom H. Stevens .......... 165

1. Introduction ...................................................... 165
2. Results ............................................................ 167
  2.1. No single domain of the vacuolar integral membrane protein DPAP B is required for vacuolar delivery .......... 167
  2.2. The cytoplasmic domain of DPAP A is necessary and sufficient for its localization to a late Golgi compartment ...... 169
  2.3. Vps1p, which is required for the sorting of soluble vacuolar glycoproteins, shares extensive similarity with a subfamily of GTP-binding proteins . . 170
  2.4. Vps1p binds and hydrolyzes GTP ................................ 172
  2.5. Mutational analysis suggests that Vps1p is composed of two functionally distinct domains .......................... 173
3. Discussion ......................................................... 177
  3.1. Targeting of integral membrane proteins in the secretory pathway of Saccharomyces cerevisiae ............................. 177
  3.2. Vps1p, which is a GTPase required for the sorting of soluble vacuolar proteins, is composed of two functionally distinct domains .......................... 179
References .......................................................... 181

Part D Peroxisomes

Chapter 15. Defining components required for peroxisome assembly in Saccharomyces cerevisiae
Jörg Höhfeld, Daphne Mertens, Franziska F. Wiebel and Wolf-H. Kunau ... 185

1. Introduction ...................................................... 185
2. Results ............................................................ 187
  2.1. Peroxisomal mutants of Saccharomyces cerevisiae ........... 187
    2.1.1. Defects in peroxisome formation (type I pas mutants: pas1, pas2, pas3, pas5) ............................................. 189
    2.1.2. Defects in peroxisome proliferation (type II pas mutants: pas4 and pas6) ..................................................... 191
    2.1.3. Defects in import of 3-oxoacyl-CoA thiolase (type III pas mutant: pas7) ..................................................... 191
  2.2. Cloning of peroxisomal genes ................................ 191
  2.3. Sequence analysis ............................................ 192
2.3.1. PAS1 .................................................. 192
2.3.2. PAS2 .................................................. 194
2.3.3. PAS4 .................................................. 194

2.4. Identification and characterization of the gene products ................. 195
2.5. Analysis of the function of the cloned genes for peroxisome biogenesis .............................................. 196
  2.5.1. Overexpression of PAS4 .................................. 196
  2.5.2. Site directed mutagenesis ................................. 197
  2.5.3. Conditional peroxisomal mutants ......................... 197

2.6. Fusion proteins as tools for further investigations .................................................. 199

3. Discussion ........................................................................ 200
  3.1. Peroxisomal mutants as a tool to dissect peroxisome biogenesis .... 200
  3.2. Do peroxisomal prestructures exist in type I pas mutants? ........ 202
  3.3. Are type I pas mutants peroxisomal import mutants? ............... 203
  3.4. Do more peroxisomal import routes exist other than the
       SKL-mediated pathway? ............................................. 203
  3.5. Are type II pas mutants affected in peroxisome proliferation? ... 204

4. Conclusions ...................................................................... 205

References ........................................................................ 205

Chapter 16. Structure and assembly of peroxisomal membrane proteins
Joel M. Goodman, Lisa J. Garrard and Mark T. McCammon ...................... 209

1. Introduction ..................................................................... 210

2. Results ........................................................................... 210
  2.1. Assembly of peroxisomal proteins ............................. 212
  2.2. Proliferation of peroxisomal components ................... 213
  2.3. Structure and composition of peroxisomal membranes ....... 213
  2.4. Cloning of genes encoding membrane proteins .............. 214
  2.5. Expression and sorting of PMP47 ............................... 215

3. Discussion ...................................................................... 217

References ........................................................................ 219

Chapter 17. Mechanisms of transport of proteins into microbodies
Suresh Subramani .................................................................. 221

1. Introduction .................................................................... 221

2. A C-terminal tripeptide is a major targeting signal for proteins of the
   microbody matrix ......................................................... 223

