Overexpression, purification and reconstitution of Tat subunits of *Arabidopsis thaliana* into liposomes

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Contents

Abbreviations III

Summary 1

1 Introduction 3
1.1 Protein transport across the envelope membrane 4
1.2 Four protein transport pathways exist at the thylakoid membrane 5
1.3 Reconstitution 11
1.4 Goal of the work 16

2 Results 17
2.1 Overexpression and purification of TatB and TatC 17
2.1.1 Overexpression of TatB and TatC 17
2.1.2 Purification of $TatB_{Strep}$ 18
2.1.3 Purification of $TatC_{His6}$ 21
2.1.3.1 Detergent screening for the solubilization of $TatC_{His6}$ from the membrane fraction 22
2.1.3.2 Ni-NTA affinity chromatography of $TatC_{His6}$ solubilized from IB Fraction 24
2.1.3.3 Purification of $TatC_{His6}$ with RP-HPLC 25
2.2 Preparation of liposomes 28
2.3 Reconstitution of Tat proteins with three different methods 32
2.3.1 Reconstitution of Tat proteins using the spontaneous insertion method ... 32
2.3.2 Reconstitution of Tat proteins with detergent-mediated method 35
2.3.2.1 Detergent removal with dialysis 35
2.3.2.2 Detergent removal with Bio-beads 37
2.3.3 Cotranslational reconstitution 43
2.3.3.1 Cotranslational reconstitution of TatB 45
2.3.3.2 Detergent screening of translated TatC in RTS 50
2.3.3.3 Cotranslational reconstitution of TatC 52

3 Discussion 55
3.1 Overexpression and purification of TatB and TatC 55
3.2 Liposome preparation 57
3.3 Reconstitution of Tat proteins into liposomes using various methods 58
3.3.1 Spontaneous insertion 59
3.3.2 Detergent-mediated Reconstitution 60
3.3.3 Cotranslational reconstitution 64

4 Materials and Methods 70
4.1 Materials 70
4.1.1 Chemicals 70
4.1.2 Marker 70
4.1.3 Enzymes 70
4.1.4 Medium and buffer 71
4.1.5 Antibodies 71
4.1.6 Lipids 72
4.1.7 Column matrix material (resin) and empty columns 72

4.1.8 Tat protein sequences for the overexpression and RTS 72
4.1.9 cDNA clones 73
4.1.10 Bacterial strains and vectors 74
4.1.11 Oligonucleotides 74
4.1.12 Reaction kits 75
4.2 Methods 76
4.2.1 Heterologeous overexpression of Tat proteins in *E.coli* 76
4.2.1.1 Analytical overexpression (5 ml scale) 76
4.2.1.2 Preparative overexpression (500 ml scale) 76
4.2.1.3 French press and fractionation through centrifugation 77
4.2.2 Molecular biological methods 77
4.2.2.1 Preparation of electro-competent *E.coli* cells 77
4.2.2.2 Transformation of E.coli cells through electroporation 78
4.2.2.3 Cloning of tat genes into pIVEX WG (Wheat Germ) vector for RTS 78
4.2.2.4 Colony PCR 81
4.2.2.5 Sequencing 81
4.2.2.6 Cell free translation of Tat proteins using RTS 81
4.2.3 Biochemical methods 82
4.2.3.1 SDS polyacrylamide gel electrophoresis (SDS-PAGE) 82
4.2.3.2 Protein content estimation after Western 85
4.2.3.3 Coomassie colloidal staining 85
4.2.3.4 Zinc imidazole staining 86
4.2.3.5 TCA/DOC precipitation 87
4.2.4 Chromatography 87
4.2.4.1 Strep-Tactin affinity chromatography 87
4.2.4.2 Ni-NTA affinity chromatography 88
4.2.4.3 RP-HPLC (*reversed phase*-HPLC) 90
4.2.5 2D-SDS-PAGE 92
4.2.6 Liposomes 92
4.2.6.1 Liposome preparation 92
4.2.6.2 Liposome size determination through Zetasizer 94
4.2.6.3 Liposome study under TEM 94
4.2.7 Reconstitution 94
4.2.7.1 Spontaneous reconstitution 94
4.2.7.2 Detergent-mediated reconstitution A: detergent removal by dialy method 95
4.2.7.3 Detergent-mediated reconstitution B: detergent removal by Bio-Beads 96
4.2.7.4 Cotranslational reconstitution 97
4.2.8 Sucrose gradient ultra centrifugation 99
4.2.8.1 Sucrose density gradient ultra centrifugation 99
4.2.8.2 "Floating" technique 100

References 101

List of Abbreviations

mAU	Milli absorption unit
2D	two dimensional
µg	microgram
µl	microliter
AA/Bis	acrylamide / BisTris
ALB3	albino 3
Amp	ampicillin
AP	alkaline phosphatase
APH	amphiphatic helix
APS	ammonium peroxodisulfate
ATP	adenosine triphosphate
A. thaliana	*Arabidopsis thaliana*
BCIP	5-bromine-4-chlorine-3-indolylphosphate
BisTris	Bis (2-hydroxyethyl) amino-tris (hydroxymethyl) methane
BN	blue native
Brij-35	polyoxyethylen(23)laurylether often used as nonionic detergent in membrane protein study and reconstitution
$C_{12}E_8$	dodecyl octaoxethylene
$C_{12}E_9$	nonaethyleneglycol mono-n-dodecyl ether
CECF	continuous exchange cell free system
CFoII	chloroplast Fo ATP synthase subunit II
CHAPS	3-[(3-Cholamidopropyl)-dimethylammonio]-propanal sulfonate
CIP	calf intestinal phosphatase
CMC	critical micellar concentration

cpTatC	plastid homolog of bacterial TatC
CP24	the 20 kDa apoprotein of CP24 complex in photosystem II
cpSRP	chloroplast signal recognition particle
C-Terminus	carboxyl terminus
CV	column volume
DDM	n-dodecyl-β-D-maltoside
DGDG	digalactosyldiacylglycerol
DM	decyl-β-maltoside
DOC	deoxycholic acid
DTT	dithiothreitol
ECL	enhanced chemoluminescence
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
ELIP2	early light-induced protein 2
EtOH	ethanol
g	gram
h	hour
GTP	guanosine-5'-triphosphate
GUV	giant unilamellar vesicle
GuadCl	guanidium hydrochloride
HABA	2-(4'-hydroxy-benzeneazo) benzoic acid
Hcf106	plastid homolog of bacterial TatB
HEPES	N-2-hydroxyl ethylpiperazine-N'-2-ethansulfonic acid
His6-tag	hexahistidine-tag
HRP	horseradish peroxidase
IBs	inclusion bodies
IgG	immune globulin of class G
kDa	kilo Dalton
λ	wavelength Lambda

l	liter
LB	Luria Bertoni
LDAO	N-dodecyl-N,N-dimethylamine-N-oxide
LHC	light harvesting complex
LHCP	light harvesting chlorophyII a/b binding protein
LMV	large multilamellar vesicle
LS	N-lauroyl sarcosine
LUV	large unilamellar vesicle
M	molar
mg	milligram
MGDG	monogalactosyldiacylglycerol
min	minute
ml	milliliter
mM	millimolar
MS	Mass spectrometry
MW	molecular weight
N-Terminus	amino terminus
NBT	nitroblue tetrazolium
ng	nanogram
NG	nonyl-b-D-glucopyranoside
nm	nanometer
NTP	nukleoside-5'-triphosphate
OD	optical density
OGP	octyl-β-D-glucopyranoside
OGTP	octyl-β-D-thioglucopyranoside
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PC	phosphatidyl choline
PG	phosphatidyl glycerine

pH	negative decimal logarithm of the hydrogen ion
pI	isoelectric point
pIVEX	plasmid of in vitro expression
PsaG	photosystem I subunit G
PsaK	photosystem I subunit K
PsbW	photosystem II subunit W
PsbX	photosystem II subunit X
psi	press unit, pounds per square inch, 1000 psi ≈ 68.95 bar
PVDF	polyvinylidene fluoride
RPEV	reversed phase evaporation
RP-HPLC	reversed phase - high performance liquid chromatography
rpm	rounds per minute
Rsat	detergent/lipid ratio in detergent saturated vesicles
Rsol	detergent/lipid ratio in mixed lipid-detergent micelles
RT	room temperature
RTS	rapid translation system (Roche or 5 PRIME, germany)
s.	see
SDS	sodium dodecylsulfate
SDS-PAA-Gel	SDS-polyacrylamide gel
Sec	secretory
SL	sulfoquinovosyl diglyceride
STD	stroma-targeting domain
SPP	stromal processing peptidase
SRP	signal recognition particle
Strep-tag	oligopeptide of sequence WSHPQFEK
SUV	small unilamellar vesicle
Tat	twin arginin translocase
TCA	trichloroacetic acid
TEM	transmission electron microscopy

TEMED	N,N,N',N'-Tetramethylethylendiamine
Tha4	plastid homolog of bacterial TatA
TIC	translocon at the inner envelope of chloroplasts
TMH	transmembrane helix
TOC	translocon at the outer envelope of chloroplasts
TPP	thylakoidal processing peptidase
Tricin	N-Tris-(hydroxymethyl)-methylglycine
Tris	Tris-(hydroxymethyl)-aminomethane
TTD	thylakoid targeting domain
V	volt
Vol	volume
v/v	volume per volume
w/v	mass per volume
WG	wheat germ

Table of amino acids with one-letter and three-letter abbreviations

A	Ala	Alanine	M	Met	Methionine
C	Cys	Cysteine	N	Asn	Asparagine
D	Asp	Aspartic acid	P	Pro	Proline
E	Glu	Glutamic acid	Q	Gln	Glutamine
F	Phe	Phenylalanine	R	Arg	Arginine
G	Gly	Glycine	S	Ser	Serine
H	His	Histidine	T	Thr	Threonine
I	Ile	Isoleucine	V	Val	Valine
K	Lys	Lysine	W	Trp	Tryptophan
L	Leu	Leucine	Y	Tyr	Tyrosine

Summary

The twin arginine translocation (Tat) machinery which is capable of transporting folded proteins across lipid bilayers operates in the thylakoid membrane of plant chloroplasts as well as in the cytoplasmic membrane of bacteria. One way to study the mechanism of the Tat transport pathway is to reconstitute the overexpressed and purified Tat proteins into artificial liposomes, which mimic the natural environment of the Tat transport machinery. The work presented in this thesis comprises initial attempts to find out suitable methods and experimental conditions for the reconstitution of Tat proteins into liposomes.

i) Preparation of Tat proteins.

The quantity of the authentic Tat proteins in thylakoid membranes is too low to isolate sufficient material for functional studies or even structural characterization. In this work Tat proteins were obtained either after overexpression in *E. coli* cells, followed by purification of Tat proteins under denaturing or native conditions, or through a cell free translation system.

Approximately 50% of the overexpressed $TatB_{Strep}$ was found in the cytosolic fraction. In contrast, the majority of the overexpressed $TatC_{His6}$ was found in membrane- and IBs-fraction. A detergent screening was performed to solubilize the $TatC_{His6}$ from membrane fractions. Among 12 detergents tested, the best solubility was achieved with 2% lauroylsarcosine. The non-ionic detergents Triton X-100, Digitonin and DM showed similar solubilisation properties for $TatC_{His6}$, but were more suitable for the reconstitution experiments than LS. The overall quantity of $TatC_{His6}$ recovered from IBs was low and the majority of proteins obtained in the elution fractions from Ni-NTA affinity chromatography were contaminants. Nevertheless, these small amounts of $TatC_{His6}$ were further enriched and purified from other proteins with RP-HPLC.

However, one common problem was observed for overexpressed TatB as well as TatC. In aqueous buffers supplemented with detergents both proteins, especially the overexpressed TatC, were only stable for a short time span. In order to overcome this obstacle, TatB and TatC were generated with a cell-free system, which, in the presence

of lipid vesicles, even allowed combining overexpression and insertion of the proteins into liposomes.

ii) Production of liposomes with various compositions.

In contrast to most eukaryotic membranes, which contain a high proportion of the zwitterionic phosphatidylcholine (PC), thylakoid membranes contain only about 3% PC and, among other lipids, about 77% neutral galactosyldiglycerides (~51% monogalactosyldiglycerides (MGDG) and 26% digalactosyldiglyceride (DGDG)).

In this thesis, liposomes were prepared either with thylakoid-like lipid composition or with pure phosphatidylcholine (PC). The morphology and stability of liposomes with different compositions were investigated with the transmission electron microscopy. Although liposomes with a thylakoid-like lipid composition are supposed to provide a more "natural" environment for the reconstituted Tat proteins, they were very unstable and tended to aggregate easily in aqueous solutions, which complicated the analysis of the reconstitution experiments. Therefore, PC-liposomes, which are more stable and easier to generate and purify, were mostly used for initial reconstitution studies in this thesis.

iii) Reconstitution of purified Tat proteins into liposomes applying three different methods.

There are various methods to insert membrane proteins into liposomes. In this thesis the reconstitution of the overexpressed and purified Tat proteins into preformed liposomes was investigated with three methods: the spontaneous, the detergent-mediated, and the cotranslational insertion. The insertion of Tat protein into preformed liposomes was done either using each protein separately or combining different Tat proteins.

TatA was readily inserted into preformed liposomes through the spontaneous insertion method. Likewise, TatA reconstitution was achieved also with the detergent-mediated method. Due to the lower stability, TatB and TatC overexpressed in *E. coli* were unable to be reconstituted with the method of spontaneous insertion. Improved reconstitution of TatB and TatC was achieved with the detergent-mediated method. The cotranslational reconstitution with the RTS cell free system was found to be the most promising method for the very hydrophobic TatC protein. Possible improvements for the reconstitution of Tat proteins with the detergent-mediated and the cotranslational insertion method are discussed.

1. Introduction

Chloroplasts are organelles found in plant cells and eukaryotic algae capable of conducting photosynthesis, which are considered to have originated from cyanobacteria through endosymbiosis (Gould *et al.* 2008; Lane and Archibald, 2008). In green plants, outer envelope membranes, inner envelope membranes, and thylakoid membranes of the chloroplasts confine three distinct intermembrane compartments of the organell – intermembrane space, stroma, and thylakoid lumen (Figure 1.1). Compared to the 3200 genes (Kaneko *et al.*, 1996) of free-living cyanobacteria, chloroplasts have a considerably reduced own genome of only 200 - 300 genes, because most of the genes have been transferred to the nucleus of the host cell or were lost. Among the estimated 3500 proteins in a functional chloroplast, only about 100 proteins are encoded by the plastid genome (The Arabidopsis Genome Initiative, 2000; Emanuelsson *et al.*, 2000). To make the organelles function properly, proteins synthesized in the cytosol have to be transported into the chloroplast through protein transport machineries located in the membranes of the organell (Keegstra and Cline, 1999; Jarvis and Robinson, 2004).

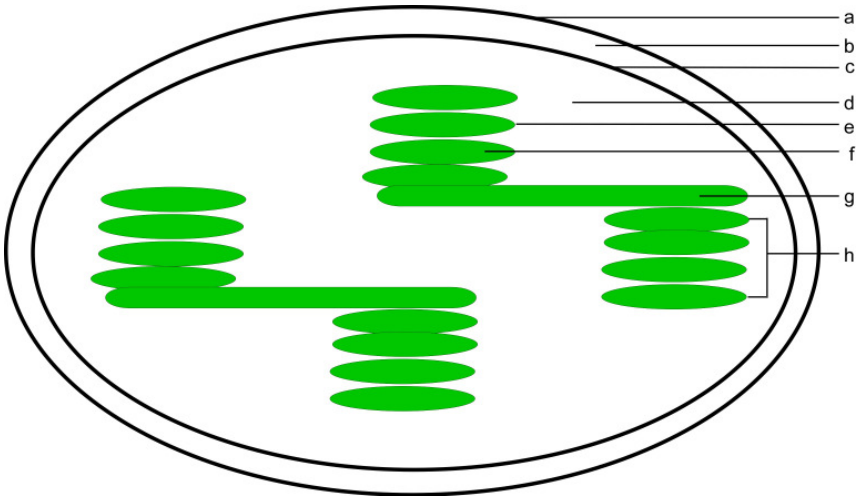


Figure 1.1: Structure of chloroplast. a) outer envelope membrane; b) intermembrane space; c) inner envelope membrane; d) stroma; e) thylakoid membrane; f) thylakoid lumen; g) stroma lamella of the thylakoid membrane; h) grana stacks of the thylakoid membrane.

1.1. Protein transport across the envelope membranes

The nucleus encoded plastid-targeting proteins, the "precursors", are synthesized in the cytosol. Their N-terminal ends contain the transport signals, which are called transit peptides (Bruce, 2000), composed of approximate 50 amino acid residues (von Heijne *et al.*, 1989).

Proteins destined for the stroma contain a stroma-targeting domain (STD). Their transport across the envelope membranes is mediated by two translocator complexes at the envelope membranes: TOC- and TIC-complexes (TOC = *translocon at the outer envelope of chloroplasts*; TIC = *translocon at the inner envelope of chloroplasts*) (Cline *et al.*, 1993; Gutensohn *et al.*, 2006; Jarvis, 2008). After reaching into the stroma, the transit peptide is proteolytically removed by a large monomeric enzyme called stromal processing peptidase (SPP) (Figure 1.2 A) (Robinson and Ellis, 1984; Robinson *et al.*, 1994).

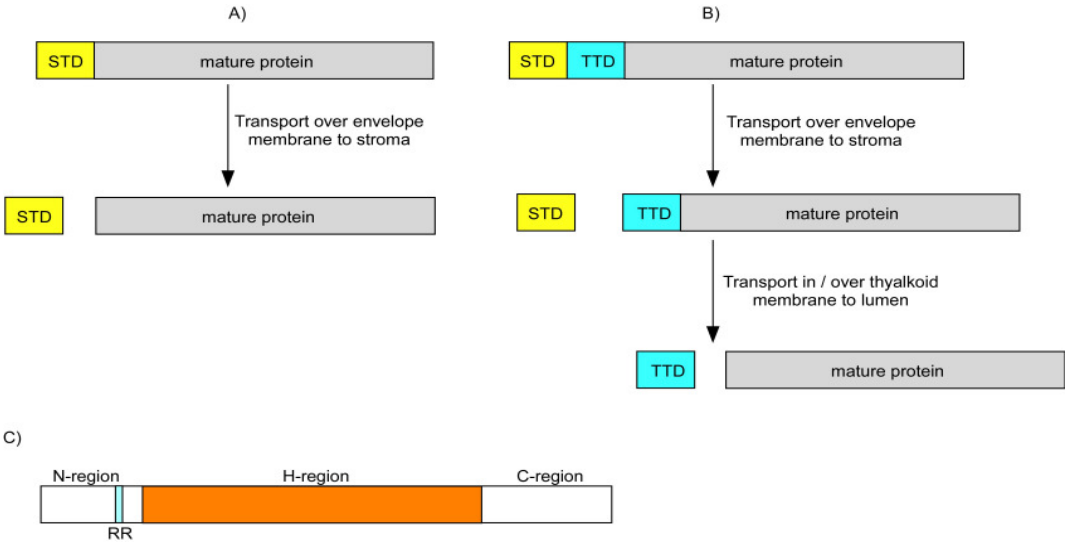


Figure 1.2: Overview of precursor transit peptides. A) **S**ingle transit peptides: precursor proteins carrying a stroma targeting domain (STD) are transported across the envelope membrane into the stroma, where the transit peptide is cleaved by a stromal processing peptidase (SPP); B) Bipartite transit peptides: They are composed of a stroma targeting domain (STD) and a thylakoid targeting domain (TTD). After arriving in the stroma, the STD of precursor proteins is removed by stromal processing peptidase (SPP), the intermediate protein is further transported into the thylakoid membrane or across the thylakoid membrane into the lumen, followed by cleavage of the signal peptide (TTD) by thylakoidal processing peptidase (TPP). C) Structure of a thylakoid targeting signal peptide specific for substrates from the twin arginine translocase (Tat)-dependent pathway. Its structure is highly conserved and contains generally three regions: a N-region carrying several positively charged amino acid residues, a hydrophobic H-region and a polar amino acid residues containing C-region. RR indicates a characteristic twin pair of Arg residues localized immediately adjacent to the H-region.

1.2. Four protein transport pathways exist at the thylakoid membrane

Proteins destined for thylakoids contain a thylakoid targeting sequence, which can be a bipartite targeting sequence composed of a stroma targeting domain (STD) and an adjacent thylakoid targeting domain (TTD), generally named as signal peptide (Smeekens *et al.*, 1986; Ko and Cashmore, 1989; Hageman *et al.*, 1990). These proteins are first transported into the stroma and their STDs are removed by SPP. The resulting stromal intermediates are then further translocated into the thylakoid membranes or across the membranes into the thylakoid lumen. After translocation the TTD is cleaved by a thylakoidal processing peptidase (TPP) (Figure 1.2 B) (Kirwin *et al.*, 1987) to produce a mature protein. The TTD contains three highly conserved homolog blocks (von Heijne *et al.*, 1989; von Heijne, 1990): a positively charged hydrophilic N-terminal end (N-region), a hydrophobic central region (H-region), and a polar C-terminal end (C-region). In the C-region, an Ala-X-Ala motive at positions -3 to -1 of the cleavage site is frequently found (Figure 1.2 C).

Some proteins destined for the thylakoid membrane have a single transit peptide, and the thylakoid targeting information is integrated in the mature protein (Viitanen *et al.*, 1988; Madueño *et al.*, 1994), which will not be cleaved by TPP. Proteins transported by SRP transport pathway belong to this kind, such as LHCP (the apoprotein of LHC-II complex) and CP24 (the 20 kDa apoprotein of CP24 complex in photosystem II).

In contrast to the transport across the envelope membranes via the common Toc/Tic complexes, at least four independent and substrate-specific transport pathways have been described for the protein transport into or across the thylakoid membrane: spontaneous, signal recognition particle (SRP)-, secretory (Sec)-, and twin arginine translocase (Tat)-dependent pathway (Figure 1.3) (Jarvis and Robinson, 2004; Gutensohn *et al.*, 2006; Schünemann, 2007; Aldridge *et al.*, 2009). The Sec- and Tat-dependent pathways attribute mainly for transporting proteins into the thylakoid lumen, while the spontaneous and the SRP-dependent pathways are responsible for insertion of proteins into the thylakoid membrane (Aldridge *et al.*, 2009).

Spontaneous insertion pathway

The spontaneous insertion pathway was first described for the insertion of CFoII protein, a subunit of ATP-synthase, which has one trans-membrane span (Michl *et al.*, 1994). The insertion is performed spontaneously, neither stroma factors nor nucleoside triphosphates or a proton gradient are necessary. Other proteins with single trans-membrane span using this spontaneous insertion route include PsbW and PsbX subunits of the photosystem II (Kim *et al.*, 1998; Thompson *et al.*, 1999), PsaK and PsaG of photosystem I (Mant *et al.*, 2001). For PsaG the positive charges in its stroma-exposed loop region were found to be essential for the insertion (Zygadlo *et al.*, 2006). PsbS and ELIP2 are transported into chloroplasts either through the assisted pathways (SRP- and Sec-pathway) or through spontaneous insertion in absence of functional SRP- or Sec-system (Kim *et al.*, 1999).

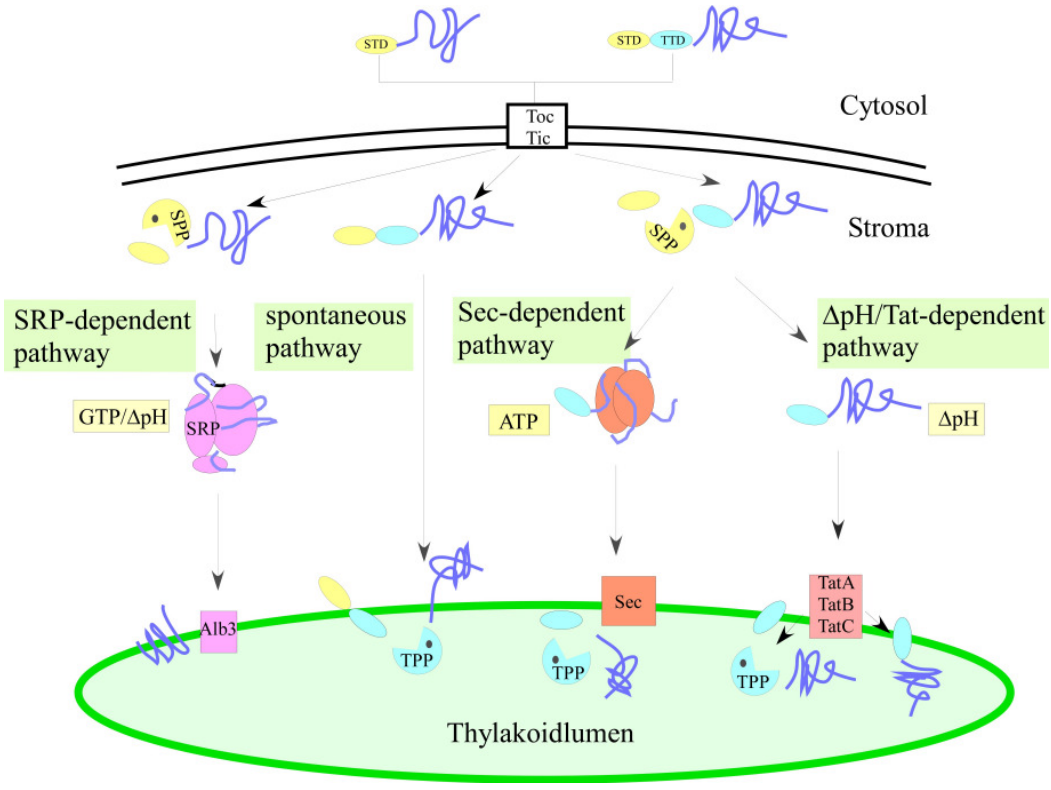


Figure 1.3: Protein transport pathways into and within the thylakoid. The precursor protein is first transported across the envelope membranes into the stroma through the TOC / TIC translocon complexes. With exception of the spontaneous pathway, the stroma targeting transit peptide (STD) is removed by the stromal processing peptidase (SPP). The resulting intermediate protein is further transported into the thylakoid membrane or across the thyalkoid membrane into the lumen using one of the four transport pathways.

SRP-dependent pathway

The plastid SRP-dependent pathway has homologues in both prokaryotes and eukaryotes. In higher plants this pathway is post-translational for nucleus encoded precursor proteins and does not require RNA components, in contrast to the bacterial SRP pathway (Li *et al.*, 1995). The mostly studied transport substrates of this pathway are proteins of the LHCP family, which are pigment binding proteins localized in the thylakoid membrane of chloroplasts and form the light harvesting antenna complexes (Klimmek *et al.*, 2006). LHCP synthesized in the cytoplasm with an N-terminal transit peptide is first transported into the chloroplast (Cashmore, 1984), following removal of the STD by the SPP, and the mature protein is targeted to the thylakoid membrane under direction of the thylakoid targeting signals localized within the mature protein (Viitanen *et al.*, 1988). Three stroma components play a central role in this SRP pathway: cpSRP54 (Franklin and Hoffman, 1993; Li *et al.*, 1995), cpSRP43 (Schünemann *et al.*, 1998), and cpFtsY (Kogata *et al.*, 1999). Additionally, ALB3 (Albino 3), an integral multi spanning protein in the thylakoid membrane, is also involved in the insertion of LHCP by the SRP pathway (Moore *et al.*, 2000). A complex of cpSRP43, cpSRP54, cpFtsY and ALB3 for proper LHCP integration can be formed in absence of substrate (Moore *et al.*, 2003). GTP hydrolysis is essential for the successful insertion of LHCP into the thylakoid membrane (Hoffman and Franklin, 1994). GTP binding domains were found in cpFtsY (Kogata *et al.*, 1999). Two GTPases in cpSRP and cpSRP receptor can interact with each other in absence of SRP-RNA to regulate the protein transport (Jaru-Ampornpan *et al.*, 2007). Although ATP and a proton gradient across the thylakoid membrane are not essential, they can greatly stimulate the LHCP integration into thylakoid membrane (Yuan, *et al.*, 2002; Schünemann, 2007).

Besides transport by the spontaneous and the SRP-dependent pathways, some proteins of the thylakoid membrane, such as Cytochrome f and Rieske protein, can also be inserted into the membrane following the other two transport pathways: Cytochrome f is a substrate of the Sec-pathway (Nohara *et al.*, 1996) and the Rieske protein is transported Tat-dependent (Molik *et al.*, 2001). However, the latter two pathways are major machineries for transporting proteins into the thylakoid lumen.

Sec-dependent pathway

The chloroplast Sec-dependent pathway evolved from the general bacterial secretory pathway. The Sec translocon in *E. coli* is composed of at least three subunits: SecA, SecE and SecY (Akimaru *et al.*, 1991). SecA has an ATPase activity and binds the transit peptide of precursor proteins, while SecE and SecY form the channel for substrates transport (Dalbey and Chen, 2004). The translocation is driven by ATP hydrolysis (Robinson *et al.*, 1994). Homologues to bacterial SecA (cpSecA), SecY (cpSecY), and SecE (cpSecE) were identified in chloroplast as cpSecA, cpSecY and SecE, respectively (Nakai *et al.*, 1994; Yuan *et al.*, 1994; Laidler *et al.*, 1995; Roy and Barkan, 1998). However, homologues to the bacterial Sec components SecB, SecG and SecD/F have not been identified in chloroplasts. The structure and function of the plastid Sec pathway is similar to that of bacteria, likewise the translocation is energetically driven by ATP hydrolysis, while a proton gradient across the thylakoid membrane is not essential (Yuan *et al.*, 1994). Similar to the bacterial Sec pathway, Sec-substrates in chloroplasts can only be transported in an unfolded state (Hynds *et al.*, 1998; Marques *et al.*, 2004).

Tat-dependent pathway

The name of the pathway comes from the characteristic Arg-Arg motif in the N-region of the signal peptide of its substrates. In strong contrast to the Sec-dependent pathway, which can transport only unfolded proteins, the Tat-dependent pathway can transport folded proteins. Protein folding is apparently an indispensible requirement for Tat transport in prokaryotes, while both folded and unfolded Tat substrates can be transported in chloroplasts (Hynds *et al.*, 1998). Bacterial Tat substrates are mostly enzymes that bind cofactors. Alternatively precursor proteins lacking cofactors but folding very quickly before translocation also require the Tat pathway (Palmer *et al.*, 2005). In addition, the Tat pathway is also capable of transporting oligomeric complexes across the thylakoid membrane (Rodrigue *et al.*, 1999).

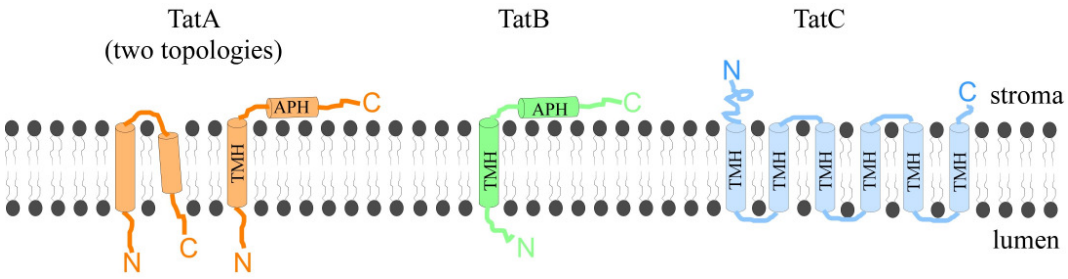


Figure 1.4: Structure of TatA, TatB and TatC of the Tat translocon. Both TatA and TatB have a single transmembrane helix (TMH) and an amphiphatic helix (APH) while TatC has six transmembrane helices with its N-and C-terminals extruding into stroma. The topology of TatA is still under discussion, two possible topologies are shown in the scheme (Gouffi *et al.*, 2004).

The Tat-dependent pathway is found in the cytoplasmic membrane of most prokaryotes and in the thylakoid membrane of chloroplasts (Robinson and Bolhuis, 2004). In bacteria, Tat translocase usually consists of three integral membrane subunits: TatA, TatB and TatC (Figure 1.4). All these three proteins have their own unique functions, although most gram-positive bacteria and archaea lack TatB (Robinson and Bolhuis, 2004). In chloroplasts homologues to the bacterial TatA, TatB, and TatC were identified: Tha4 (Mori *et al.*, 1999; Walker *et al.*, 1999), Hcf106 (Settles *et al.*, 1997) and cpTatC (Mori *et al.*, 2001), respectively. TatA (Tha4) and TatB (Hcf106) are single span membrane proteins containing an N-terminal transmembrane helix (TMH) followed by at least one short amphipathic helix (APH) and an unstructured stromal C-terminal domain (Settles *et al.*, 1997; Chanal *et al.*, 1998). The N-terminal ends of both TatA and TatB protrude into the thylakoid lumen, while the N- and C-terminals of TatC (cpTatC) with six transmembrane domains are both extruding into the stroma (Behrendt *et al.*, 2004). The topology of TatA is still under discussion, a dual topology of TatA is also predicted, in which the amphipathic helix can also change to a transmembrane helix, resulting in a conformation with both the N-terminal and C-terminal ends pointing to the thylakoid lumen (Gouffi *et al.*, 2004). TatC protein is not particularly highly conserved, but at some positions highly conserved residues are present essentially for its activity, e.g. the cytoplasmic N-terminus and the first cytoplasmic loop region of TatC protein include a number of conserved residues (Allen *et al.*, 2002; Buchanan *et al.*, 2002). The stoichiometry of TatA:TatB:TatC in the cytoplasmic membrane of *E. coli* was estimated to be approximately 20-30:1:0.4 (Berks *et al.*,

2003). However, the amount and stoichiometry of Tat subunits in chloroplasts depends on the species and the developing stages of plants (Jakob *et al.*, 2009).

Several *in vitro* studies demonstrated that Tat dependent protein translocation is driven by the ΔpH aross the thylakoid membrane (Mould *et al.*, 1991; Cline *et al.*, 1992; Klösgen *et al.*, 1992), therefore this pathway is also named as ΔpH-dependent pathway. However, in *Chlamydomonas reinhardtii* it was shown that *in vivo* elimination of ΔpH did not influence the thylakoid targeting of Tat passenger proteins (Finazzi *et al.*, 2003). It was suggested that the transmembrane electric potential might be used as an energy source too (Braun *et al*., 2007). Recently, it was demonstrated that the transmembrane electric potential was exclusively utilized for Tat transport in bacteria (Bageshwar and Musser, 2007). To obtain a deeper understanding on the exact energetic source of the Tat-dependent pathway further investigations will be necessary.

In the current translocation model of Tat pathway, Tat transport is initialized by direct insertion of the Tat substrate in the thylakoid membrane (Hou *et al.*, 2006; Shanmugham *et al.*, 2006), followed by its interaction with the receptor complex composed of TatB and TatC (Cline and Mori, 2001; Richter and Brueser, 2005). The subsequent translocation of the passenger protein depends on the transient interaction of TatA with the TatBC- substrate complex in the presence of a proton gradient across the thylakoid membrane (Mori and Cline, 2002; Alami *et al.*, 2003). Finally, after translocation of the C-terminal domain of the substrate into the thylakoid lumen, the signal peptide is removed by TPP (thyalkoid processing peptidase).

The exact function of TatA during transport is still unclear and controversial. In many transport models, hydrophilic pores are involved in the Tat translocation, which are predominantly or exclusively composed of oligomeric TatA proteins (Porcelli *et al.*, 2002; Gohlke *et al.*, 2005). However, some studies suggested that TatA might facilitate Tat transport by weakening the membrane in an unknown manner (Natale *et al.*, 2008; Jakob *et al.*, 2009). Moreover, how oligomeric complexes of TatA facilitate the transport of proteins with various sizes remains an open question (Berks *et al.*, 2003; Müller and Klösgen, 2005).

1.3. Reconstitution

Investigating membrane proteins in their native environment is often suffered from the low amount of proteins and the complexity of the natural membranes. The analysis and interpretation of results are often complicated by interferences with other membrane constituents having other functions or performing other reactions. Therefore, forming functional proteoliposomes by insertion of purified membrane proteins, either from their natural sources or after overexpression, into liposomes provides a powerful tool for elucidating both functional and structural features of desired membrane proteins *in vitro*.

Liposomes are artificially prepared vesicles of lipid bilayers. The size and the lipid components of liposomes can be adjusted. Depending on their size and lamellarity, vesicles can generally be divided into large multilamellar vesicles (LMV), small unilamellar vesicles (SUV), large unilamellar vesicles (LUV) and giant unilamellar vesicles (GUV). The unilamellar vesicles can be prepared from the "onion-like" LMV, which are formed spontaneously when a dried amphiphilic lipid film is hydrated. A detailed review on the formation of liposomes has been given by Lasic (1988). Membrane reconstitution has played an important role in functional analyses of many membrane proteins (Lund *et al.*, 1989; Putman *et al.*, 1999; Seddon *et al.*, 2004; Nozawa *et al.*, 2007). Reconstitution methods are diverse and do not work equally well with different membrane proteins (Rigaud *et al.*, 1995; Seddon *et al.*, 2004). Therefore, three frequently used reconstitution methods were investigated for the Tat proteins in this thesis.

Spontaneous reconstitution

The simplest technique of reconstitution is to incorporate membrane proteins spontaneously into preformed liposomes. The main advantage of this strategy is that the proteins are found to be oriented unidirectionally in the membrane of proteoliposomes (Eytan, 1982; Jain and Zakim, 1987). Sponteneous incorporation of membrane proteins was observed occurring preferentially into liposomes with small diameter (20 nm) (Eytan, 1982; Eytan, 1978; Caroll and Racker, 1977). The mechanisms of spontaneous reconstitution were studied in detail by Zakim and co-workers using various purified natural integral membrane proteins like bacteriorhodopsin, cytochrome oxidase, and

UDP-glucuronosyltransferase (Scotto and Zakim, 1985 and 1986; Scotto *et al.*, 1987). The efficiency of reconstitution could be enhanced by pre-sonication of liposomes or by presence of low concentration of amphiphatic contaminants like cholesterol, detergent, or fatty acids (Scotto and Zakim, 1985 and 1986; Jain and Zakim, 1987). These trace lipid impurities and sonication induced probably the features benefiting the reconstitution, such as the packing defects, reorganization of bilayers, and the phospholipid transvesicle movement (Jain and Zakim, 1987; Scotto *et al.*, 1987). However, the possibility that the tightly bound residual lipid or lipid-detergent during the protein isolation might facilitate the insertion was not discussed. The spontaneous insertion was proposed to occur through two steps: the membrane proteins inserted initially fast into a small percentage of SUVs due to the high molar lipid to protein ratio, which were subsequently fused with remaining protein-free SUVs (Scotto *et al.*, 1987). Due to this insertion mechanism, this method has the following disadvantages: heterogeneous distribution of membrane proteins in liposomes, a wide size range of the resulting proteoliposomes, and the presence of the above mentioned additives which may affect the permeability of the proteoliposomes.

Detergent-mediated reconstitution

Due to their inherent high hydrophobicity, membrane proteins are generally insoluble in aqueous solution. Detergents are normally used to stabilize membrane proteins when they are isolated and purified either from their natural sources or after overexpression (le Maire *et al.*, 2000; Garavito and Ferguson-Miller, 2001) Therefore, detergent-mediated methods are the most often used strategy for reconstitution of integral membrane proteins into unilamellar liposomes to form biologically active proteoliposomes (Racker, 1979; Eytan, 1982; Seddon *et al.*, 2004).

Using the detergent-mediated insertion, an isotropic solution of lipid-protein-detergent and lipid-detergent micelles is initially formed with an appropriate detergent. This can be accomplished either by dissolving lipids in a buffer containing the target protein and detergent or by solubilization of preformed liposomes in the presence of the membrane protein and detergent. Subsequently, by removing the detergent, liposomes with inserted target protein will be formed progressively (Rigaud *et al.*, 1995; Dolder *et al.*, 1996).

A three-stage model is generally accepted for the solubilization of liposomes with detergents (Lichtenberg, 1985; Rigaud *et al.*, 1988). Stage one: With increasing amount of detergents, the detergent molecules are first incorporated into the lipid bilayer

structures, leading to alterations in permeability of the membranes and increase of the turbidity due to membrane swelling. Stage two: At a crucial point when the membrane is saturated with detergents (R_{sat}), the membrane begins to disintegrate and lipid-detergent mixed micelles begin to form. R_{sat} corresponds to the detergent/lipid ratio in detergent saturated vesicles. Stage three: Further addition of detergent results in complete solubilization of the liposome membrane (R_{sol}) to lipid-detergent mixed micelles. R_{sol} corresponds to the detergent/lipid ratio in mixed lipid-detergent micelles.

Kragh-Hansen *et al.* (1998) assumed that the mechanism of solubilization depends on the nature of the detergent used. Two different mechanisms were described for the solubilization of liposomes with various detergents. One is the transbilayer solubilization, which is a fast process proceeding via open intermediates with detergent molecules inserting from both sides of the membrane bilayer. The other mechanism is the micellar solubilization, which goes slowly through closed vesicles with detergent molecules inserting only into the outer leaflet of liposomes and detergent-lipid micelles pinching off from the outer leaflet only. Stuart and Boekema (2007) have extended these two mechanisms from solubilization to the reformation of liposomes. They found that solubilization and reformation of liposomes proceeded via the open vesicular intermediate with Triton X-100 and via closed micelles when n-dodecyl-β-D-maltoside (DDM) was used.

Since membrane proteins need membrane bilayers for successful reconstitution, liposome solubilization and reformation with detergents following the micellar model is unlikely. When the detergent concentration exceeds R_{sat}, only micelles but no bilayer exist (Stuart *et al.*, 2004). In contrast, reconstitution into liposomes using detergents forming open bilayer structures is possible over a wide range, because bilayers are present at any point between R_{sat} and R_{sol} (Knol, 1996). Since Triton X-100 solubilized liposomes according to the transbilayer model, it was chosen for the detergent-mediated reconstitution in this thesis using detergent concentrations between R_{sat} and R_{sol}.

There are various methods to remove the detergent (Moller *et al.*, 1986; Seddon *et al.*, 2004). Which method is suitable is determined by the physicochemical properties of the detergent. One of the most important properties is the critical micelle concentration (CMC), which is defined as the concentration at which the detergent monomers begin to form micelles. Detergents with high CMCs (e.g. *n*-Octyl-β-D-glucopyranoside, cholate,

CHAPS) form generally small micelles and can be removed easily by dialysis (Allen *et al.*, 1980). In contrast, detergents with low CMCs intend to form large micelles and cannot be readily removed by dialysis. In this case, the detergent can be efficiently removed through adsorption on hydrophobic resins (e.g. BioBeads SM2) (Holloway, 1973; Ueno *et al.*, 1984). Triton X-100 belongs to this kind of detergents.

Previous works have shown that the detergent–mediated reconstitution of membrane proteins is influenced by many factors: the initial concentration of the detergent (Mimms *et al.*, 1981; Rigaud *et al.*, 1988), the rate of detergent removal (Eytan, 1982; Eytan and Broza, 1978), and the state of protein aggregation when the membrane begins to reform from micelles are suggested as crucial factors for reconstitution (Helenius, 1981). Other factors such as liposome size, lipid composition and ionic conditions play also a role in the reconstitution process (Eytan and Broza, 1978[b]; Eytan *et al.*, 1975, 1976). Despite of numerous investigations, the molecular mechanism for the detergent-mediated reconstitution is still not thoroughly understood (Stuart and Boekema, 2007).