3. Certain variants of the SKL tripeptide can also function as PTS ...... 224

4. Peroxisomal protein transport in microinjected mammalian cells. ... 224

5. Import deficiency in fibroblast cells from Zellweger’s syndrome patients. ... 225

6. An amino-terminal PTS resides in the cleaved leader peptides of the
   peroxisomal thiolases .................................................. 226

7. Selective import deficiency in Zellweger cells ..................... 226

8. Transport of membrane proteins into peroxisomes ................ 227

9. Summary ....................................................................... 227

References ........................................................................ 227
### Chapter 18. Lessons for peroxisome biogenesis from fluorescence analyses of Zellweger syndrome fibroblasts

**Paul B. Lazarow, Hugo W. Moser and Manuel J. Santos**  

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>231</td>
</tr>
<tr>
<td>Results</td>
<td>232</td>
</tr>
<tr>
<td>Discussion</td>
<td>233</td>
</tr>
<tr>
<td>3.1. Peroxisome membranes are always present: perhaps they are required for viability</td>
<td>233</td>
</tr>
<tr>
<td>3.2. The peroxisome membranes are nearly empty ghosts: these are Peroxisome IMport (PIM) mutations</td>
<td>234</td>
</tr>
<tr>
<td>3.3. Peroxisome membrane assembly has fewer requirements, or different requirements, from the packaging of peroxisome matrix proteins</td>
<td>234</td>
</tr>
<tr>
<td>3.4. Empty peroxisome membrane ghosts divide</td>
<td>235</td>
</tr>
<tr>
<td>3.5. Genetic complementation for peroxisome assembly is formally demonstrated</td>
<td>235</td>
</tr>
<tr>
<td>3.6. Preliminary partial correlation of genotype and phenotype</td>
<td>236</td>
</tr>
<tr>
<td>3.7. Future directions</td>
<td>236</td>
</tr>
</tbody>
</table>

References  

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>236</td>
</tr>
</tbody>
</table>

### Part E Mitochondria

### Chapter 19. The mitochondrial protein import machinery of Saccharomyces cerevisiae

**Victoria Hines**  

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>241</td>
</tr>
<tr>
<td>2. Components of the import machinery</td>
<td>242</td>
</tr>
<tr>
<td>2.1. Import receptors</td>
<td>242</td>
</tr>
<tr>
<td>2.2. The protein translocation channel</td>
<td>245</td>
</tr>
<tr>
<td>2.3. Refolding and processing proteins</td>
<td>245</td>
</tr>
<tr>
<td>3. The mechanism of protein import</td>
<td>246</td>
</tr>
<tr>
<td>3.1. Energy requirements</td>
<td>246</td>
</tr>
<tr>
<td>3.2. Contact sites</td>
<td>247</td>
</tr>
<tr>
<td>3.3. Protein sorting</td>
<td>248</td>
</tr>
<tr>
<td>4. Outlook</td>
<td>250</td>
</tr>
</tbody>
</table>

References  

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
</tr>
</tbody>
</table>

### Chapter 20. Protein insertion into mitochondrial outer and inner membranes via the stop-transfer sorting pathway

**Gordon C. Shore, Douglas G. Millar and Jian-Ming Li**  

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>254</td>
</tr>
<tr>
<td>2. Results and discussion</td>
<td>256</td>
</tr>
<tr>
<td>2.1. Mitochondrial topogenic sequences and stop-transfer sorting</td>
<td>256</td>
</tr>
<tr>
<td>2.2. The OMM signal-anchor sequence</td>
<td>256</td>
</tr>
<tr>
<td>2.3. Stop-transfer sorting to the inner membrane</td>
<td>257</td>
</tr>
</tbody>
</table>
Chapter 21. General and exceptional pathways of protein import into sub-mitochondrial compartments

Roland Lill, Christoph Hergersberg, Helmut Schneider, Thomas Söllner, Rosemary Stuart and Walter Neupert