Cotranslational reconstitution in cell free systems

Cell free translation has been used since the early 1950s. The protein expression was originally carried out in a single compartment batch with low production rates of recombinant proteins ranging in the nanogram or microgram scale (Winnick[a,b], 1950; Borsook, 1950). The modification of the cell free synthesis by separating the single reaction chamber into two chambers resulted in considerably higher yields of recombinant proteins (Kim and Choi, 1996; Alakov *et al.*, 1995; Spirin *et al.*, 1988). In the two-chamber system, all high molecular mass compounds of the translation machinery in the reaction solution are separated from a feeding solution containing the low molecular mass precursors by a semi-permeable membrane. With this design the reaction solution is continuously supplied with fresh precursors and energy substrates from the feeding solution, and undesired byproducts are removed from the reaction solution. This continuous exchange increases the yield of recombinant proteins to the mg scale per 1 ml feeding solution (Kigawa *et al.*, 1999). Two main sources of cell free extracts have been established: Wheat Germ extracts (Sawasaki and Endo, 2004; Madin *et al.*, 2000; Endo *et al.*, 1992; Anderson *et al.*, 1983) and *E. coli* cell extracts (Ozawa *et al.*, 2004; Torizawa *et al.*, 2004; Kigawa *et al.*, 2004; Jewett and Swartz, 2004). Both systems are comparable with respect to their productivities (Spirin, 2004). Cell free

systems are currently commercially available, for example, the so-called rapid translation system (RTS^{TM}) (Roche *or* 5 PRIME, Germany).

Recently, these cell free systems have been employed for reconstitution of membrane proteins (Klammt *et al.*, 2006; Nozawa *et al.*, 2007). Since the standard cell free systems lack a hydrophobic environment, the produced membrane proteins often precipitate. However, these precipitates can be easily solubilized with mild detergents (Ishihara *et al.*, 2005; Klammt *et al.*, 2004; Shimada *et al.*, 2004; Elbaz *et al.*, 2004; Berrier *et al.*, 2004), unlike inclusion bodies formed by heterologous overexpression, which have to be solubilized with strong detergents like SDS or denaturants in high concentration (e.g. 8 M urea).

Compared to traditional protein expression in cellular systems, the cell free translation is an "open" system that offers a variety of options to manipulate the translation conditions in order to solubilize or stabilize the synthesized membrane proteins. A hydrophobic environment can be generated during cell free synthesis by directly supplementing detergents or liposomes, or both in combination into the reaction solution (Klammt *et al.*, 2004; Klammt *et al.*, 2005; Nozawa *et al.*, 2007). In the presence of liposomes the synthesized membrane proteins can be directly reconstituted into the liposomes. Moritani *et al.* (2010) found that increasing the concentration of liposomes during protein expression increased the Connexin-43 integration into liposomes, while no increase was observed when more liposomes were added after the protein expression. Therefore, it was concluded that the insertion of synthesized membrane proteins was possibly cotranslational rather than post-translational.

Besides liposomes, the supplemented detergent and its concentration are critical for successful expression, solubilization, and reconstitution of target proteins with cell free systems too. Therefore, a detergent screening is normally indispensible for expression and membrane insertion of an individual target protein (Nozawa *et al.*, 2007; Goren and Fox, 2008; Martin *et al.*, 2009). The most frequently used detergents include Triton X-100, Brij-35, CHAPS and Digitonin.

1.4. Goal of the work

The goal of this work was to reconstitute the Tat proteins into artificial liposomes in order to get a tool allowing *in vitro* studies on the formation of the TatBC receptor complex, the binding of substrates to this complex and finally, get inside into the mechanism of protein transport on the Tat pathway. To do this, the following three tasks had to be accomplished. i) Preparation of sufficient Tat proteins. Tat proteins were obtained either after overexpression in *E.coli* cells, followed by purification of Tat proteins under denatured or native conditions, or through cell free translation. ii) Production of liposomes with various methods and constituents. Lipid composition could be either thylakoid-like, such liposomes are characterized by a high content of galacto-lipids instead of phospho-lipids, or pure phosphatidyl choline containing lipid vesicles, which are easier to generate and were used for initial studies. Furthermore, for preparation of different kind of liposomes different methods were established and standardized. iii) Reconstitution of purified Tat proteins into liposomes applying three different methods. Initially, the insertion of heterologously overexpressed and purified Tat proteins into preformed liposomes was investigated. Furthermore, the detergent-mediated reconstitution method and the cotranslational reconstitution using the RTS cell free system were intensively investigated. Subsequently, various purification methods were explored to purify and isolate the obtained liposomes with inserted Tat proteins, so-called proteoliposomes. The results in this thesis constitute an initial attempt to find out suitable methods for reconstitution of Tat proteins into liposomes.

2. Results

To date, three Tat components have been identified in the thylakoidal Tat transport pathway: TatA (Tha4), TatB (Hcf106), and TatC (cpTatC) (Settles 1997, Walker 1999, Mori 2001). The protein transport on Tat pathway is initiated by the direct insertion of substrate protein in the target membrane, followed by the interaction with the oligomeric TatBC receptor complex. Subsequent translocation of the substrate proteins is dependent on TatA which transiently joins the TatBC complex in the presence of the Tat substrate and the thylakoidal pH gradient across the membrane (Mori 2002, Alami 2003). The translocation ends up with the cleavage of the signal peptide of the substrate protein.

The quantity of the authentic Tat proteins in thylakoid membrane is too low to isolate sufficient material for functional and structural studies. In order to get enough Tat proteins, we tried to overexpress the thylakoidal membrane proteins in *E.coli*, which were then purified by various biochemical methods. One way to study the mechanism of the Tat transport pathway is to reconstitute the purified Tat proteins in artificial liposomes, which mimic the natural environment of the Tat transport pathway. So, during my PhD the focus of my work was the purification of recombinant Tat proteins and their reconstitution into liposomes with various reconstitution methods.

2.1 Overexpression and purification of TatB and TatC

2.1.1 Overexpression of TatB and TatC

The purification of Tat proteins started with TatB and TatC, the two components of the TatBC receptor complex. To make the subsequent purification easier, both TatB and TatC were fused with tags at the C-terminal ends: TatB with a Strep II-tag and TatC with a His_6-tag, respectively. The constructs pBW-AT-tatB_{Strep} and pBW-AT-tatC_{His6} were used to overexpress the $TatB_{Strep}$ and $TatC_{His6}$ in *E.coli* BL21 strain grown on LB_{amp} (Wilms, 2000). The bacteria clones were prepared by our collaborator (T. Brüser, the Institute of Microbiology, Martin-Luther University). The bacteria were grown

aerobically in 37°C LB medium in the presence of 50 µg/ml ampicillin until reaching an OD_{600nm} of 0.5, then the expression of Tat proteins was induced by addition of 0.2% (w/v) rhamnose for further 3 h. The cells were harvested by centrifugation for 20 min, at 4000 rpm (Sorvall, ST-H750) and disrupted by two cycles of French press at 1000 psi. The inclusion bodies (IBs) fraction was pelleted from the cell suspension by centrifugation (30 min, 10000 rpm, Sorvall SL-50T) and the resulting supernatant was separated further into the cytosol (supernatant)- and membrane-fraction (pellet) by ultra centrifugation for 1 h at 28000 rpm (Beckman SW28). Through these steps the cell suspension was separated into three fractions: cytosol-, membrane-, and IBs-fraction.

2.1.2 Purification of $TatB_{Strep}$

The expressed $TatB_{Strep}$ with its single transmembrane helix was soluble and approximately 50% of the protein was found in the cytosol fraction. Figure 2.1 shows the purification of $TatB_{Strep}$ from the cytosol fraction with affinity chromatography, after which lots of contaminants were still present in the $TatB_{Strep}$ fraction. So, aliquots from this fraction were further purified under denaturing conditions through the *reversed phase*-HPLC (RP-HPLC) and 2D gel electrophoresis.

The 30 ml cytosol fraction was first applied to a 2 ml Strep-II affinity chromatography column equilibrated with washing buffer. After several washing steps the $TatB_{Strep}$ protein was eluted with 2.5 mM Desthiobiotin. Five eluates with each of 1 ml were collected. 15 µl aliquots from each fraction were incubated with 5 µl 4x SDS Laemmli sample buffer and loaded to a 15% SDS-PAA gel. After electrophoresis the gel was stained by coomassie-colloidal (Figure 2.1A).

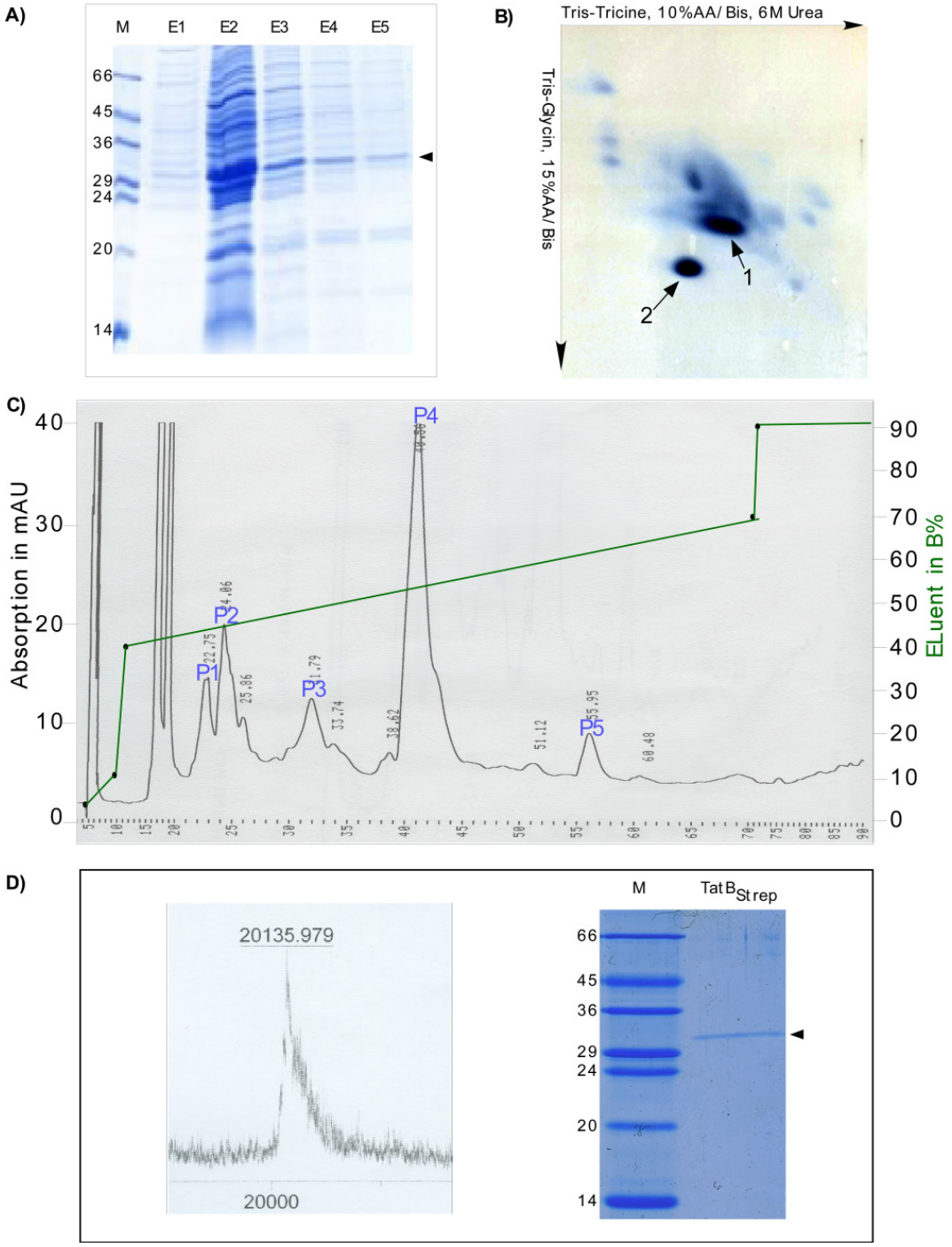


Figure 2.1: Purification of $TatB_{Strep}$ though affinity chromatography, RP-HPLC and 2D electrophoresis. A) Coomassie colloidal staining of proteins purified by Strep-II affinity chromatography from the cytosol fraction. 200 µl aliquots from each fraction were precipitated with acetone, the resultant pellets were resolubilized in 100 µl 4x Laemmli sample buffer and boiled at 95°C for 5 min, then 20 µl from it were loaded to a 15% SDS-PAA gel. After electrophoresis, the gel was stained with coomassie-colloidal; B) Zinc imidazol staining after purification by the 2D electrophoresis. The 1. dimension: Tris-Tricine PAGE, 10% AA /Bis, 6

M urea, 50 µl sample was loaded in the 1. dimension. After the electrophoresis the whole lane was cut out and put above the 2. dimension (Tris-Glycine PAGE, 15% AA / Bis). Arrow 1 represents a soluble contaminant, while arrow 2 designates purified $TatB_{Strep}$. C) RP-HPLC chromatogram of the E2 fraction after the Strep-Tactin affinity chromatography. The horizontal axis shows the time scale, the left vertical axis the absorption in mAU, and the right vertical axis the percentage of eluent B. The green line in the chromatogram is the gradient of the eluent B. D) MALDI-TOF spectrum and coomassie colloidal staining of the highly pure $TatB_{Strep}$; 1 µl aliquot of the peak 4 was analyzed by the mass spectrum and 12 µl aliquot was loaded in the 15% SDS-PAA gel. E: elution; M:marker; P:peak.
The RP-HPLC ran under the following conditions:
column: EC 250/10 Nucleosil 500-7 C3 PPN
sample: 2 ml $TatB_{Strep}$ (E2 of cytosol fraction)
eluent A: H_2O + 0.05% trifluoroacetic acid
eluent B: acetonitrile + 0.05% trifluoroacetic acid
flow speed: 3 ml/Min
gradient (in B%): 1-10 min with 10%, 10-11 min to 40%, 11-70 min to 70%, 70-71 min to 90%, 71-90 min with 90%
detection: 220 nm

Figure 2.1A shows that $TatB_{Strep}$ proteins largely accumulated in the second elution fraction. The $TatB_{Strep}$ has a molecular weight of 20.1 kDa, but it ran at 29 kDa in the gel. The deviation was possibly caused by excessive acidic and hydroxyl side groups, which bind less SDS molecules during incubation with Laemmli sample buffer. As a result, the mass-charge ratio was changed and $TatB_{Strep}$ proteins run slowly in the electrophoresis showing a larger apparent molecular weight. The major part of $TatB_{Strep}$ eluted in the E2 fraction, while in other four fractions only small amount of $TatB_{Step}$ were detectable. The Strep-II affinity chromatography is a native purification method, allowing the protein to stay in its native folded structure after purification. However, there were still lots of contaminants in E2 fraction after the purification through the Strep-II affinity chromatography. That`s why aliquots from this fraction were further purified through following two denatured purification methods.

RP-HPLC (*reversed phase*-HPLC) was used to further remove the contaminants in the E2 and E3 fraction after the affinity chromatography. The RP-HPLC column is filled with alkylated silica, and has a non-polar stationary phase and an aqueous moderately polar mobile phase. With the RP-HPLC proteins are separated according to their hydrophobicity. The more hydrophobic a protein is, the longer it is retarded on the column. In my experiment the column was eluted with a water/acetonitrile gradient.

The E2 from the Strep-II affinity chromatography (Figure 2.1A) was loaded to a semi preparative RP-HPLC column. The separation was performed under the conditions described in Figure 2.1. In the RP-HPLC chromatogram (Figure 2.1C) the peaks at the

very beginning represent salts and buffer components. The P1-P3 correspond to other proteins than $TatB_{Strep}$ in the sample. The 38-47 fractions of the big peak P4 contained $TatB_{Strep}$ proteins, which were dried in vacuum and resolubilized in 60 µl buffer (10 mM NaH_2PO_3 / 0,01%SDS, pH 7,2). 1µl aliquot from it was analyzed with mass spectrometry (Figure 2.1D, left) (Dr. Angelika Schierhorn, Max-Planck-research center "Enzymology of protein folding", Halle). The verified molecular weight of $TatB_{Strep}$ is 20136 Da, which is in accordance with the calculated value 20161 Da. The difference was possibly due to a point mutation (Pro → Ala) in the protein. 12 µl aliquot $TatB_{Strep}$ was also loaded in a 15% SDS-PAA gel and stained with coomassie colloidal, showing high purity of the purified $TatB_{Strep}$ (Figure 2.1D, right).

As an alternative to the RP-HPLC method, two-dimensional SDS-PAGE (2D gel electrophoresis, Rais et al, 2004) can also be used to purify the $TatB_{Strep}$ proteins. We have optimized an efficient 2D system for the purification of $TatB_{Strep}$. The gel used for the first dimension electrophoresis contains 10% AA/Bis, Tris-Tricine and 6 M Urea. After the separation by the first dimension, the whole lane was cut out and put horizontally above the second dimension (15% AA/Bis, Tris-Glycin). After the 2D electrophoresis the hydrophobic proteins were expected to be located above the diagonal (Figure 2.1B) while other water soluble proteins were detected near the diagonal. The gel was stained with zinc imidazol solution, $TatB_{Strep}$ was detected as a single spot (spot 2) below the diagonal and the spot 1 on the diagonal indicated a hydrophilic contaminant in the sample.

2.1.3 Purification of $TatC_{His6}$

After overexpession the cells were disrupted by two cycles of French press at 1000 psi and fractionated by centrifugation steps as described for $TatB_{Strep}$ (see Chapter 2.1.1). The membrane fraction and IBs fraction contain most of overexpressed $TatC_{His6}$. A His_6-tag fused to the C-terminal end of the TatC is to ease the purification of the overexpressed $TatC_{His6}$ protein. Additionally, compared to the Strep-tag, the His_6-tag can tolerate more detergents and denaturing additives in higher concentration. For example, up to 2% Triton X-100 and 6 M guanidium hydrochloride (GuadCl) can be used in the Ni-NTA affinity chromatography, which is not the case with the Strep-Tactin affinity chromatography.

Because membrane proteins are embedded in membrane bilayers, detergents are necessary to solubilize and isolate the membrane proteins while maintaining their structural and functional properties. With its six transmembrane helices TatC is more hydrophobic than $TatB_{Strep}$ and tends to aggregate in aqueous solutions. Various detergents were tested to solubilize the TatC proteins from the membrane fraction. The choice of detergents had to be governed not only by their ability of solubilization, but also by their compatibility with biochemical and structural studies.

2.1.3.1 Detergent screening for the solubilization of $TatC_{His6}$ from the membrane fraction

In order to find out a suitable detergent for the solubilization, a detergent screening was performed. The membrane pellet was resuspended in 2 ml H_2O supplemented with protease inhibitors (pepstatin, leupeptin and AEBSF). 12 x 40 µl aliquots from the suspension were taken out, and 10 µl 10% detergents (final concentration was 2% (w/v)) were added. In the screening 12 different detergents often used in the solubilization of membrane proteins were tested. The solubilization was achieved by rotating the samples at 4°C for 2 h. The insoluble proteins were precipitated by ultra centrifugation (1 h, 4°C, 55 000 rpm, MLA-80). An aliquot of 18 µl from each supernatant was taken out and incubated with 6 µl 4xSDS Laemmli sample buffer. All samples were not boiled and directly loaded to a 15% SDS-PAA gel. For comparison an aliquot of 18 µl from the start suspension was incubated directly with 6 µl 4xLaemmli sample buffer without the following ultra centrifugation step and co-loaded in the gel. The 1x Laemmli sample buffer contains about 1% (w/v) SDS, which can theoretically solubilize the total $TatC_{His6}$ from the membrane in the sample.

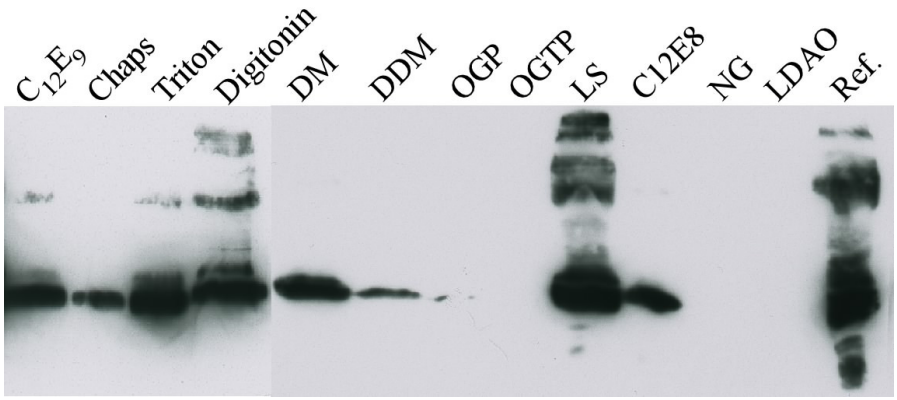


Figure 2.2: Detergent screening for the solubilization of $TatC_{His6}$ from the membrane fraction. $TatC_{His6}$ in the membrane fraction was solubilized using 12 different detergents with a final concentration of 2% (w/v) at 4°C for 2 h. The soluble $TatC_{His6}$ in the supernatants was analyzed on a 15% SDS-PAA gel. After the electrophoresis, the proteins were blotted to a PVDF membrane and followed by immune detection with an anti-TatC antibody. Ref: 18 µl aliquot of the start membrane suspension was incubated with 6 µl 4x Laemmli sample buffer without previous ultra centrifugation treatment; DM: Decyl-ß-maltoside; DDM: Dodecyl-ß-maltoside; OGP: Octyl-ß-D-glucopyranoside; OGTP: Octyl-ß-D-thioglucopyranoside; LS: N-lauroyl sarcosine; C12E8: Dodecyl octaoxyethylene; NG: Nonyl-ß-D-glucopyranoside; C12E9: Nonaethyleneglycol mono-n-Dodecyl Ether; LDAO: N-dodecyl-N,N-dimethylamine-N-oxide.

The results of the detergent screening are shown in Figure 2.2. The $TatC_{His6}$ protein in the membrane fraction shows the best solubility with 2% LS. The amount of solubilized $TatC_{His6}$ was almost the same as that in the reference. Due to the high concentration of $TatC_{His6}$ in the LS lane, there were also dimer and oligomers of the $TatC_{His6}$ protein to be seen. Detergents like Triton X-100, digitonin and DM have similar solubilization properties for $TatC_{His6}$. Although these three detergents were less efficient than LS, they were much milder and used frequently in the membrane protein studies. Digitonin, a mild detergent, does not destroy the protein complex structure and is often used in native gel systems for protein complex studies. Triton X-100 and DM are nonionic detergents which are widely used in the membrane protein solubilization. Especially Triton X-100 is also often applied for the detergent-mediated reconstitution of membrane proteins into liposomes (more details in the Chapter 2.3.2). $C_{12}E_8$, $C_{12}E_9$ and CHAPS can solubilize the $TatC_{His6}$ proteins with less efficiency. Little or no TatC signals were detectable in the lanes with other detergents (DDM, OGP, OGTP, and NG), indicating that they are not suitable for the solubilization of $TatC_{His6}$ from the membrane fraction. The choice of a detergent depends both on the efficiency of the

solubilization and on its compatibility for the subsequent procedure, like the purification step and the following reconstitution.

2.1.3.2 Ni-NTA affinity chromatography of the $TatC_{His6}$ solubilized from IBs fraction

The IBs fraction of the overexpression of the Tat_{His6} was solubilized in 6 M GuaHCl. Insoluble particles were precipitated by centrifugation. The supernatant containing the $TatC_{His6}$ was loaded to a Ni-NTA affinity chromatography. Four elution fractions with each 2 ml were collected, and analyzed with a 15% SDS-PAA gel with coomassie colloidal staining and the Western blotting.

It was shown in SDS-PAA gel electrophoresis with the coomassie colloidal staining (Figure 2.3A) that after the Ni-NTA affinity chromatography the eluted proteins were accumulated mostly in the E2 and a small amount in E3 fractions. In these two fractions a thick band with a molecular weight of approximately 31 kDa was detected, which was likely correlated to the expressed $TatC_{His6}$. To verify this, a second gel was blotted to a PVDF membrane and detected with anti-TatC antibodies (Figure 2.3B), on which a strong TatC signal was detected at a similar position. It was difficult to correlate the thick band on the coomassie colloidal stained gel (Figure 2.3A) with the TatC signals in the Western (Figure 2.3B). Therefore the Western membrane was stained with coomassie (Figure 2.3C). The result indicated that the thick band with a molecular weight of 31 kDa in Figure 2.3A was not the $TatC_{His6}$ but a strong contaminant. Since many other contaminants were still present in the elution fractions after the affinity chromatography, additional purification steps were necessary. Same as for the purification of $TatB_{Strep}$, the RP-HPLC was used to further remove the contaminants from the elution fractions.

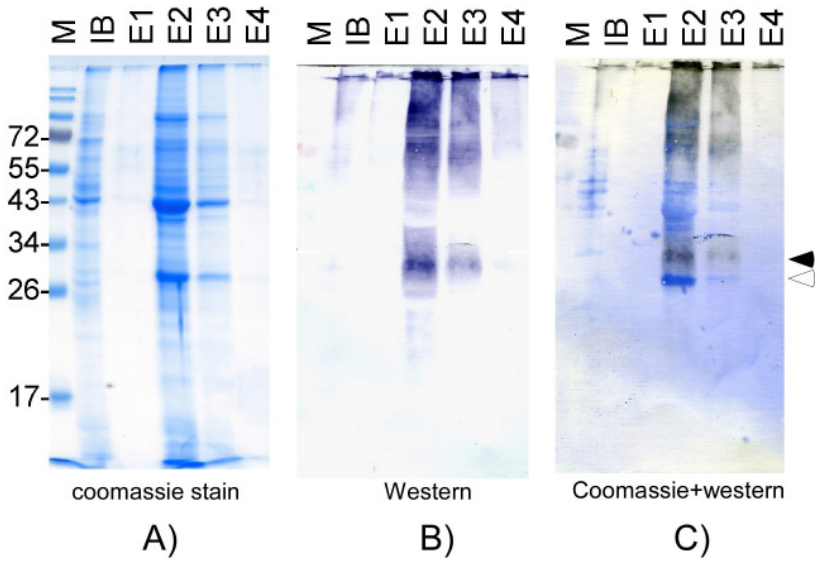


Figure 2.3: Ni-NTA affinity chromatography of TatC-His$_6$ solubilized from IBs fraction. 12 µl aliquots from the elution fractions (and the total IBs fraction) were incubated with 4 µl 4 x SDS Laemmli sample buffer and loaded to a 15% SDS-PAA gel without boiling. 3 µl prestained protein marker was co-loaded in the gels. The same samples were loaded in two gels. After electrophoresis one gel was stained with coomassie colloidal to check the purity of $TatC_{His6}$ after the chromatography (A), the other gel was blotted to a PVDF membrane, followed by an immune detection with anti-TatC antibodies (B). The coomassie staining of the Western membrane is shown in (C). The black arrow points to the TatCHis6 band detected with an anti-TatC antibody while the hollow arrow the strong contaminant appeared in the coomassie stainng.

2.1.3.3 Purification of TatC-His$_6$ with RP-HPLC

The E2 fraction after Ni-NTA affinity chromatography was loaded to the RP-HPLC. Because TatC with its six transmembrane helices is much more hydrophobic than TatB, a three-eluent system (H_2O / acetonitrile / *n*-propanol, detailed conditions see the figure legend of Figure 2.4) was used to purify the $TatC_{His6}$ in the RP-HPLC. The addition of the organic solvent *n*-propanol as the third eluent increases the hydrophobic property of the eluent, and thus could elute the $TatC_{His6}$ proteins easier, which were tightly bound on the column.

The peaks at the very beginning of the chromatogram (Figure 2.4A) resulted from the salts and buffer compounds passing the column without retardation. In the fractions 16-30 the less hydrophobic proteins were eluted from the column. At the fraction 76 a very sharp peak appeared, which perhaps contained the $TatC_{His6}$ proteins. This fraction was dried in vacuum and then resolubilized in 200 µl buffer (10 mM NaH_2PO_3 / 0.01%SDS,

pH 7,2). Aliquots from the solution were analyzed by coomassie colloidal staining and Western analysis respectively. The protein lane in the coomassie colloidal staining (Figure 2.4B) was rather pure and only one band with the molecular weight of approximately 31 kDa was visible. Compared with Figure 2.3A, the result showed that the RP-HPLC could efficiently remove the contaminants from the $TatC_{His6}$ protein. The gel was also blotted to a PVDF membrane, following detection with anti-TatC antibodies, and a strong TatC band at 31 kDa appeared. The smeared signals above the TatC band resulted from the high concentration of TatC, which tends to form aggregates at higher concentrations (Figure 2.4B).

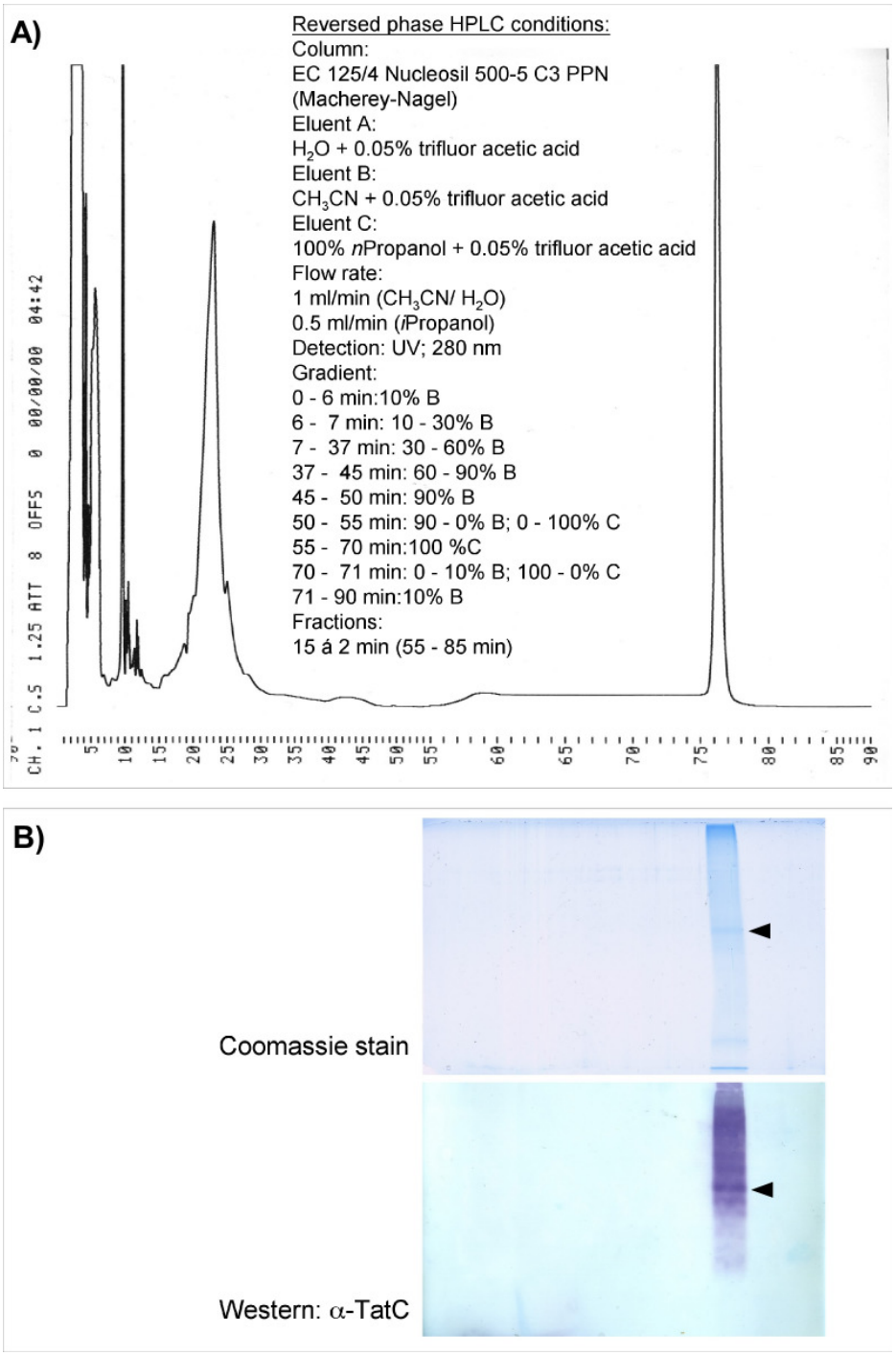


Figure 2.4: RP-HPLC purification of $TatC_{His6}$. A) RP-HPLC chromatogram of the E2 fraction obtained from the Ni-NTA affinity chromatography. The horizontal axis shows the number of the retention time, and the insert the conditions in RP-HPLC. B) Coomassie colloidal staining and Western analysis of the peak (fraction 76) in the chromatogram. After the RP-HPLC the fraction 76 was dried in vacuum and then resolubilized in 200 µl. 30 µl aliquot from it was incubated with 10 µl 4xLaemmli sample buffer, finally loaded to a 15% SDS-PAA gel without previous boiling.

2.2 Preparation of liposomes

The understanding of the structure of the Tat translocase integrated in thylakoid membrane is indispensable for investigations on the mechanism of the Tat transport pathway. Since the authentic thylakoid membranes present a plethora of various proteins, they are not suitable for *in vitro* studies on a particular integrated protein. Instead, the reconstituted proteoliposome system is useful for the study of membrane protein function. Generally, purified and solubilized membrane proteins will be inserted into lipid bilayers of artificial lipid vesicles which are also called liposomes. The lipids of liposomes can be extracted from membranes (for example, *E.coli* cell membranes or thylakoid membranes) or prepared from synthetic lipids. According to their size and structure, liposomes are generally divided into three sorts: multi lamellar vesicles (MLVs), large unilamellar vesicles (LUVs) and small unilamellar vesicles (SUVs). With their multilamellar structure, MLVs are not suitable for the import- and export-analysis of proteins over the translocator in the membrane. SUVs are less stable than LUVs because of the stress imposed by their curvature. So LUVs are mostly used for reconstitution experiments.

The lipid composition of the thylakoid membrane is unique among eukaryotic membranes because it contains about 77% neutral galactosyldiglycerides (Douce and Joyard, 1979), of which 51% are monogalactosyldiglycerides (MGDG) and 26% digalactosyldiglyceride (DGDG). These lipids are uncharged and slightly polar (Gounaris and Barber, 1983). In sharp contrast to most eukaryotic membranes which contain a high proportion of the zwitterionic phosphatidylcholine (PC), the thylakoid membranes contain only about 3% PC. Additionally, two acidic lipids are also present in the thylakoid membranes in low proportion: 7% sulfoquinovosyldiglyceide (SQDG) and 5% phosphatidylglycerol (PG).

There are numerous methods to obtain liposomes. The step common to all methods for preparing liposomes consists in evaporating the organic solvent in which the lipids are dissolved and then dispersing the lipids in an aqueous solution (buffered or unbuffered). The procedures for preparation differ in terms of the manner in which the lipids are dispersed and can be classified as follows: a) Hydration of a thin lipid layer: This is the original method of Bangham *et al.* (1965). Starting with the organic solution of the

constituent lipids, a thin lipid film is prepared through removal of the organic solvent by means of evaporation (at reduced pressure in a rotary evaporator). The dry lipid film deposited on the wall of the flask is hydrated by adding an aqueous solution and vortexing. b) Reverse phase evaporation: First a lipid film is prepared by removing organic solvent, then redissolved in a second organic solvent, for example Freon II (Sprague, 1984). H_2O is added to the solution and the mixture is vortexed strongly till an emulsion formed. Liposomes are formed after the removal of Freon II by warming it at RT. c) Solvent injection. The lipids dissolved in an organic solvent like ethanol are injected slowly into a lukewarm aqueous solution (Batzri and Korn, 1973). The drawbacks of the method are that the size is heterogeneous (30-110 nm), liposomes are very dilute, and it is difficult to remove all ethanol, which may inactivate various biologically macromolecules, even in low amount (Batzri and Korn, 1973).

In this work a modified procedure based on the method of "hydration of a thin lipid layer" was used as the standard protocol for the preparation of the PC liposomes, which is schematically represented in Figure 2.5. Briefly, lipids dissolved in chloroform were dried by rotational evaporation and pumped under vacuum over night to remove residual chloroform. Subsequently, the dry lipid film was resuspended in reconstitution buffer by vortexing for 30 min at RT. Liposomes were formed spontaneously from the lipid film by this hydration step. Finally, the liposome suspension was extruded through a polycarbonate membrane with a pore size of 200 nm to get a homogeneous size of the liposomes of about 200 nm. The size and the form of the prepared liposomes was then analyzed using the transmission electron microscopy (TEM) (cooperation with Lianbing Zhang, MPI of Microstructure Physics, Halle).

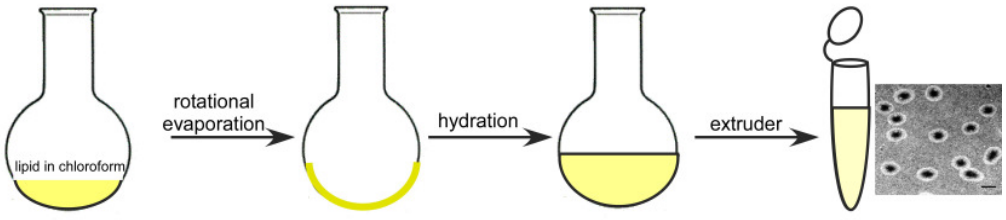


Figure 2.5. **Schematic representation of the standard protocol for liposome preparation.** As an example, the TEM image of prepared PC liposomes was shown on the right (scale bar: 200 nm).

Since the lipid composition influences the liposome structure significantly, liposomes with different lipid compositions were prepared. First, vesicles were prepared from 100% PC according to the above described protocol. PC liposomes are the most stable ones and widely used in reconstitution studies. The second sample was prepared with galactolipids. It contained 40 % MGDG, 30% DGDG, and 30% PC, which roughly mimics the natural lipid composition of the thylakoid membranes. These galactosyl liposomes were prepared with the rappid reverse phase evaporation method (detailed procedure in Chapter 4.2.6.1), a method that is reported to work better than the standard liposome preparation method which is mostly used for preparation of PC liposomes (Sprague 1984). For comparison, 5 µl suspension from authentic thylakoid membranes of *Arabidopsis thaliana* was also analyzed with TEM. For visualization of the liposomes under TEM, all samples were negatively stained with 2 % (w/v) uranyl acetate (detailed procedure in Chapter 4.2.6.3).

The TEM images of liposomes with different lipid composition are shown in Figure 2.6. The liposomes totally composed of PC were spherical with an average diameter of 200 nm (Figure 2.6A). Under TEM the lipid bilayers of the liposomes are visualized through the negative staining as white circles, in which the black dots represent the lumen of the liposomes. The size of the PC liposomes was homogenous and little lipid aggregates were detected. The structure of the authentic thylakoid membranes, which contain about 77 % galactolipids but only 3 % PC, were not spherical like the PC liposomes, but stacked tubules (Figure 2.6C). A very interesting observation is that the structure of the liposomes composed of 40 % MGDG, 30 % DGDG, and 30 % PC was very similar to the authentic thylakoid membranes (Figure 2.6B). Aside from the similar stacked tubular structure, there were lots of lipid aggregates present in the sample of the galactosyl liposomes. Since the presence of galactolipids (MGDG and DGDG) greatly changed the shape of the liposomes, the tubular structure very probably resulted from the high concentration of galactolipids as given in the authentic thylakoid membranes.

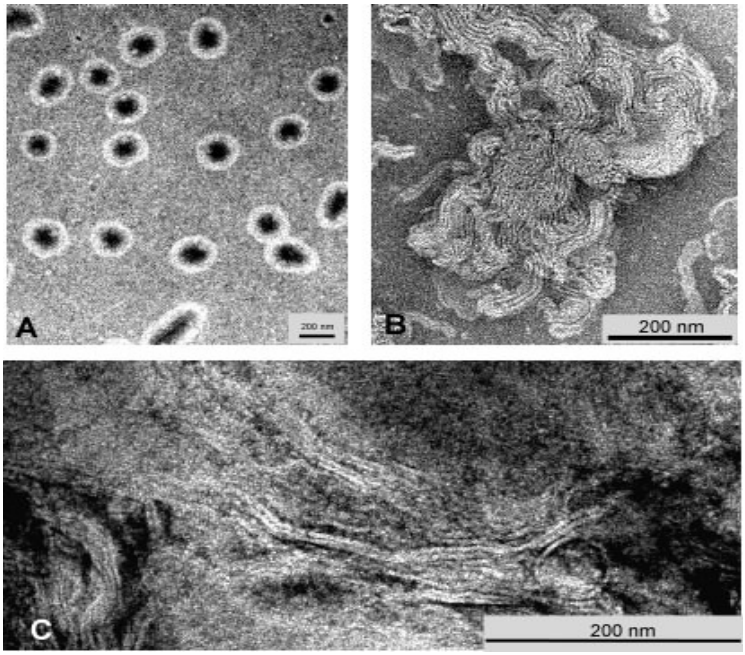


Figure 2.6: TEM images of liposomes prepared with various lipid compositions. A) Liposomes composed of 100 % PC; B) Liposomes composed of 40 % MGDG, 30 % DGDG and 30 % PC (thylakoid-like lipid composition); C) Authentic thylakoid membranes of *Arabidopsis thaliana*; PC: phosphatidyl choline; MGDG: monogalactosyldiglycerides; DGDG: digalactosyldiglyceride.

As the ideal system for reconstitution experiments, liposomes with a similar lipid composition as found in the thylakoid membranes are supposed to be prepared, which will provide a more "natural" environment for investigations on the transport mechanism of Tat pathway. However, liposomes prepared with the thylakoid-like lipid composition tend to aggregate easily in aqueous solutions, which make such liposomes very unstable and very difficult to be prepared qualitatively for reconstitution experiments. In contrast to the galactosyl-liposomes, the PC liposomes are much easier to be prepared and very stable. They stay intact at 4°C for several weeks in most buffered solutions, which makes them a generally used system for reconstitution of membrane proteins. Therefore, the PC liposomes were prepared and used at the preliminary stage of the reconstitution of the TatB and TatC in this work.

2.3 Reconstitution of Tat proteins with three different methods

There are three mostly used reconstitution methods: (1) the spontaneous insertion (Jain *et al.*, 1978), (2) the detergent-mediated reconstitution (Rigaud *et al.*, 1995), and (3) the recently appeared cotranslational reconstitution (Nozawa *et al.*, 2007). All these three reconstitution methods were tested in this work. The working conditions of the three reconstitution methods have been optimized and standardized.

In the first two methods, the recombinant Tat proteins were first purified and then used for the reconstitution experiment. Compared to TatB and TatC, TatA is the smallest membrane protein which contains only one transmembrane helix. The purification of TatA was more successful than that of the other two Tat proteins. Therefore, in order to find an efficient reconstitution method and further optimize it, TatA was always used as the model protein for the reconstitution experiments. The second method has several variants which use different methods to remove detergents. In this work, dialysis and physical adsorption with Bio-beads were tested for the detergents removal. The third reconstitution method is quite different from the other three methods. In the presence of liposomes, nascent translated membrane proteins can be directly inserted into liposome bilayers without tedious protein purification steps. Since protein translation and its reconstitution into liposomes are carried out simultaneously, this method is named as cotranslational reconstitution.