1. The general pathways for protein import into sub-mitochondrial compartments. 265
2. Exceptional pathways of protein import. 268
3. MOM19 is imported into the OM without the aid of surface receptors. 269
4. Cytochrome c heme lyase is imported directly through the OM via a non-conservative sorting pathway. 271
5. Perspectives. 274

References. 275

Part F Chloroplasts

Chapter 22. Targeting of proteins into and across the chloroplastic envelope

H.-M. Li, S.E. Perry and K. Keegstra

1. Introduction. 280
2. Transport across the envelope membranes. 281
   2.1. Binding of precursors to the chloroplastic surface. 281
   2.2. Translocation of precursor across the envelope membranes. 283
3. Targeting of proteins into the envelope membranes. 284
   3.1. Targeting to the outer envelope membrane. 284
   3.2. Targeting to the inner envelope membrane. 286
4. Summary and future prospects. 287

References. 287

Chapter 23. Transport of proteins into the thylakoids of higher plant chloroplasts

Colin Robinson

1. Introduction. 289
2. Results. 291
   2.1. Development of an in vitro assay for the import of proteins by isolated thylakoids. 291
   2.2. Energy requirements for the import of proteins into isolated thylakoids. 292
   2.3. Events in the stroma. 292
   2.4. Maturation of imported thylakoid lumen proteins. 295
3. Discussion. 295

References. 296
Chapter 24. Comparison of two different protein translocation mechanisms into chloroplasts
Jürgen Soll, Heike Alefsen, Birgit Böckler, Birgit Kerber, Michael Salomon and Karin Waegemann...

1. Introduction .................................................................................. 299
2. Results and discussion .................................................................. 299
   2.1. Import characteristics of pSSU and OEP 7 ......................... 299
   2.2. Specificity and mechanism of OEP 7 insertion .................. 301
References ....................................................................................... 306

Part G  Chaperones

Chapter 25. DnaJ homologs and protein transport
Takao Kurihara and Pamela A. Silver ............................................. 309

1. Introduction ................................................................................. 309
   1.1. Stimulation of protein transport by HSP70s and additional factors 309
   1.2. *E. coli* DnaJ and GrpE function with and regulate bacterial HSP70 (DnaK) .. 310
      1.2.1. Bacteriophage λ and P1 replication and protein complex disassembly ...... 311
      1.2.2. Refolding of thermally inactivated λcI857 repressor ...................... 312
      1.2.3. Proteolysis of puromycin-generated polypeptide fragments ............. 312
      1.2.4. Stimulation of DnaK ATPase activity by DnaJ and GrpE ............... 313
2. Results .......................................................................................... 313
   2.1. DnaJ homologs .................................................................... 313
      2.1.1. Bacterial DnaJ homologs ....................................................... 316
      2.1.2. DnaJ homologs in the yeast *Saccharomyces cerevisiae* .......... 316
      2.1.3. SCJ1 ............................................................................. 316
      2.1.4. YDJ1/MAS5 .................................................................. 317
      2.1.5. SIS1 ............................................................................. 318
      2.1.6. NPL1/SEC63 ................................................................. 319
      2.2. The J-region ....................................................................... 321
         2.2.1. The NPL1/SEC63 J-region: localization to the ER lumen ........ 321
         2.2.2. Genetic evidence for J-region role in KAR2 interaction and ER translocation .... 323
3. Discussion .................................................................................... 323
   3.1. Model for NPL1/SEC63 function ........................................ 323
   3.2. DnaJ homologs, J-regions and protein transport ............. 324
References ....................................................................................... 325

Chapter 26. Chaperonin-mediated protein folding
Arthur L. Horwich, Shari Caplan, Joseph S. Wall and F.-Ulrich Hartl .... 329

1. Introduction ................................................................................. 329
2. In vivo analysis of chaperonin function ..................................... 330
3. Role of hsp60 in biogenesis of mitochondrial-encoded proteins .... 331
4. Chaperonin-mediated folding reconstituted in vitro ............... 333
Comparison of two different protein translocation mechanisms into chloroplasts