After the reconstitution, in the RTS reaction mixture besides proteoliposomes there are many contaminants such as the translation co-factors, uninserted free Tat proteins and protein- or lipid- aggregates. Using the following purification methods proteoliposomes with Tat proteins can be purified which are basically free from contaminants: ultra centrifugation, gel filtration or sucrose gradient floating technique.

2.3.1 Reconstitution of Tat proteins using the spontaneous insertion method

In this method, the purified recombinant Tat proteins are added to the preformed liposomes, the spontaneous insertion is achieved by shortly vortexing the protein-

liposome mixture and incubating it on ice for 30 min. The proteoliposomes, the liposomes with the inserted proteins in their bilayers, can be purified and isolated with several centrifugation and wash steps.

In this experiment, 1 ml 1mg/ml five-lipid liposomes (galactosyl liposomes: 51% MGDG, 26% DGDG, 4% PC, 7% SL, 9% PG) were prepared by rappid reverse phase evaporation method (see Chapter 4.2.6.1). Purified TatA, TatB and TatC were tested both separately and in combination as TatBC and TatABC for the insertion. The Tat proteins were added to the liposome suspension, shortly vortexed, and then incubated on ice for 30 min. Protein- and lipid-aggregates were removed by a centrifugation at 10,000 g for 10 min. The centrifugation steps are shown in Figure 2.7. The supernatant contained uninserted soluble proteins (free Tat proteins), liposomes without proteins, and proteoliposomes. Following the transfer of this supernatant to a new reaction tube, the proteoliposomes were pelletized by ultra centrifugation at 100,000 g at 4°C for 30 min. The proteoliposome pellet was then washed three times with each 100 µl reconstitution buffer by repeating resuspension and pelletizing. The final washed proteoliposome pellet was resuspended in 100 µl reconstitution buffer. The supernantant, the three washing fractions and the final proteoliposome-pellet were analyzed in SDS-PAA gel. As negative control, the procedure with the identical steps was performed without liposomes. The gels were blotted to PVDF membranes, followed by an immune detection with the corresponding antibodies.

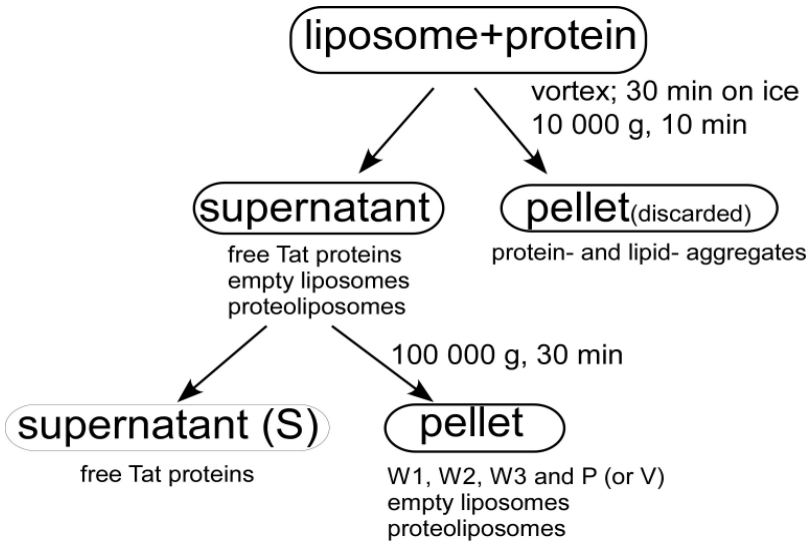


Figure 2.7: Schema for the purification of proteoliposomes by the centrifugation.

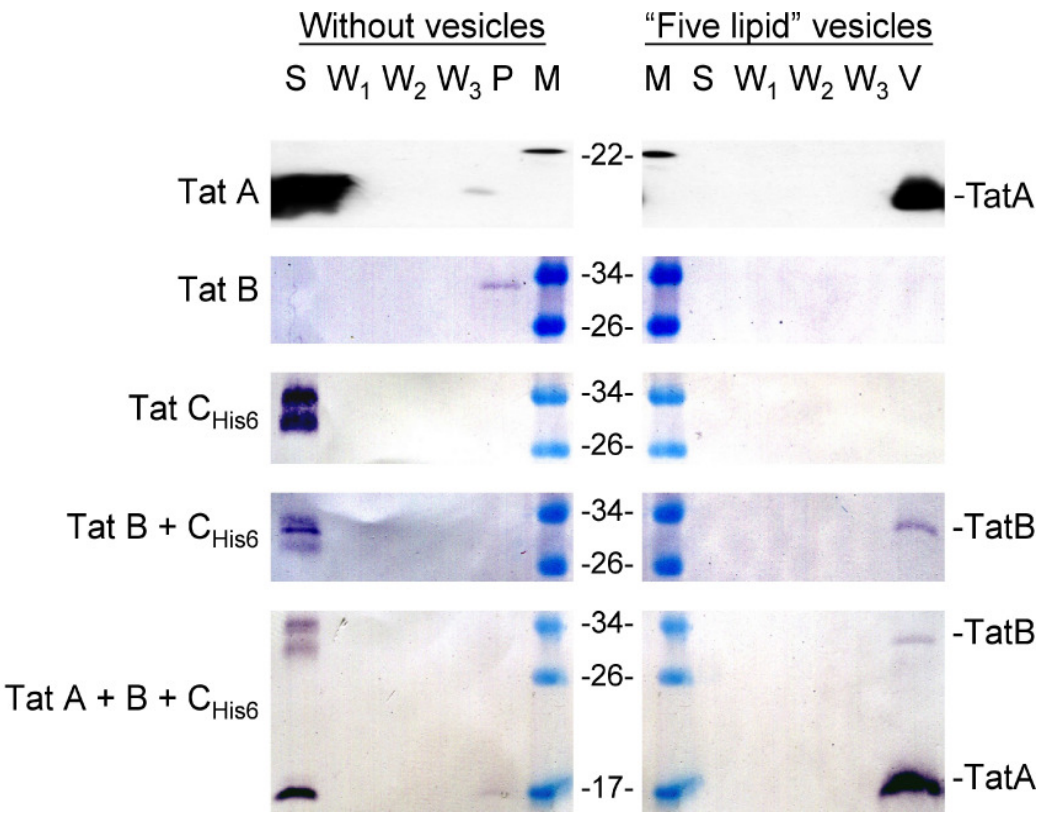


Figure 2.8: Spontaneous reconstitution of TatA, TatB, and TatC proteins into liposomes. On the left is the negative control experiment, and on the right the results of the reconstitution in the presence of liposomes. The free Tat protein-containing supernatant was concentrated by acetone precipitation and the resultant pellet was resolubilized in 100 µl 2xLaemmli sample buffer. 10 µl aliquots from each washing step and the pellet suspension were incubated with 4 µl 4xLaemmli sample buffer, then boiled at 95°C for 5 min (except samples with TatC) and loaded together with 10 µl aliquot from the supernatant in 15% SDS-PAA gels. The gels were blotted to PVDF membranes, followed by an immune detection with corresponding antibodies. M: protein marker; S: supernatant; W1-3: wash step 1-3; P: pellet; V: vesicles (liposomes).

The insertion experiment of TatA (Figure 2.8) showed that in the absence of liposomes strong TatA signals were detected in the supernatant lane, very weak TatA band in the pellet lane, and no signals in the wash fractions, while in the presence of liposomes the TatA signals were found only in the proteoliposomes. This means most of the TatA proteins were inserted in the liposomes. The weak TatA band in the pellet lane in the control experiment was originated probably from a small amount of aggregated TatA proteins.

The insertion of TatB proteins ran oddly, no protein signals were detected both in the S lane without the liposomes and also in the P lane with the liposomes. The TatB proteins

for this insertion experiment were possibly insoluble and precipitated already in the first centrifugation step. The weak TatB band in the P lane in control experiment was perhaps, like TatA, the TatB aggregates, which were pelleted.

In the control experiment of TatC bands were detected in the S lane, this indicated, a part of TatC proteins was soluble. However, in the presence of liposomes no signals were found, perhaps the small amount of soluble TatC proteins precipitated in the presence of liposomes. The insertion of TatB and TatC were not so efficient as that with TatA, this perhaps because TatA is the smallest one.

In the thylakoid membrane Tat proteins exist as TatBC receptor complex, and a TatABC complex in the presence of a substrate and a proton gradient during the translocation process. Perhaps the insertion of Tat proteins can be more efficient if they are added simultaneously. So, in the other two insertion experiments the two Tat proteins of the receptor complex (TatB and TatC), and all three Tat proteins (TatA, TatB and TatC) were added to the liposomes suspension. Only a weak TatB band was visible in the proteoliposome lane while no TatC signal was detected in both cases. This result reveals that the spontaneous insertion method is more suitable for TatA. To achieve better reconstitution results of TatB and TatC, other reconstitution methods are further studied.

2.3.2 Reconstitution of Tat proteins with detergent-mediated method

2.3.2.1 Detergent removal with dialysis

In this method the lipids are dissolved in a buffer containing the target protein and a detergent. The liposomes will be formed after the detergent removal, with the membrane proteins reconstituted in the bilayers (proteoliposomes). A schematic presentation of the procedure is illustrated in Figure 2.9.

In this experiment the mild nonionic detergent octyl glucopyranoside (OGP) was used. It has a high critical micellar concentration (CMC) about 0.7% (w/v), and can be easily removed by dialysis (Allen, 1980). Tat proteins were diluted to 1 µg / ml with the reconstitution buffer containing 1% (w/v) detergent. The detergent-protein containing solution (1 ml) was added to 1 mg of dried five-lipid (galactolipids) film and the galactolipids were dissolved by gently vortexing at 25°C. The sample was then placed in a dialysis tube with the molecular weigh cut off of 3.5 kDa and dialyzed for 24 h at 4°C against 1000 volumes (1 l) of the reconstitution buffer which was changed once

after 16 h. After the dialysis the concentration of the residual OGP was far below its CMC, so liposomes with the inserted Tat proteins were formed (Tat proteoliposomes). However, the formed proteoliposomes were mostly multilamellar. To get unilamellar proteoliposomes, they were treated with extrusion, which is a quick and simple one-step procedure that produces homogenous unilamellar liposomes by forcing aqueous suspensions of lipid through polycarbonate filters with defined pore size. In this work, liposomes were extruded 19 times through two stacked polycarbonate filters with a pore size of 200 nm. In the solution, besides proteoliposomes there are also free Tat proteins and aggregates, which were removed by several centrifugation steps as described in Figure 2.7. The supernatants, the three wash steps and the pellet were analyzed with the Western blotting. Control experiments in absence of liposomes were performed with the identical procedures.

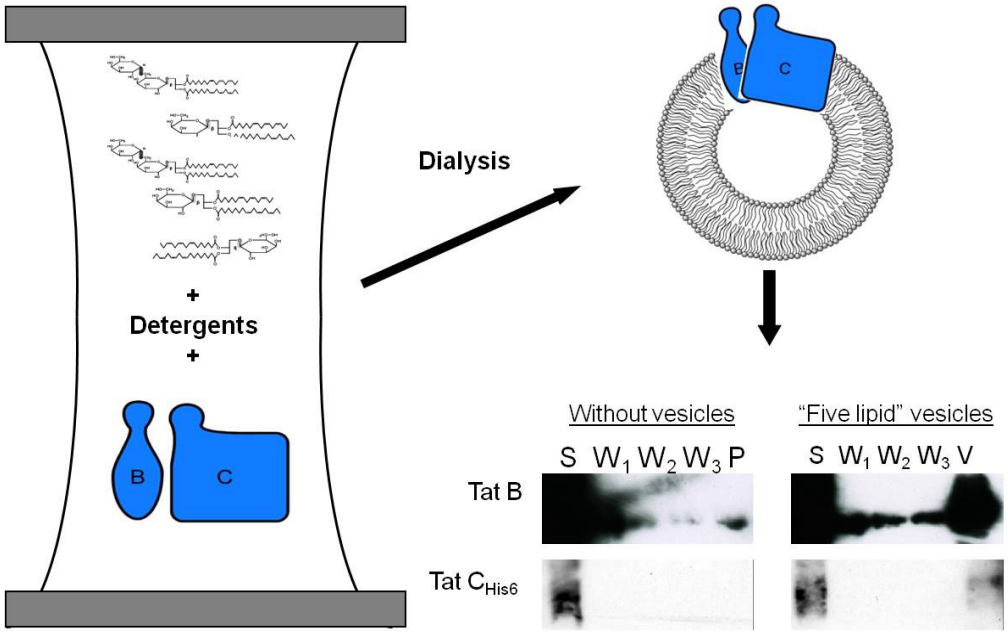


Figure 2.9: The reconstitution of TatB and TatC in galactolipid liposomes using the dialysis method. The proteoliposomes were purified and isolated through several centrifugation steps. The free Tat protein-containing supernatant was concentrated by acetone precipitation and the resultant pellet was resolubilized in 100 µl 2xLaemmli sample buffer. 10 µl aliquots from each washing step and the pellet suspension were incubated with 4 µl 4xLaemmli sample buffer, then boiled at 95°C for 5 min (except samples with TatC) and loaded together with 10 µl aliquot from the in 100 µl Laemmli sample buffer resuspended supernatant in 15% SDS-PAA gels. The gels were blotted to PVDF membranes, followed by an immune detection with the corresponding antibodies. M: protein marker; S: supernatant; W1-3: wash step 1-3; P: pellet; V: vesicles (proteoliposomes).

In the Western Blotting shown in Figure 2.9, the signals for TatB were very strong in the supernatants both in the control and in the experiment with the liposomes. In the control the TatB signal appeared as a small spot in the pellet, that means most TatB proteins were soluble and only little amount of TatB proteins was precipitated in the ultra centrifugation steps. In the presence of the liposomes, the TatB signal is much stronger in the vesicle fraction, demonstrating that a certain amount of TatB was inserted into the liposomes.

In the case of TatC, the signals in the supernatant in both the control and reconstitution experiment were much weaker than that of the reconstitution of the TatB protein. TatC proteins were unstable and tended to aggregate. The aggregates of the protein were removed already by the first centrifugation step, so that less intense signals appeared from soluble TatC. Even with the limited amount of soluble TatC available for the reconstitution, some TatC protein was inserted into the liposomes, visible as a faint band in the vesicle fraction in the presence of liposomes, while no TatC signals in the pellet were detectable in the control. Although the insertion efficiency for both TatB and TatC was not high, some proteins were indeed inserted in the liposomes by this method. Compared to the spontaneous insertion, the dialysis reconstitution method works better for the larger membrane proteins.

In order to further increase the insertion efficiency for TatB and especially TatC, the protocol of the dialysis method has been optimized by changing some parameters, such as the lipid-protein ratio, the concentration of the detergent OGP (data not shown), however, no better insertion results were achieved. Since the results with this method were not satisfying, the reconstitution with another considered technique (the detergent-mediated method) was then investigated.

2.3.2.2 Detergent-removal with Bio-beads

Detergent-mediated reconstitution is the most frequently used strategy for the proteoliposome preparation (Helenius and Simons, 1975). In this method the reconstitution is initiated by adding detergent to a solution of preformed liposomes. Membrane proteins are then added into the detergent-lipid-solution during the solubilization of liposomes. Upon removal of the detergent the liposomes containing

incorporated membrane proteins in the bilayers were progressive reformed as the proteoliposomes.

Depending on the nature and the concentration of detergents, the process of liposome solubilization by different detergents can be described as a "three stage model" (Lichtenberg, 1983, 1985), which can be followed through the turbidity measurement of the detergent-liposome suspension with a photometer. When at constant concentration of liposomes the detergent concentration is increased, the three stages of solubilization process can be distinguished. In the stage one, increasing the concentration of detergents up to a point where detergent micelles begin to form, the phospholipid bilayers become "saturated" with the detergent. At this “saturated” point the molar ratio of the detergent to the lipid is defined as R_{sat}. The turbidity of the solution reaches its maximal value. During the stage two, further increasing the concentration of the detergent forces the "saturated" liposomes to go through a structural transition from a lamellar structure to a population of phospholipid-detergent micelles. The transition is not infinitely sharp, so in this stage the detergent "saturated" bilayers coexist with micelles. This stage is characterized by a decrease of turbidity due to the gradual solubilization of the liposomes. In the stage three the phospholipids are completely solubilized to micelles, leading to an optically transparent solution. At this point the molar ratio of the detergent/lipid is defined as R_{sol}.

Liposome solubilization with Triton X-100

Triton X-100 is the mostly used detergent for the detergent-mediated reconstitution, since it is a nonionic mild detergent, which can solubilize the liposomes through a partially opened vesicular structure (Stuart, 2007). Membrane proteins were added to partially opened liposomes with a detergent concentration lying between the R_{sat} and R_{sol}, which can be determined by a titration experiment as following. Briefly, 1 ml 4 mg/ml PC liposomes were prepared according to the standard protocol. 10 % (w/v) Triton X-100 were added stepwise to liposomes solution, and incubated after each addition for 10 min at RT. The solubilization process was visualized by measuring the turbidity at 540 nm with a spectrophotometer. The OD_{540nm} values were plotted against the detergent concentration (Figure 2.10). The R_{sat} and R_{sol} correspond to the a- and b-values on the horizontal axis with maximal and minimal OD_{540nm} values, respectively. An aliquot after the addition of 2 mg Triton X-100 was taken out for TEM. In the TEM

image (inset of Figure 2.10), it becomes visible that Triton X-100 partially maintained the bilayer structure of the liposomes and the solubilization goes through an open vesicle, which benefits the insertion of the membrane proteins. For 1 ml 4 mg/ml PC liposomes 20 µl 10% Triton X-100 (equals to 2 mg) was used for the solubilization of liposomes in my reconstitution experiment.

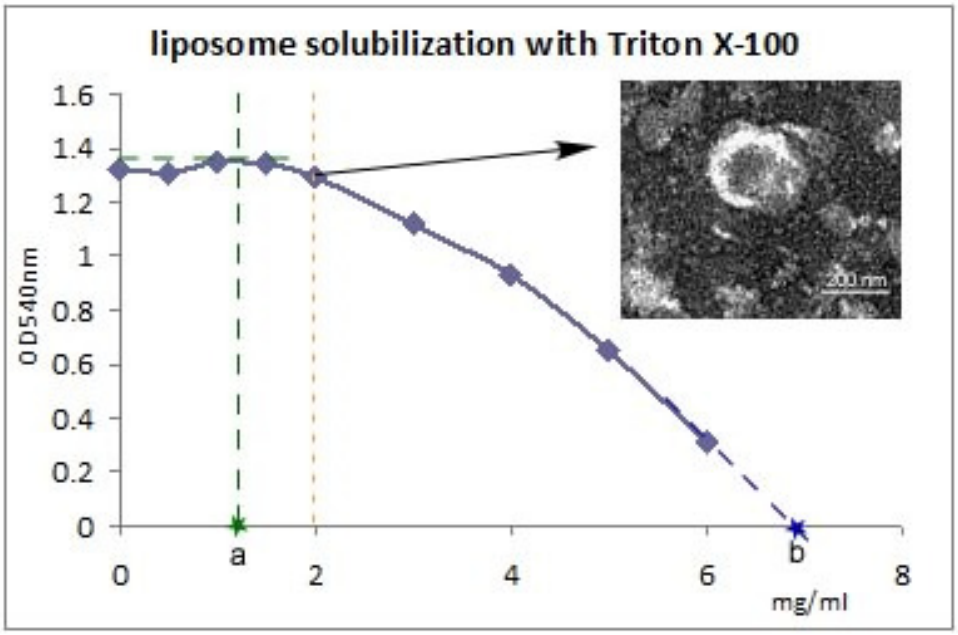


Figure 2.10: Solubilization of 1 ml 4 mg/ml PC liposome with 20 µl 10% (w/v) Triton X-100. Aliquots of 10 % (w/v) Triton X-100 were added stepwise to the liposome solution, the process of the solubilization was followed by the turbidity measurement in a photometer. The values on the horizontal axis with the maximal and minimal turbidity at OD_{540nm} correspond to the R_{sat} and R_{sol} respectively (labeled with a green (a) and blue star (b) correspondingly). 5 µl aliquot from the point at which the liposomes were solubilized with 2 mg Triton X-100 was taken out for the TEM (inset: solubilization with 2 mg Triton X-100, an open vesicle).

The reformation of liposomes with the incorporated proteins in the bilayer is achieved through removal of the detergent from the lipid-detergent-protein suspension. With its low CMC (0.2 mM) value Triton X-100 cannot be removed by the traditional methods, such as gel filtration and dialysis, which are suitable for removing monomeric molecules of detergents with high CMC. Holloway *et al.* reported a simple, rapid, and mild procedure for removing Triton X-100 detergent by using the Bio-Beads SM-2 adsorbent, which can remove detergents by hydrophobic interaction. The protocol is

slightly modified in this work. The procedure for the reconstitution with the detergent-mediated method is schematically shown in Figure 2.11.

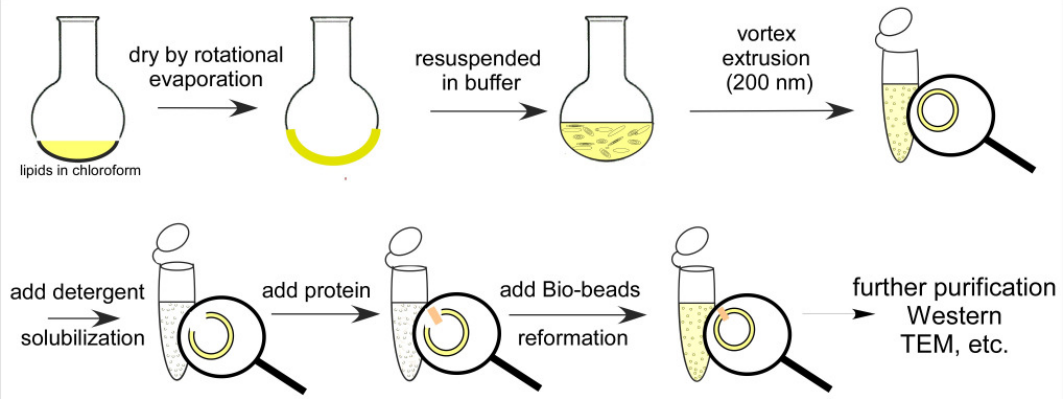


Figure 2.11: Scheme for the reconstitution using the detergent-mediated method

For the reconstitution, 1 ml 4 mg/ml PC liposomes with a diameter of 200 nm were prepared according to the standard protocol. Solubilization of the liposomes was accomplished by adding 20 µl 10% (w/v, equal to 2 mg) Triton-X-100 to the solution, followed by 1 h incubation at RT under continuously stirring until the solubilization reaches an equilibrium state, at which the OD_{540nm} value of the solution became stable. Finally the Tat proteins were added and incubated at RT for 30 min under continuously stirring. For the removal of Triton X-100, 80 mg Bio-Beads SM2, which were extensively washed with acetone/methanol before use as described (Holloway, 1973), were added to the lipid-detergent-protein suspension, and the mixture was stirred at RT for 2 h. A second portion of 80 mg Bio Beads was added for an additional incubation for 2 h at 4°C to remove the residual detergent. With the removal of Triton X-100 the proteoliposomes were progressively reformed. The contaminants like protein aggregates in the solution were removed through various methods, such as centrifugation, gel filtration, sucrose floating technique.

Recontitution of TatA with the detergent-mediated method

The reconstitution using the detergent-mediated method was first investigated with overexpressed $TatA_{His6}$ proteins which were solubilized in 1% (w/v) Triton X-100. The reconstitution was carried out according to the procedure described in Figure 2.11. 200 µl $TatA_{His6}$ was added and the final 1 ml reconstitution mixture was ultra centrifugated at 150000g, 4°C for 1 h (MLA-80). The pellet was resuspended in 200 µl reconstitution buffer, which was then carefully overlaid on a discontinuous sucrose density gradient (10% - 40%) (Figure 2.12A). The preparation of the gradient was described in detail (see Chapter 4.2.8.1). After the ultra centrifugation at 150 000 g, 4°C for 16 h (rotor MLS-50), 22 fractions with each 200 µl were collected from the top of the ultra centrifugation tube. The pellet was resolubilized with 200 µl 2x SDS Laemmli sample buffer. All fractions and the pellet were analyzed in Western blotting. As a control experiment, $TatA_{His6}$ without the liposomes was subjected to the ultra centrifugation under identical conditions.

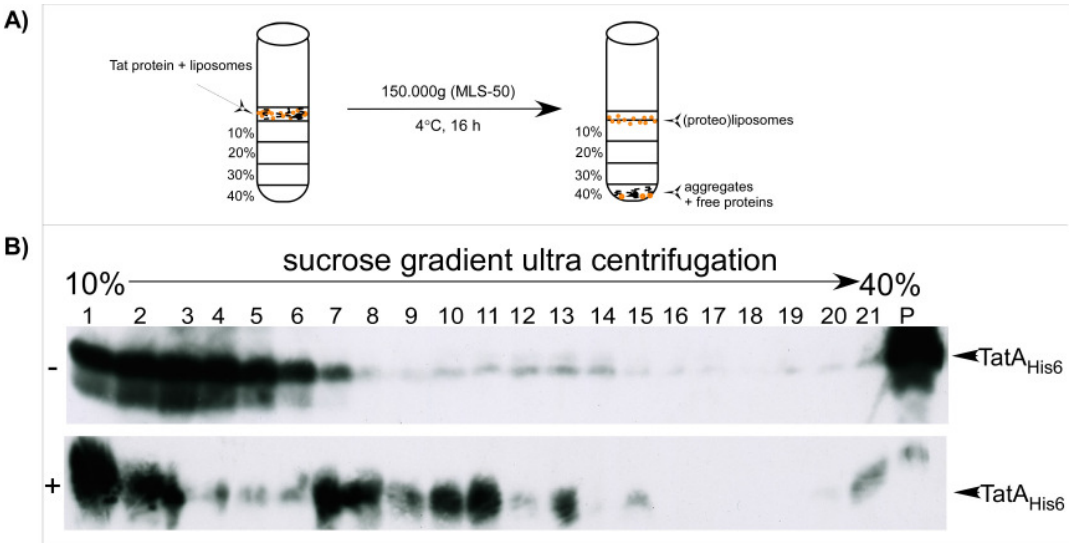


Figure 2.12: Reconstitution of $TatA_{His6}$ with the detergent-mediated method. A) schema for the sucrose gradient ultra centrifugation; The percentage of sucrose gradients are shown on the left of the centrifugation tube, and the centrifugation conditions are listed near the arrow. B) Western of the fractions obtained after sucrose gradient ultra centrifugation. The fraction numbers are written above the film. The percentage numbers correspond to the sucrose gradients. 18 µl aliquot from each fraction was incubated with 6 µl 4x SDS Laemmli sample buffer, boiled at 95°C for 5 min and loaded in two 15% SDS-PAA gels. After electrophoresis the gels were blotted to PVDF membranes and immune detected with polyclonal anti-TatA antibodies. +: with PC liposomes; -: without PC liposomes (control); P: pellet.

The results of the Western Blotting are shown in Figure 2.12B. In the control experiment strong TatA signals were found in the fractions 1-7, which approximately corresponded to the gradient layer with 10% sucrose. Since no liposomes were added in this control experiment, these TatA signals were not from the proteoliposomes but the soluble $TatA_{His6}$ which has a low molecular weight and remained in the upper layers of the gradient after the ultra centrifugation. A very strong TatA signal was also detected in the pellet (P) in the control experiment, which originated possibly from the TatA aggregates.

In the reconstitution experiment, the TatA signals appeared in the fractions 1, 2 and 7-13. The fractions 3-6 and the fractions higher than 14 showed no or very weak signals of TatA. In the presence of the liposomes, only weak signals appeared in the fraction 21 and the pellet. The signals of the fractions 7-13 are probably due to the formation of the proteoliposomes. However, the interpretation was interfered by the possible presence of free protein in the upper fractions, as demonstrated within the control.

Recontitution of TatC

The detergent-mediated reconstitution method is reported to work also well for the large membrane proteins, which is advantageous for the reconstitution of TatC. Since $TatC_{His6}$, overexpressed in *E. coli*, was unstable and always precipitated during the purification steps, the insertion of TatC with detergent-mediated reconstitution method was carried out with $TatC_{His6}$ synthesized in the rapid translation system (RTS). To avoid the possible precipitation, the synthesized $TatC_{His6}$ was not further purified before the insertion with the detergent-mediated method.

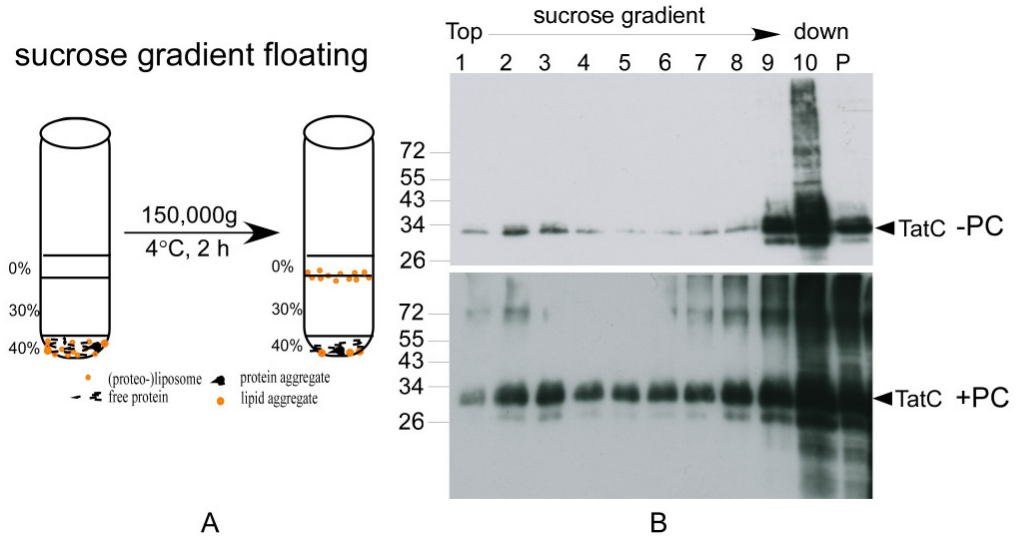


Figure 2.13: Reconstitution of 50 µl RTS translated $TatC_{His6}$ into 4 mg/ml PC liposomes using the detergent-mediated method. A) scheme of the sucrose gradient floating. B) Western Blotting analysis of the fractions obtained after sucrose gradient floating. +PC: with PC liposomes; -PC: without PC liposomes (control).

To avoid the possible coexistence of free protein and the proteoliposomes in the upper layer, a reversed floating direction was used by the ultra centrifugation with the sucrose gradient, which is illustrated in Figure 2.13A. By this floating technique, the proteoliposomes and liposomes with the lower density floated to the upper layers of the sucrose gradients, while the uninserted proteins and lipid aggregates with a higher density stayed at the bottom of the tube with the sucrose concentration of 40%.

After the centrifugation, the fractions were analyzed by the Western blotting. The results are shown in Figure 2.13B. Without the liposomes, the $TatC_{His6}$ accumulated in the bottom fractions. In fractions 1-8 the TatC signals were very weak without the liposomes, which turned much stronger in the presence of the PC liposomes. However, there was no obvious separation between the reconstituted and the uninserted $TatC_{His6}$.

2.3.3 Cotranslational reconstitution

By the other three methods of the reconstitution, which are described in the previous parts, the recombinant Tat proteins have to be overexpressed in *E. coli* and purified with various biochemical methods before they were used for the reconstitution experiments. Because of the large molecular size and its six transmembrane helices, the expressed

TatC was precipitated already during the preliminary purification steps, and the final amount of pure TatC was only in µg scale, which was not sufficient for reconstitution in the next step. Although the overexpression and the purification of recombinant TatA and TatB were much better, the purification of TatA and TatB were carried out under denatured condition. An efficient refolding will be required for the further reconstitution. The refolding protocol for TatA and TatB in our labor is still under optimization.

To circumvent the above mentioned problems with the low stability, the cell free translation system is a good alternative (Katzen *et al.,* 2005). The Wheat Germ extract was used to produce Tat proteins *in vitro*. Compared to the conventional overexpression system, such as the *E. coli* system, the cell free system possesses many advantages for the overexpression and reconstitution of membrane proteins. For example, small amounts of detergents can be added to the translation reaction mixture directly to increase the solubility of the expressed proteins, without considering the toxicity of the detergents to host cells. Most importantly, liposomes can also be added in the translation reaction, which enables the nascently translated proteins to be directly inserted into the liposomes (Elbaz *et al.,* 2004, Klammt *et al.,* 2006, Nozawa 2007). Since protein translation and its reconstitution into liposomes occur spontaneously, this method is named as "cotranslational reconstitution". Another advantage of this method is that the tedious purification and refolding steps are unnecessary.

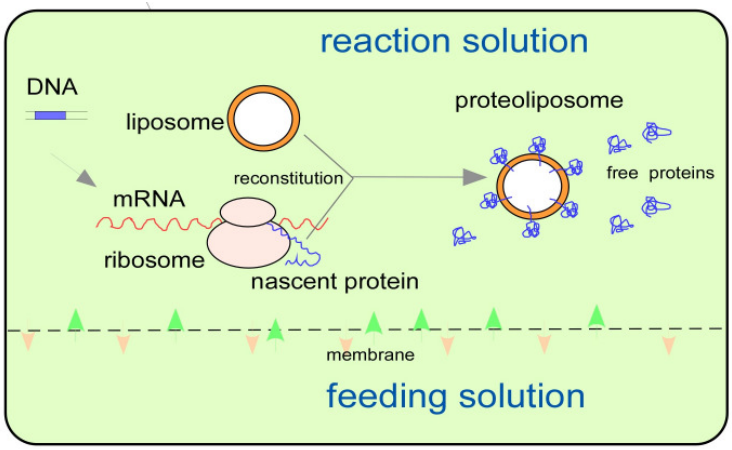


Figure 2.14: Scheme of the cotranslational reconstitution. 4 mg/ml PC-liposomes were added at a final concentration of 4 mg/ml into a 50 µl cell free translation reaction. The reaction device is composed of two chambers, one for reaction solution and the other for feeding solution, which are separated from each other by a semi-permeable membrane.

TatB and TatC, the relatively larger subunits, were investigated with the cotranslational reconstitution method. Before the cotranslation was started, PC liposomes with the concentration of 4 mg/ml were prepared according to the standard protocol. The Tat cDNAs were cloned in the pIVEX 1.3 vectors, which were used in the cell free reaction. Plasmid constructs were in detail described in the section of "Materials and Methods". The synthesis of the Tat protein with the Wheat Germ cell free translation kit (rapid translation system (RTS)) was performed according to the instruction manual with slight modifications. The procedure was schematically presented in Figure 2.14. Briefly, 50 µl reaction mixture (15 µl Wheat Germ lysate, 4 µl amino acids, 1 µl methionin, 2 µg tatpIVEX1.3 WG plasmid, 10 µl PC liposomes, H_2O) and 1 ml feeding mixture (80 µl amino acids, 20 µl methionin, 900 µl feeding mix) were prepared. The translation mixture and the feeding mixture were carefully set into the reaction chamber and the feeding chamber, which are separated by a semi-permeable membrane. With the two-chamber device substrates and energy components needed for a sustained reaction are continuously supplied via the semi-permeable membrane. At the same time, potentially inhibitory reaction by-products are diluted since they diffuse through the same membrane into the 1ml feeding compartment. The Wheat Germ RTS reaction ran at 24°C, 900 rpm for 24 h. After the reaction, the proteoliposomes were purified from the reaction mixture by the sucrose gradient floating technique shown in Figure 2.13A.

2.3.3.1 Cotranslational reconstitution of TatB

In order to check if this cotranslational reconstitution system works, the TatB was first tested, since its single transmembrane helix is theoretically easier to be reconstituted into the liposomes. As described previously and in the Materials and Methods, 2 µg tatBpIVEX1.3 vector and 10 µl 20 mg/ml PC liposomes (final concentration: 4 mg/ml) were added to the Wheat Germ extract reaction mixture. As negative control, another reaction with all other components except the PC liposomes was started in parallel. After 24 h reaction, the reaction mixture of 45 µl was put under a discontinuous sucrose gradient (0% - 40%) in an ultra centrifugation tube. After centrifugation (2 h, 150.000 g at 4°C), the proteoliposomes and liposomes with the lower density floated to the upper layers of the sucrose gradients. The uninserted TatB proteins and lipid aggregates of higher density stayed at the bottom of the tube with the sucrose concentration of 40%.

10 fractions (each of 200 µl) were carefully collected from the top of the tubes to the bottom. After the fractionation, the pellets in the ultra centrifugation tubes were resuspended in 200 µl two-fold SDS Laemmli sample buffer, which was, together with the gradient fractions, analyzed in the following procedure.

Aliquots from all the fractions were loaded to a 15% SDS-PAA gel. The proteins were then transferred to a PVDF membrane and detected by Western Blotting with polyclonal anti-TatB antibodies. The protein content of each fraction was determined by comparing the signal intensities on the Western Blotting. The liposome content of each fraction was estimated by the count number in the Zeta-sizer instrument. It is an instrument using the light scattering technique to measure the size of nanoparticles (like liposomes) and also being used to determine the presence of protein aggregates. The relative amount of nanoparticles will be given as the Kcount number in the measurement. According to the protein content and the liposome content in each fraction the reconstitution efficiency by this cotranslational method was estimated. The results of the cotranslational reconstitution of the TatB were shown in Figure 2.15.

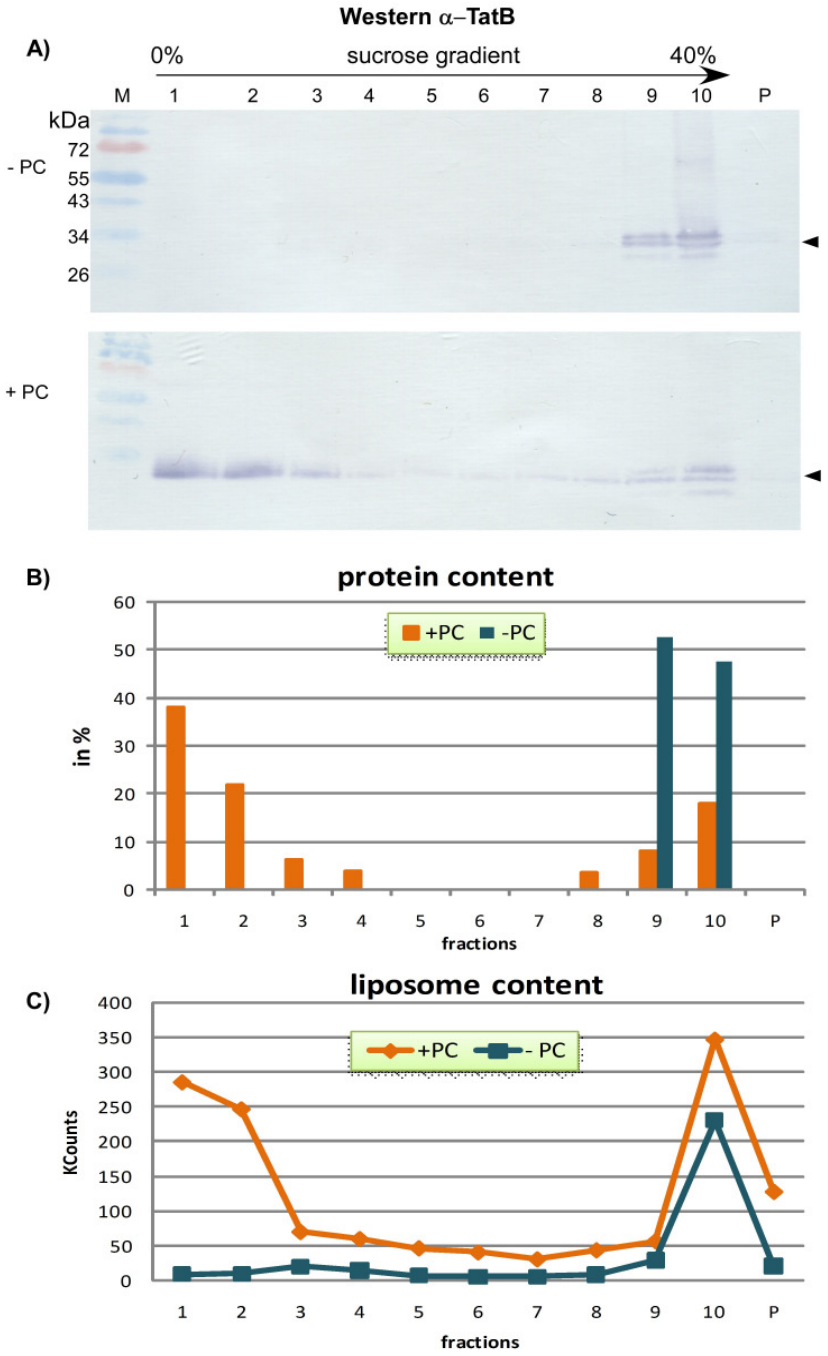


Figure 2.15: Cotranslational reconstitution of TatB. A) SDS-PAGE analysis of all fractions obtained from the sucrose floating, the numbers above the membrane were the fraction numbers, the sucrose gradient concentration was shown in percentage above the membrane. The upper membrane is the control reaction containing no liposomes (-PC), the lower one contains 4 mg/ml PC liposomes (+PC) ; B) TatB protein content in the fractions estimated from the band intensities in the Western using the software *2D-Image master*. The protein content of each fraction was plotted as percentage of the total protein amount in all fractions against the fraction numbers; C) Liposome content in the fractions measured by count number in Zetasizer instrument, and was shown for different fraction.

Figure 2.15A shows the results of the Western Blotting. It is shown that in the control reaction without liposomes, all synthesized TatB was only detectable in the lowest two fractions (fraction 9 and 10) of the sucrose gradient after the ultra centrifugation for 2 h. There was no detectable TatB in the pellet. With the PC liposomes in the reaction mixture, the nascently translated TatB was expected to be inserted into the bilayer membrane of the liposome during the translation (Klammt et al. 2006, Nozawa 2007). After sucrose gradient ultra centrifugation, a white liposome layer in the upper gradient was visible. Correspondingly to this observation, the intensity of the TatB signals decreased in the fraction 9 and 10 of the reaction with the liposomes. Instead, strong signals were observed in the upper two fractions (fraction 1 and 2). There was a weak signal of TatB detectable in the fraction 3 and negligible in the fraction 4-8.

The protein content was determined by measuring the signal intensity of the TatB. The total intensity from all fractions was set to be 100%. The percentage of each intensity was then plotted against the fraction number and shown in Figure 2.15B. In the reaction with liposomes, approximate 60% of the synthesized TatB were in the fractions 1 and 2, while less than 30% of the TatB remained in the bottom fractions 9 and 10.