JÜRGEN SOLL¹, HEIKE ALEFSEN², BIRGIT BÖCKLER¹, BIRGIT KERBER³, MICHAEL SALOMON² and KARIN WAEGEMANN¹

¹Botanisches Institut, Universität Kiel, Olshausenstraße 40 W-2300 Kiel 1, Germany,  
²Botanisches Institut, Universität München, Menzinger Straße 67 W-8000 München 19, Germany and ³Fachrichtung Botanik, Universität des Saarlandes, W-6600 Saarbrücken, Germany

1. Introduction

Chloroplasts are highly structured plant specific organelles. They possess three discrete membrane systems which differ in composition and function, i.e. the outer/inner envelope and the thylakoid membranes. In addition three solute spaces can be distinguished, i.e. the space between the envelope membranes, the stroma and the thylakoid lumen [1]. While most of the chloroplastic proteins, which are synthesized as precursors in the cytosol, seem to follow a common route of translocation into the organelle, proteins of the outer envelope, which is in direct contact with the cytosol, are inserted (imported) by a very different and distinct mechanism [2,3].

A typical polypeptide destined for the inside of the organelle, possesses a cleavable target sequence, retains a loosely folded conformation with the help of molecular chaperones, is recognized by proteinaceous receptors on the organellar surface, requires low concentration (μM) ATP for binding but high concentrations (mM) for complete translocation through the membranes [4,5]. Outer envelope polypeptides (OEP) studied so far, do not possess a cleavable target sequence, do not require protease sensitive receptors on the organellar surface and do not require ATP for either binding or insertion into the outer envelope [3,6].

2. Results and discussion

2.1. Import characteristics of pSSU and OEP 7

A typical import experiment for a plastidic precursor protein destined for the inside
Fig. 1. Characteristics of pSSU import into chloroplasts. pSSU translation product (lane 1) is imported into chloroplasts and processed to its mature form (lane 2). SSU appears protease protected inside the organelle after thermolysin treatment (lane 3). Chloroplasts pretreated with thermolysin bind pSSU only to a very small extent in a non-productive way (lane 4). The import assay was either not depleted (lane 5) or depleted of ATP (lane 6) through the action of apyrase. Methods are described by Waegemann and Soll [15].

of the organelle is shown in Fig. 1. The precursor protein (pSSU) binds to the chloroplast outer envelope, it is subsequently translocated inside the organelle, processed to its mature form and protected against externally added protease. Chloroplasts pretreated by thermolysin, a protease which only digests surface exposed polypeptides [7,8], bind pSSU only to a very small extent in a non-productive manner [2]. Similar results are obtained if ATP is removed from the import incubation mixture by the ATP hydrolyzing enzyme apyrase. Binding is greatly reduced and import not observed, demonstrating the ATP dependence of binding as well as translocation [5].

The insertion (import) of OEP 7 seems to follow a quite distinct pathway. No shift in molecular weight can be observed between the translation product and the inserted form, demonstrating the absence of a cleavable transit sequence [3]. Translocation experiments carried out in the light, i.e. in the presence of ATP, show no greater OEP 7 translocation efficiency than those carried out in the dark, i.e. in the absence of ATP (Fig. 2). Neither apyrase treatment nor the simultaneous inclusion of a non-hydrolysable ATP analog, adenylylimidodiphosphate, influenced the yield of OEP 7

Fig. 2. Characteristics of OEP 7 import (insertion) into chloroplasts. OEP 7 translation product (lane 1) was incubated with intact chloroplasts either in the light (lane 2), i.e. presence of ATP, or in the dark (lane 3), i.e. absence of ATP (translation product was apyrase treated). Protease treatment after import yields a protease protected breakdown product (lane 4). Pretreatment of intact chloroplasts by thermolysin does not influence the efficiency of OEP 7 import (lane 5). OEP 7 is localized in the envelope membranes (lanes 6, 7). Lanes 8 and 9 show a silver stained gel of envelope membranes either not treated (lane 8) or treated (lane 9) with thermolysin. Protease treatment of imported OEP 7 and OEP 7 in situ gives identical proteolytic breakdown products (compare lanes 4 and 9). Methods are described by Salomon et al. [3].
insertion into the outer envelope membrane of chloroplasts. Translocation of OEP 7 followed by thermolysin treatment resulted in a lower molecular weight breakdown product identical to that found if envelope membranes were treated with protease (Fig. 2). OEP 7 contains only the N-terminal methionine, which is not removed by either maturation or thermolysin treatment. This clearly indicates that the N-terminus is exposed to the intermembrane space and therefore protease protected, while the C-terminus is on the cytosolic leaflet of the membrane and susceptible to external protease. Analysis of the amino acid sequence of OEP 7 corroborates these results and predicts only one membrane span [3]. The outline of this translocation route is supported by findings described in [6] where an identical insertion mechanism is described for an outer envelope protein of pea chloroplasts.

2.2. Specificity and mechanism of OEP 7 insertion

The import route outlined above for OEP 7 is very distinct and also differs from that described for proteins localized in the outer mitochondrial membrane, which require protease sensitive receptors and ATP for correct routing and efficient translocation [9]. Experiments were carried out to address the problems of specificity and mechanism of OEP 7 import. In an initial experiment, chloroplasts and mitochondria, both isolated from pea leaves, were incubated in the same import assay with OEP 7 translation product. After completion of the import reaction, chloroplasts were separated from mitochondria by differential centrifugation and each organelle type analyzed, respectively. The results (Fig. 3) demonstrate that OEP 7 binds to the surface of chloroplasts as well as mitochondria. Treatment of the organelles with thermolysin, however, clearly demonstrates that OEP 7 integrates only into the outer envelope of chloroplasts in the proper way but not into the outer membrane of mitochondria as judged from the protease protected breakdown product.

It has been shown that precursor proteins have to retain a loosely folded (transport competent) conformation in order to be translocated through the import apparatus of either mitochondria [9,10] or chloroplasts [4]. They do this with the help of

```
<table>
<thead>
<tr>
<th>Chloropl.</th>
<th>Mitoch.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protease</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>
```

Fig. 3. OEP 7 specifically inserts into chloroplast but not mitochondrial membranes. Chloroplasts and mitochondria, isolated from pea leaves, were incubated in the same incubation assay with OEP 7 translation product. After completion of the reaction, organelles were separated by differential centrifugation and either not treated or treated by thermolysin prior to SDS-PAGE and fluorography.
Fig. 4. Efficient insertion of OEP 7 into chloroplasts requires a special conformation. OEP 7 was synthesized either at 26 °C (■) or 37 °C (□) in a reticulocyte lysate system. Only OEP 7 synthesized at 26 °C inserts efficiently into the outer envelope of chloroplasts (not shown). OEP 7 synthesized at 26 °C (■) is more susceptible to trypsin treatment (units/ml) than OEP 7 synthesized at 37 °C (□).

molecular chaperones, e.g. hsc70 [11,12]. We therefore addressed the question whether it was possible to distinguish between import competent and import incompetent OEP 7.

This was indeed found to be the case. OEP 7 synthesized in a reticulocyte lysate system at 37 °C under improper cofactor conditions and prolonged reaction time did, no longer insert into the outer envelope of chloroplasts (not shown). Trypsin treatment of import incompetent OEP 7 demonstrates that it is much less sensitive to protease than the import competent form of OEP 7 (Fig. 4). The data strongly indicate that OEP 7 like other precursor polypeptides needs to retain a special conformation until it has been inserted into the outer envelope membrane. The outer chloroplast envelope has a unique lipid and protein composition in comparison not only to other organelar membranes exposed to the cytosol but also to the other membrane systems of the chloroplast. Phosphatidylcholine for example is not present in thylakoids. Monogalactosyldiglyceride and digalactosyldiglyceride which are exclusively found in plastidal membrane systems are also major lipid constituents of the outer envelope [1]. The specificity of OEP 7 insertion into the outer envelope could therefore be, at least in part, due to the specific lipid composition.