The liposome content was measured by Zetasizer. The Kcount number (represent a measurement for liposome content) was plotted against the fractions as shown in Figure 2.15C. The liposome peaks in the two upper fractions (1 and 2) was detectable in the reaction with the PC liposomes. Otherwise, there were no peaks in the fractions 1-9. The peaks in the fraction 10 appeared in both cases, in the absence of liposomes the signal originated largely from TatB aggregates, while in the presence of liposomes representing protein aggregates and lipid aggregates. Differently, the fraction 9 (Figure 2.15B) of the control experiment contained mostly soluble TatB because low KCounts number (Figure 2.15C) was measured with Zetasizer in this fraction.

According to the results shown in Figure 2.15B and C, the TatB content was consistent to the liposome content. The synthesized TatB and the liposomes both accumulated in the first two upper fractions. These facts give a strong indication that the TatB signals in the first two fractions were due to the formation of the TatB proteoliposomes. There were also small amounts of TatB detected in the fraction 3 and 4. Altogether more than 70% of the total translated TatB were inserted into the liposomes by this cotranslational reconstitution method.

Besides the high efficiency of the reconstitution, the purity of the TatB protein in the upper fraction has to be examined. For this purpose, a gel after the SDS-PAGE was stained. To do that, an aliquot of 9 µl from each fraction was incubated with 3 µl four-fold Laemmli sample buffer and loaded in two 15% SDS-PAA gels. After electrophoresis the two gels were fixed and stained in the coomassie colloidal solution. The results of the SDS-PAGE are shown in Figure 2.16.

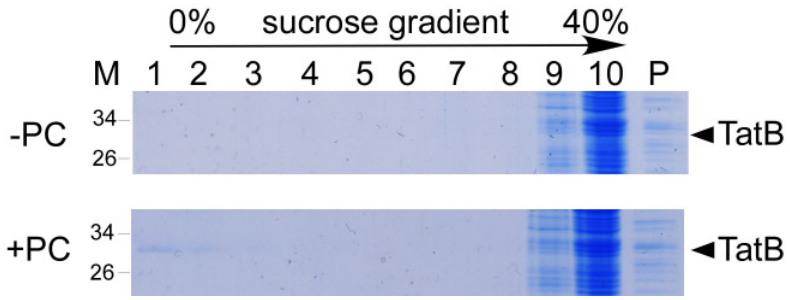


Figure 2.16. SDS-PAGE analysis of the fractions obtained after the sucrose gradient floating with the coomassie colloidal staining. M: protein marker; P: pellet; **+/-**PC: with / without addition of the PC liposomes in the reaction mixture

Figure 2.16 shows that proteins in the reaction without addition of the PC liposomes accumulated in the last two fractions and in the pellet. These proteins originated from the RTS kit and the synthesized TatB. Without the liposomes, all protein remained in the bottom fractions and could not "float" into the upper fractions of the sucrose gradient during the ultra centrifugation. In contrast, there were two bands with the molecular weight of about 29 kDa (the same as TatB) appeared in the first two fractions in the reaction with the PC liposomes. Compared to the bottom fractions, the first two fractions were rather pure.

The results shown in Figure 2.15 and 2.16 illustrates that the TatB signals in the upper fractions originated from the TatB proteoliposomes. Additionally, it was demonstrated that the floating technique can isolate and purify the proteoliposomes simultaneously. With the RTS and the following floating technique, the expression, the purification, and the reconstitution of TatB can be performed in one simple step. With the cotranslational reconstitution, the problems with the instability of the recombinant proteins during the

tedious purification steps, which occurred by the overexpression in *E. coli* system, can be avoided.

2.3.3.2 Detergent screening of translated TatC in RTS

Within the *E. coli* system, the overexpressed TatC was insoluble and unstable, which impedes its reconstitution into liposomes. Based on the results with the TatB, the cell free translation system based on the eukaryotic Wheat Germ extract may provide a new route to produce soluble TatC and reconstitute it into liposomes.

With its six transmembrane helices, TatC may tend more likely to aggregate, which can still complicate its reconstitution in the cell free system. Therefore, the synthesis of the TatC in the cell free translation system was performed with addition of different detergents into the reaction mixture.

In order to find out under which conditions the translated TatC was soluble, two mild detergents were tested: Triton X-100 and Brij35. Triton and Brij35 are mild nonionic detergents which were usually used in the solubilization of membrane proteins. Two translation reactions were performed with addition of the detergents. An additional reaction without detergent was used as the control for comparison. For the translation, 2 µg tatCpIVEX 1.3 WG plasmid and Triton X-100 or Brij35 with a final concentration of 0.2% (w/v) and 0.1% (w/v), respectively, were added to the reactions, which were premixed according to the manufacture's manual. The reactions ran at 24°C, 900 rpm for 24 h.

Subsequently, an aliquot of 1 µl was taken out from each of the three reactions, incubated with 9 µl 1 x SDS Laemmli sample buffer for 5 min at RT, and put on ice afterwards. Another aliquot of 1µl was diluted with 9 µl sterile dd. H_2O, ultra centrifugated with 150.000 g for 1 h. The supernatants were then carefully taken out and mixed with 3 µl fourfold concentrated SDS Laemmli sample buffer; the pellets were resuspended in 10 µl twofold concentrated SDS Laemmli sample buffer. All the samples were loaded to a 15% SDS gel for the electrophoresis. After the proteins were transferred to a PVDF membrane, the detection of the synthesized TatC was performed with polyclonal anti-TatC antibodies. Figure 2.17 shows the results of the immune detection.

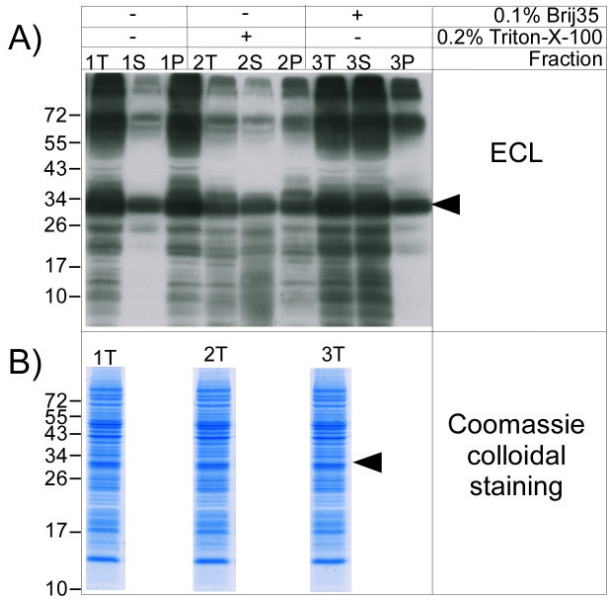


Figure 2.17: Solubilization test of cell free translated TatC with detergents Triton X-100 and Brij35. A) Western analysis. After the translation, 1 µl aliquot was incubated with 9 µl 1x Laemmli sample buffer (labeled as T), another 1 µl aliquot was added with 9 µl H_2O and ultra centrifugated to obtain a supernatant (S) and pellet (P), which were incubated with 3 µl 4x (or 9 µl 1x) Laemmli sample buffer and loaded together with T to the 15% SDS-PAA gel. The proteins were transferred to a PVDF membrane and detected with polyclonal anti-TatC antibodies; B) Coomassie colloidal staining. 1 µl aliquot from each translation was loaded to the 15% SDS-PAA gel, which was stained with coomassie colloidal solution. The black arrows point to the TatC proteins. T: total translation, S: supernatant, P: pellet.

Without the detergents, the TatC (MW 34 kDa, and apparent MW of approximately 29 kDa in the SDS-PAA gel.) was mostly found in the pellet fraction, only small amount in the supernatant. Upon addition of Triton X-100, the solubility of the synthesized TatC was increased and the distribution of TatC was almost equally in the supernatant and pellet fraction. However, the addition of Triton X-100 influenced the translation efficiency. Compared to the control reaction, the presence of Triton X-100 reduced the total amount of the synthesized TatC.

With the addition of Brij35, the translated TatC proteins largely accumulated in the supernatant fraction, indicating that the addition of Brij35 in the reaction mixture improved the solubility of the synthesized TatC protein. In contrast to Triton X-100, Brij35 showed no obvious effect on the translation efficiency. On account of these observations, Brij35 was chosen to assist the synthesis and the reconstitution of TatC in the cell free system.

In all three reactions, multiple signals of the synthesized TatC were observed. It may indicate the presence of the dimer- and multimer of the TatC. With the coomassie colloidal staining, the protein pattern of all three reactions is similar and there is no very apparent thick TatC band observed with the MW of 34 kDa.

2.3.3.3 Cotranslational reconstitution of TatC

There were two ideas to get soluble TatC proteins reconstituted into liposomes, carrying TatC in its functional form. One possibility was with the addition of liposomes to the translation reaction. It is possible that the nascently translated TatC can be inserted directly in the liposome bilayers in the neighborhood, before it aggregated. The second one was with both the detergent Brij35 and liposomes in the reaction: The detergent enables a prolonged solubility of the nascently translated TatC, which in turn improves the possibility that TatC can be inserted into the lipid bilayers.

For the synthesis of the TatC protein with the RTS, 2 µg tatCpIVEX1.3 WG plasmid, 10 µl 20 mg/ml PC liposomes (diameter: 200 nm), and Brij35 in different concentrations were added to the premixed translation reaction mixtures. As shown in the Figure 2.17, the addition of 0.1% (w/v) Brij35 did not influence the translation efficiency itself but increase the solubility of the translated TatC. So 0.1% (w/v) Brij35 was added to the translation reaction. One reaction without the Brij35 and another one with neither liposomes nor Brij35 were used as control translations for comparison.

The translation and the following floating were performed as described for TatB. After the sucrose gradient floating, the sucrose gradient was fractionated in ten fractions each of 200 µl and the pellet was resuspended in 200 µl 2 x SDS Laemmli sample buffer. Aliquots from all the fractions and pellets were loaded to a 15% SDS-PAA gel and analyzed with the subsequent immune detection.

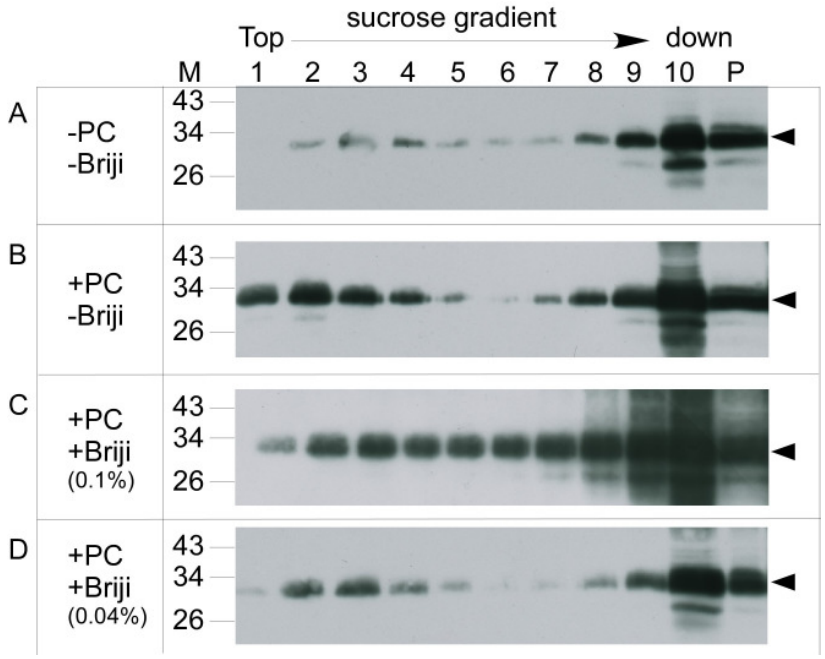


Figure 2.18: **Cotranslational reconstitution of TatC under different conditions.** After the floating of the translation reaction, the samples were fractionated and analyzed in the SDS PAGEs, which were then detected by the anti-TatC antibodies. A) the control translation in the absence of liposomes and detergent. B) the translation with liposomes only. C) the translation with liposomes and 0.1% Brij35. D) the translation with liposomes and 0.04% Brij35.

Similar as found for the cotranslational reconstitution of the TatB, the proteoliposomes with inserted TatC should be found in the upper layers of the sucrose gradient after the ultra centrifugation at 150.000 g for 2 h. In the control reaction without the liposomes and Brij35 (Figure 2.18A), there were only very weak TatC signals in the fraction 2-7. The fraction 1 showed no signal of the TatC. Without the liposomes, most of the synthesized TatC still remained at the bottom of the gradient.

With 4 mg/ml (final concentration) PC liposomes in the translation reaction, strong TatC signals are found in the fraction 1-4 from the upper layers of the gradient (Figure 2.18B). There were still lots of TatC in the fraction 10 and the pellet. Without the addition of the detergent, the nascently translated TatC was mainly insoluble and difficult to be inserted into the liposomes.

To increase the solubility of the TatC proteins for the reconstitution, the detergent Brij35 was added to the translation reactions. According to the results of the detergent test, Brij35 with final concentration of 0.1% (w/v) was added to the translation (Figure

2.18C). The total amount of soluble TatC in fractions 1-9 was considerably increased compared to the amount of soluble TatC in fractions (1-4 and 7-9) in Figure 2.18B, which indicated the increased solubility of the synthesized TatC in presence of Brij35. However, the signals in the fractions 2-7 showed a fast equal distribution of TatC in these fractions and an unsuccessful separation of proteoliposomes was achieved. Only a trace of TatC could be detected in the proteoliposome representing fractions (fraction 1and 2, Figure 2.18B). Brij35 in 0.1% probably affected the liposomes and their "floating" behaviour along the gradient. To exclude this possibility, the concentration of Brij35 was reduced to 0.04%. With the reduced concentration of Brij35, an apparent separation of TatC could be observed between the upper and the bottom fraction with effectively reduced signals in the fraction 6 and 7 (Figure 2.18D). However, the solubility of TatC and the reconstitution efficiency were not increased as expected. To optimize the cotranslational reconstitution of TatC, perhaps a concentration of Brij35 between 0.1% and 0.04% is under consideration.

To insert more TatC into the liposomes, the conditions of this cotranslational reconstitution method should be further optimized. Other detergents for solubilization of TatC can be tested and screened. Maybe TatC with its six transmembrane helices is not suitable to be inserted into the liposomes using this cotranslational reconstitution method. Other reconstitution methods should be undertaken, such as the detergent-mediated method, in Figure 2.13B the cell free translated TatC seemed to be inserted in the liposomes to some extent, however, further optimizations are necessary.

3. Discussion

3.1. Overexpression and purification of TatB and TatC

Unlike soluble proteins, heterologous overexpression of membrane proteins is more complicated in traditional expression systems, such as *E. coli*. Due to the hydrophobic nature of membrane proteins, which normally need a membrane system to stabilize their folded or active form, heterologous overexpression of membrane proteins, especially of helical bundle membrane proteins, often encounter problems such as low expression level, instability, incorrect folding, and difficulty of refolding (Kiefer, 2003; Wagner *et al.*, 2006).

Compared with $TatC_{His6}$, the overexpression of $TatB_{Strep}$ was more successful, with approximately 50% of the overexpressed TatB found as soluble protein in the cytosolic fraction. In contrast, the majority of the overexpressed TatC was found in the membrane- and IBs-fraction. This difference may result from the different size and hydrophobicity of TatB and TatC. TatB with one single transmembrane helix is less hydrophobic and becomes more easily expressed as soluble protein in *E. coli* than TatC that carries six putative transmembrane helices. It is reasonable, that the more hydrophobic TatC needs to be inserted into a membrane after overexpression, otherwise it tends to form insoluble IBs.

The soluble fraction of TatB obtained from the bacterial cytosol was efficiently purified using affinity chromatography, RP-HPLC or 2D-electrophoresis. The analysis with the MALDI-TOF mass spectroscopy confirmed the identity of the purified TatB protein. Interestingly, the behaviour of TatB in the double SDS-PAGE deviated from the behaviour that was expected for a typical membrane protein. Normally, hydrophobic membrane proteins migrate faster in low crosslinked gel systems, and, following a standard SDS-PAGE according to Laemmli (Laemmli, 1970) in the second dimension, the spots for membrane proteins are found above the diagonal line formed by the various protein spots. However, in our case, the TatB was found below the diagonal line formed by the contaminating proteins. This paradoxal behaviour of TatB cannot be

explained so far, but it is in line with some other exceptional properties observed for this protein. For example, by analysing thylakoid membrane proteins in RP-HPLC, TatB with its single transmembrane helix elutes later from the column than proteins of the light harvesting complex carrying three or more putative transmembrane spans (Mehnert, 2006).

A detergent screening was performed to find suitable conditions for solubilization of $TatC_{His6}$ from membrane fractions. Among 12 detergents tested, the best solubility was achieved with 2% LS. The amount of solubilized $TatC_{His6}$ was almost the same as that in the reference sample containing the protein solubilized in Laemmli sample buffer. Furthermore, dimers and oligomers of the $TatC_{His6}$ protein were detectable. This phenomenon always was observed when the concentration of $TatC_{His6}$ exceeded a certain value. Unfortunately, it was not possible to prevent this by adding higher concentrations of urea (up to 6M) and/or reducing agents like ß-mercaptoethanol (up to 30%) into the Laemmli sample buffer.

Triton X-100, Digitonin and DM showed similar solubilization properties for $TatC_{His6}$, but were less efficient than LS. However, it should be noticed that these three detergents are much milder and used more frequently in membrane protein studies. Digitonin, which is commonly considered as the mildest detergent so far, does not destroy the protein complex structure and is often used in native gel systems for protein complex studies. Triton X-100 and DM are likewise nonionic detergents, which are widely used in the membrane protein solubilization. Additionally, Triton X-100 is also often applied in protocols for the detergent-mediated reconstitution of membrane proteins into liposomes (Paternostre *et al.*, 1988; Rigaud *et al.*, 1995; Seddon *et al.*, 2004). For a final choice of a detergent from these four candidates, not only the efficiency in membrane protein solubilization but also its compatibility with the subsequent steps like protein purification and reconstitution has to be considered.

After purification of $TatC_{His6}$ solubilized from the IBs-fraction with Ni-NTA affinity chromatography, the protein was detectable by Western analysis, but not as the distinct band in Coomassie stained SDS-PAA gels (Figure 2.3). Therefore, the overall quantity of $TatC_{His6}$ recovered from IBs was low and the majority of proteins obtained in the elution fractions from Ni-NTA affinity chromatography were contaminants. Nevertheless, these small amounts of $TatC_{His6}$ can be further enriched and purified from other contaminants with RP-HPLC (Figure 2.4).

Another problem was observed regarding the stability of the overexpressed TatC proteins, which were only stable for a short time span. After solubilization and purification the proteins were stored either at 4°C or frozen at -20°C. However, in both cases the amount of $TatC_{His6}$ still dissolved in the buffer decreased considerably after one week. Obviously this effect was not caused by contaminating proteases because the addition of a cocktail of protease inhibitors did not improve the stability of the protein. More likely, the very hydrophobic $TatC_{His6}$ irreversibly adsorbs to the hydrophobic surface of the polypropylene tubes. Consequently, the losses were somewhat reduced when the protein was stored in glass vessels.

3.2. Liposome preparation

As the platform of reconstitution experiments, liposomes are important tools for successful insertion of desired membrane proteins into lipid bilayers. Liposome preparation by hydration of a thin lipid film was the first method described in detail (Bangham *et al.*, 1965) and is still the most widely used method in literature. In this thesis liposomes with desired size and composition were prepared with this method, followed by sequential extrusion through polycarbonate membranes with defined pore size.

Using this approach, spherical liposomes with a well-defined size distribution can be prepared easily with phosphatidyl choline (PC) as the single lipid component. The prepared PC liposomes were stable at least for two weeks at 4 °C. During storage of the liposome solution no change of turbidity or precipitation was observed. The shape and size of prepared PC-liposomes were analyzed using transmission electron microscopy (TEM) and Zetasizer measurements.

In order to imitate the authentic thylakoid membrane, liposomes were prepared containing 70% galactolipids (MGDG and DGDG) and 30% PC. An interesting observation was that such liposomes with a high content of galactolipids did not show a spherical shape, but formed stacked tubular structures. The TEM analysis also revealed similar structures when isolated authentic thylakoid membranes of *Arabidopsis thaliana*, which contain about 77% galactolipids, were analyzed. We assume that the high amount of MGDG causes this unusual structure. It was reported that MGDG alone cannot form bilayers due to its cone-shaped molecular structure, but its presence influences the membrane curvature (McIntosh and Simon, 2006) and together with

DGDG regulates membrane stability and flexibility (Lavalmartin and Troton, 1990; Garab and Mustardy, 1999). It is also known that the lipid composition influences the size and the shape of formed liposomes (Szoka and Papahadjopoulos, 1980). The observed similarities between the authentic thylakoid membrane structure and the liposomes containing a high content of galactolipids may refer to a natural property of such galactolipid-liposomes to form structures with such an unusual morphology.
However, it must be noted that in the course of TEM analysis the sample of the liposomes has to be dried on the carbon coated TEM grids before doing the negative staining. Since the morphology of the liposomes may alter during this drying process, other microscopic methods, such as freeze fracture electron microscopy, should be used for comparison in order to confirm or verify the results on the morphology of prepared liposomes.

Due to the easy preparation of spherical PC-liposomes, which proved to be convenient for purification and analysis of membrane proteins, the initial reconstitution experiments were done with this kind of liposomes. However, it must be considered that the size and the shape of liposomes can affect the efficiency of reconstitution of specific membrane proteins (Eytan, 1982). There are also reports about the influence of lipid composition on the activities of reconstituted enzymes (Magdalou *et al.,* 1982; Zakim and Edmondson, 1982). Especially, MGDG is reported to be important for the function of proteins inserted into liposomes as shown for the light-harvesting complex of photosystem II (LHCII) from the thylakoid membrane (Kruijff, 1997; Bruce, 1998, Zhou *et al.*, 2009; Schaller *et al.*, 2010). Therefore, galactolipid-liposomes should be included into the reconstitution studies in order to address the question if their specific morphology, which needs further investigation and verification, might be important for the activity of reconstituted Tat-complex.