Most likely the interaction of OEP 7 with other outer envelope proteins aids the insertion specificity. This can be deduced from experiments presented in Fig. 5. Purified chloroplast membranes, i.e. outer envelope, inner envelope and thylakoids, were incubated with OEP 7 translation product. Only the interaction of OEP 7 with outer envelope membrane vesicles, either pretreated with or without protease, resulted in the correct insertion of the protein into the membrane bilayer. OEP 7 also bound to the other chloroplast membranes but we could not detect the typical proteolytic breakdown product, indicating that OEP 7 was either surface exposed and thus protease sensitive or inserted incorrectly into the membranes.
It has been shown that thylakoid membranes are isolated as outside-out vesicles [13]. The same was found to be the case for isolated outer envelope membranes [14]. These findings are important to interpret the described data correctly. Outside-out envelope vesicles were also used to study their interaction with pSSU, a normal precursor [15]. As in the organellar system, pSSU requires ATP and protease sensitive receptors to bind to the envelope surface (Fig. 6). The interaction between pSSU and the isolated envelope membrane does not halt at the binding stage but pSSU is partly inserted into the translocation apparatus as characterized by protease protected translocation intermediates [15]. Isolated outer envelope membranes therefore contain at least part of the chloroplast import machinery in a functionally active manner. Early events in binding and translocation can thus be analyzed in this isolated and partially purified system in vitro.

Our results indicated that the precursor was not translocated into the inside of the vesicle but was stuck in the transport apparatus. Solubilization of precursor loaded outer envelope membrane vesicles followed by sucrose density centrifugation resulted in the isolation of a membrane fraction with precursor protein still bound to it (Fig. 7).
A membrane complex loaded with pSSU can be isolated from outer envelope membranes. Purified outer envelopes were incubated with pSSU translation product, re-isolated, solubilized by digitonin and subjected to fractionation on a linear sucrose density gradient. pSSU distribution was determined by liquid scintillation counting (graph) or SDS-PAGE and fluorography (insert). Free pSSU stays on top of the gradient while complex bound pSSU migrates to higher density in the sucrose density gradient. Methods as described by Waegemann and Soll [15].

When the same experiment was carried out using OEP 7 translation product, no radiolabelled protein was detectable in fractions 14–18 of the sucrose gradient (compare Fig. 7) (not shown). This might indicate that OEP 7 does not enter the common import apparatus to be inserted into the outer envelope.

The membrane fraction recovered from the sucrose density gradient was shown to contain all the proteins necessary for a transit sequence and ATP dependent insertion of pSSU into the isolated complex [16]. The interaction of pSSU with the isolated complex also gave rise to the transport intermediates described for the chloroplast system. Isolation of an active import apparatus represents a major advantage to study the function of single components in the translocation event. So far we have identified an outer envelope localized hsc70 homologue and OEP 86 as constituents of the isolated import complex. The hsc70 homologue localized in the import apparatus could act in sequence with its cytosolic and stromal counterparts in an unidirectional import process [15,16].

The polypeptide composition of the isolated import apparatus together with results from crosslink studies imply the involvement, either direct or indirect, of a number of proteins in the translocation event. A schematic view of the different transport pathways into chloroplasts is depicted in Fig. 8.

The major envelope protein which was described as the master receptor for chloroplast protein import [17] and subsequently found to be identical to the phosphate-triose phosphate translocator of the inner envelope [18,19] is neither found in isolated outer envelope membranes which are active in pSSU recognition and insertion nor in the isolated import complex. Together with data presented in [20]
we conclude that a receptor for chloroplastic precursor proteins still remains to be identified.

**Acknowledgements**

We thank U.-I. Flügge, Würzburg, for part of the data presented in Fig. 2, K. Keegstra, Madison, for providing cDNA clones of pSSU and SSU. This work was supported by the Deutsche Forschungsgemeinschaft.
References