3.3. Reconstitution of Tat proteins through three methods

In order to study the mechanism of Tat transport pathway, the purified recombinant Tat subunits were inserted into artificial liposomes, which can provide a native membrane similar environment that offers a tool to analyze many properties of membrane proteins, for example, the interaction between proteins, the topology of proteins in membrane

bilayers and substrate binding. Tat proteins were inserted either separately or together with other subunits into liposomes. There are many methods to insert membrane proteins into liposomes. As already described in the introduction part, the mostly frequently used methods can be divided into three groups: the spontaneous, the detergent-mediated and the cotranslational reconstitution. All the three methods were intensively tested with Tat subunits, and the results of the reconstitution experiments will be discussed here.

3.3.1. Spontaneous insertion

Since the spontaneous insertion of proteins into preformed liposomes is the most simple and easy method, the reconstitution experiments of the three Tat proteins (TatA, TatB and TatC) were started with this approach. The Tat proteins were inserted either separately or together in different combinations (Figure 2.8) into liposomes, which were prepared in this experiment in composition and molar ratios of the components according to the five most abundant thylakoid lipids. The three recombinant Tat proteins were purified under denaturing condition before.

The best insertion with this method was observed for TatA, which was completely pelletized with liposomes. Due to the previously discussed instability of TatB and TatC, the process of aggregation of these two proteins occured before or during the insertion process. The aggregates were pelletized by the first centrifugation step and discarded. Therefore, there were no detectable signals during the separate insertion of TatB and TatC. However, when TatB was incubated together with TatC, there were signals of TatB in the vesicle fraction. This could be an indication of a better and extended stability of TatB in the presence of TatC, which facilitated the insertion of TatB.

Possible improvements to insert TatB and TatC using this method could be presonication of liposomes prior to the addition of proteins or addition of “impurities” into the liposome preparation (e.g. cholesterol). These treatments are reported to enhance the protein insertion by inducing packing defects in the lipid bilayer (Scotto and Zakim, 1986; Scotto *et al.*, 1987). Scotto *et al.* (1987) also reported that preinserted proteins enhanced the subsequent spontaneous insertions of other proteins. Since TatA is easily reconstituted with this method, liposomes with preinserted TatA might be purified and tested for subsequent insertion of TatB and TatC.

Besides the above-mentioned possibilities, there are many other parameters that should be tested and verified, in order to optimize the conditions of this "simple" reconstitution method. For example, the initial lipid/protein ratio, the liposome sizes, the incubation temperature, and the lipid composition of liposomes are all important for an efficient reconstitution. It was reported that higher temperatures than 4°C improved the protein insertion (Knol *et al.*, 1996), probably due to the enhanced fluidity of the lipid bilayer at higher temperatures (Hjelmeland, 1980). Although the spontaneous insertion was not widely used in recent years, a modified method has been still applied in a few works. For example, Zhou *et al.* (2009) reconstituted components of LHCII with freeze-thaw treatments of the protein/liposome mixture, followed by sonication. Those modified procedures can also be considered for the reconstitution of Tat proteins. Our previous work showed that TatB could also be efficiently reconstituted into five-lipids liposomes under varied conditions, e.g. overnight incubation at 25°C (Li, Diplom Thesis).

In order to provide a bilayer environment for the proteins that resembles the natural situation, five-lipids liposomes were used for this reconstitution. According to the discussion on the liposome preparation, the PC-liposomes and the galactolipid-liposomes may have different effects on the insertion efficiency. It is worth to perform spontaneous reconstitution of Tat proteins also with the PC-liposomes and compare the results with that of the five-lipids liposomes, which may gain useful basic information for other reconstitution methods and for the activity and functionality tests in later experiments.

3.3.2. Detergent-mediated reconstitution

Dialysis:

Since the high CMC of octyl-D-glucopyranoside (OGP) facilitates its removal from a lipid-membrane protein-detergent mixture by dialysis (Schwarz *et al.*, 1984) and due to the fact that there are several successful reconstitutions of proteins with OGP (Rigaud *et al.*, 1988; Parmar *et al.*, 1997; Angrand *et al.*, 1997), this detergent was used for the reconstitution of TatB and TatC.

With this method TatB was apparently better reconstituted than TatC. A strong signal was detected in the vesicle fraction, which was not the case in control assays without lipids. Although there was considerable amount TatB in the vesicle fraction, it was also still detectable in the last washing fraction, which makes it difficult to exclude the

possibility that some TatB just sticked on the surfaces of the liposomes, rather than inserted into the lipid bilayer.

In contrast to TatB, only a small part of TatC appeared in the vesicle fraction. The reasons for the much less efficient insertion of TatC might be its larger size, its much higher hydrophobicity and instability. In addition, according to the results of the detergent screening for the solubilization of overexpressed TatC from the membrane fraction, OGP is not a suitable detergent for solubilization of TatC. Insufficient solubilization of TatC might be another highly possible reason for its poor reconstitution.

In our first attempts to use dialysis for reconstitution, the detergent-protein mixture was used to rehydrate a lipid film. This procedure deviates from the widely used protocol, in that a detergent is added to a solution of preformed liposomes to solubilize them, followed by addition of membrane proteins to the detergent-lipid-solution, and subsequent detergent removal (Levy *et al.*, 1992; Angrand *et al.*, 1997). The use of buffered protein-detergent mixtures to rehydrate a lipid film, as done in our experiments, may facilitate, at least to some extent, the spontaneous formation of liposomes (Parmar *et al.*, 1999). However, such rapidly formed liposomes may encapsulate some protein- or detergent-molecules, which will impede the removal of the detergent and the insertion of the proteins. The encapsulated protein molecules will also pelletized with liposomes and interfere the interpretation of the signals in the vesicle fraction. Generally, preformed liposomes may be more suitable for future insertion of membrane proteins because it has been observed that proteins tend to have a more homogeneous orientation when reconstituted with preformed liposomes rather than reconstituted from a suspension of mixed micelles (Eytan, 1982; Rigaud *et al.*, 1995).

Bio-beads:

Detergents with a relative low CMC like Triton X-100 (CMC ≈ 0.02% (w/v)) cannot be efficiently removed from the protein-lipid-detergent mixture by dialysis. In addition, it has been reported that a slow removal of the detergent favors protein denaturation and/or self-aggregation and thus reduces the reconstitution efficiency (Levy *et al.*, 1992; Parmar *et al.*, 1999). Therefore, removal of detergents with Bio-beads through physical adsorption was tried for the reconstitution. According to the literature, Bio-beads are capable of removing all kinds of detergents (Rigaud *et al.*, 1995) and have minimal adsorption capacity towards lipids and proteins (Holloway, 1973; Levy *et al.*, 1990).

The non-ionic detergent Triton-X 100 was chosen to solubilize the preformed PC-liposomes, since it is a relatively mild detergent and widely used for reconstitution of membrane proteins (Seddon *et al*., 2004). More important, it showed a good ability to solubilize overexpressed TatC, which may help to improve the so far unsatisfying reconstitution results of TatC.

The solubilization of PC-liposomes with Triton X-100 was controlled by turbidity measurements on a spectrophotometer and TEM studies. The TEM analysis revealed that the liposomes existed as partially open vesicles at a Triton X-100 concentration of 2 mg/ml (~ 0.2 % (w/v)). This behaviour corresponds to the proposed mechanism for solubilization and reformation of liposomes with Triton X-100, which proceeds through an open vesicular intermediate (Stuart and Boekema, 2007).

The reconstitution procedure was carried out first with the most stable Tat subunit, TatA, which was reconstituted already successfully with the method of spontaneous insertion. The separation of the proteoliposomes from free TatA was performed with the sucrose gradient centrifugation applying a top-to-bottom migration direction. Only trace amounts of TatA were detectable in the pellet after sucrose density gradient centrifugation of the reconstitution sample, showing that most of the protein successfully inserted into the liposomes.

The pattern of the TatA-containing fractions changed in the presence of the liposomes, compared to a control experiment done in the absence of liposomes, showing that proteoliposomes were formed that migrated in the expected size range of the sucrose gradient. However, some small liposomes or proteoliposomes might retain together with free TatA in the upper fractions. Therefore, in the presence of liposomes the signals in the first two fractions could come from the free TatA or from some small proteoliposomes with inserted TatA, which makes a clear and reliable interpretation impossible.

Interestingly, with the method of protein insertion into partially opened vesicles the reconstitution of TatC, which did not work so far, showed some improvements when applying a TatC sample produced with the Wheat Germ cell-free translation system. In order to separate the uninserted proteins from the proteoliposomes and thus avoid their co-migration within the same fractions, a floating technique with a discontinuous sucrose gradient was performed as described by Brown and Rose (1992) (Figure 2.13A). Using the floating technique, soluble proteins and aggregates remain in bottom

fraction after the ultra centrifugation, while liposomes and proteoliposomes float to the upper part of the gradient with low density.

Without liposomes, TatC remained in the bottom part and only traces of it appeared in the upper fractions of lower density. The TatC protein obtained by *in vitro* translation with the cell-free system in the presence of Triton X-100 showed a greatly enhanced solubility and stability, the major part of TatC was found as soluble protein and did not aggregate in the pellet. The presence of trace amounts of TatC in the upper fractions may be due to residual lipid-particles originating from the cell-free translation system, which might be tightly bound to the translated TatC. Analysis with the Zetasizer proved the existence of liposome-like particles in the original reaction solution of the cell-free system. According to Brown and Rose (1992), such protein-lipid components are generally able to float to the lower sucrose density gradient during centrifugation.

The amount of TatC in the upper part of the sucrose density gradient increased in the presence of the liposomes. However, a sharp separation of the putative proteoliposomes from uninserted protein was not achieved. One possible reason is the heterogeneous size distribution of liposomes. There are many indications of size change of liposomes during the process of detergent removal, depending on the temperature, the detergent, and the speed of detergent subtraction (Rigaud *et al.*, 1995; Walde and Ichikawa, 2001). Even in the presence of liposomes, there was still a considerable amount of soluble TatC remaining in the bottom of the gradient. In further experiments it should become examined if an increase of the liposome concentration or a decrease of the TatC concentration can improve the reconstitution.

Altogether, it is possible to reconstitute all three Tat proteins using the detergent-mediated method. Especially for TatB and TatC, the detergent-mediated reconstitution is more suitable and promising. The solubility and stability of TatC was improved in the presence of Triton X-100, which is helpful for further reconstitution of TatC also with other methods. Since there are several parameters which are critical for a successful detergent-mediated reconstitution, such as the temperature, the initial lipid/protein/detergent ratio, and the rate of detergent removal, a lot of optimizations are required for each step and each protein. For example, higher temperature may enhance the insertion of protein and the removal rate of detergent, but also may facilitate the liposome fusion and lower the stability of protein (Rigaud *et al.*, 1995; Walde and Ichikawa, 2001). The rate of detergent removal influences the size of formed liposomes

(Levy *et al.* 1990). Large liposomes are formed by slow removal, whereas smaller ones formed by fast removal. The rate of detergent removal also plays an important role for the final protein orientation in the liposome and the membrane protein activity (Rigaud *et al.*, 1995). For example, a high activity of reconstituted Ca^{2+}-ATPase could be achieved only by rapid detergent removal (Levy *et al.*, 1992), while several other proteins preferred slower removal rates (Dierks and Krämer, 1988; Eytan, 1982; Knol *et al.*, 1996).

3.3.3. Cotranslational reconstitution

In this method, protein translation was performed in a Wheat Germ cell-free translation system. Compared with the heterologous overexpression in *E. coli*, the Wheat Germ extract used in this cell-free system provides an eukaryotic system of a higher plant, which is expected to be more compatible with the desired Tat subunits. In the presence of liposomes, the translated proteins need no more isolation and purification because they are expected to be inserted directly into liposomes during translation. Therefore, the reconstitution process and translation are carried out simultaneously. Since TatA was already successfully reconstituted with the previous methods, only TatB and TatC were reconstituted with this method.

Cotranslational reconstitution of TatB

In the cotranslational reconstitution of TatB (Figure 2.15A) the proteoliposomes appeared in the upper fractions after the purification with the sucrose floating technique. More than 60% of the translated TatB proteins were inserted into liposomes, and only small amounts of synthesized TatB retained in the bottom fractions either as free soluble TatB or TatB aggregates. In the control experiment without liposomes the translated TatB was mostly found in the bottom fractions. According to the band intensities in Western blots, the total amount of synthesized TatB in the presence of liposomes was apparently higher than that in absence of liposomes. The liposomes in the cell free translation system can probably stimulate the translation. Withdrawal of TatB into the liposomes prevents an accumulation in the soluble fraction which might precipitate when exceeding the limit of solubility. Such precipitated TatB in turn might disturb the functionality of the whole translation system and reduce the rate of protein synthesis.

In Western analysis (Figure 2.15A) the signal for the TatB proteins obtained from the bottom fractions of the sucrose gradient (fraction 9 and 10) splitted into three bands, this phenomenon was observed both with and without liposomes. The three bands were located closely to each other, and have molecular weights of 30 kDa, 29 kDa and 27 kDa, respectively. In contrast to this, TatB proteins in proteoliposomes (fractions 1 to 4, in the presence of liposomes) emerged as a single band with a molecular weight of 29 kDa. This phenomenon can perhaps be explained as follows. The translated TatB proteins have possibly three different conformations, which run differently in SDS-PAGE, resulting in the three bands in the bottom fractions. However, not all these three different TatB conformations were inserted into liposomes with equal efficiency, but only TatB with an apparent molecular weight of 29 kDa could be reconstituted efficiently. Perhaps this conformation benefits the protein-lipid interactions.

Besides separating the proteoliposomes from uninserted proteins, the sucrose floating technique can also simultaneously purify the proteoliposomes as shown in the gel stained with the coomasie colloidal dye (Figure 2.16). The single band of TatB proteoliposomes in fraction 1 indicated that the proteoliposomes were efficiently purified from the translation mix, which has numerous ingredients. The coomasie staining reveals also another advantage provided by the cotranslational reconstitution method: In contrast to the detergent-mediated reconstitution that relies on partially opened liposomes during insertion, preformed liposomes facilitated only the incorporation of membrane proteins in the bilayers but no other contaminating proteins can be included into the lumen of liposomes. Those contaminants are extremely difficult to be eliminated and will disturb the further analysis of proteoliposomes. Compared to the spontaneous reconstitution, which is carried out post-translationally and generally only suitable for small membrane proteins (e.g. TatA, Figure 2.8), this reconstitution method is performed cotranslationally and hence, an alternative for reconstitution of larger membrane proteins. The reconstitution of Connexin-43 with this method has demonstrated that an increase of liposome concentration during the translation enhances the insertion of the protein, while a post-translational increase resulted in the protein aggregation and precipitation (Moritani *et al.*, 2010). Therefore, the reconstitution with this method is rather cotranslational than post-translational. Although the detailed mechanism of the insertion is unclear, a chaperone-like function of liposomes was proposed, by which the stability and the reconstitution of proteins were possibly

enhanced by direct interactions of the liposomes with the nascent protein (Moritani *et al.*, 2010). Besides the factors for protein synthesis, many other factors are present in the extract-based cell-free systems. It has been observed that the Wheat Germ extracts (WGE) systems are capable of protein posphorylation through their endogenous kinase activities (Nakamura, 1993). Similarly, other factors and machineries may be also present, which facilitate the cotranslational or post-translational protein modifications, and even their insertion.

Although the reconstitution efficiency of TatB is rather high (~ 60%), there are still small amounts of TatB as free soluble protein in the bottom fraction (Figure 2.15, fraction 9). In the cotranslational experiment of TatB described here, the liposomes were probably already saturated. Therefore, in future experiments the concentration of liposomes in the cell free synthesis experiment could be raised to check if the insertion efficiency also increases. Additionally, in fraction 10 (Figure 2.15) were still TatB aggregates, which probably could be reduced by the addition of small amounts of detergents, which do not solubilize the liposomes.

Cotranslational reconstitution of TatC

Since small amounts of TatC produced with the cell-free system were reconstituted with the detergent-mediated method, we likewise tested its suitability for membrane insertion using the method of cotranslational reconstitution. The addition of detergents to the translational solution was intended to increase the solubility and stability of TatC. However, the added detergent must be compatible with the cell-free system and not impede the translation. Several studies have examined the compatibility of some frequently used detergents (Klammt *et al.*, 2005; Nozawa *et al.*, 2007). A good compatibility has been observed with Brij35 and several other detergents. Since Triton X-100 increased the solubility of TatC and facilitated its insertion in our previous studies using the detergent-mediated reconstitution, its influence on the cell-free translation of TatC was investigated too. Likewise, the influence of Brij35 on the translation of TatC was also tested.

A detergent concentration of 0.2% Triton X-100 decreased the translational efficiency of TatC. In contrast, 0.1% Brij35 even slightly enhanced the efficiency and increased the solubility significantly. Similar effects were observed for these two detergents

during the cell-free translation of the phosphoenolpyruvate/phosphate translocator 1 (PPT1) of *Arabidopsis thaliana* (Nozawa *et al.*, 2007).

In the absence of liposomes (Figure 2.18A) weak TatC bands appeared in the upper fractions (fractions 2-5) of the sucrose gradient after floating, similar as found in the detergent-mediated reconstitution of TatC translated in the Wheat Germ cell free system.

When preformed liposomes were added to the translation system, the amount of TatC in the upper fractions significantly increased (Figure 2.18B). There was a sharp separation of the Tat protein between the upper and the bottom fractions. The main amounts of proteoliposomes should be harvested in the first two upper fractions. Like with TatB, the total translation of the protein was enhanced in the presence of the liposomes. However, the efficiency of reconstitution of TatC was much lower than that of TatB under the same conditions. One possible reason is that TatC has a larger molecular weight and carries six transmembrane helices, instead of only one for TatB. Translated TatC proteins probably already form their secondary or tertiary structure before insertion, which hinders its efficient reconstitution. Another reason might be that, in contrast to TatB, the translated TatC protein was insoluble and precipitated easily after translation. If this is the reason lowering the efficiency of reconstitution, increasing the solubility of the translated protein should rise the reconstitution efficiency. In order to increase the solubility of TatC, small amounts of detergent Brij35 were added into the reactions (Figure 2.17A). The reconstitution of TatC after addition of Brij35 at two concentrations was not improved as expected.

Instead of the typical distribution pattern observed after the addition of liposomes, TatC signals were continuously distributed over almost all fractions in the presence of 0.1% Brij35 (Figure 2.18C). In this case, Brij35 has possibly disrupted the structure of liposomes, resulting in an abnormal floating behavior of the proteoliposomes. With reduced amounts of Brij35 (Figure 2.18D) the proteoliposomes were separated from uninserted proteins, however, only reduced concentrations of TatC were detected. This means, Brij35 affects generally the structure of liposomes. The influence of Brij35 on the structure of liposome should be analyzed with pure liposomes. Other compatible detergents should be also taken in consideration, such as Digitonin. Even Triton X-100 should not be excluded at this stage, since it shows good solubilization properties towards TatC. In this study only one concentration was tested, a lower concentration

might have less negative effects on the translation, but could preserve a still good solubility.

Besides the protein solubility, other factors influencing the efficiency of reconstitution should be studied in future. Like for TatB, there was still a lot of soluble and uninserted TatC in the bottom fractions of the sucrose gradient. Higher reconstitution efficiency may be achieved by increasing the liposome concentration (Moritani *et al.*, 2010). Moreover, since in the Tat transport pathway TatB and TatC proteins form a complex and act together as receptor, simultaneously expression and insertion of TatB and TatC as a complex may benefit the process of reconstitution.

With the survey of publications for membrane protein reconstitution, no single method, procedure, or strategy is likely to work equally well for all proteins. The protocol of reconstitution can only be optimized and standardized individually for one or, at least, one type of desired protein. The results described in this thesis cover a lot of methodical approaches. As already discussed in the sections above, many optimizations and adjustments are still required for each step, for example, from the protein production and its solubilization, from the composition of lipids for the liposome preparation, from choice of the detergent to its removal, and so on. Since a previous step will influence the subsequent procedure and all factors may have their effects on the final products, for the first attempts to reconstitute a protein, we intended to try all possible strategies at first to get a rough overview, instead to fix on one single method. Only by this way it is possible to find some parameters, which are critical and must be considered from the very beginning, for example, the choice of the detergent or the lipid composition for liposomes. In contrast, the factors gathered by optimizing of one step may be not compatible for the subsequent steps.

Based on the preliminary results of this work, several proposals are made in the following, which may be helpful for the future experiments:

1. Both the detergent-mediated and the cotranslational reconstitution are promising for TatB and TatC. The first one bases on a relative simple system and offers more choices towards possible compatible detergents. The latter one combines the expression and the reconstitution of proteins in one step, which is a great advantage for less stable proteins, such as TatC.

2. Technically, the detergent removal works better with the Bio-beads than with the dialysis, since the regulation of the removal speed is much more convenient. The sucrose floating is more suitable for the separation of the proteoliposomes, since the free protein, aggregates, and proteoliposomes will be theoretically separated in distinct fractions.
3. Instead of reconstitution of only one single Tat subunit, simultaneously expression and insertion of two or three Tat proteins may facilitate their reconstitution efficiency all together, since the protein-protein interactions could also influence protein conformation which affects in turn the protein-lipid interaction and consequently the insertion.

   There are indications that previous insertion of proteins into liposomes enhances the subsequent reconstitution of other membrane proteins (Rigaud *et al.*, 1995; Scotto *et al.*, 1987). Using proteoliposomes, instead of protein-free liposomes, may increase the reconstitution efficiency. For example, first, reconstitution of TatA into liposomes using the method of spontaneous insertion, and then using the formed TatA-liposomes for reconstitution of TatB and/or TatC with the cotranslational method could facilitate the reconstitution of the TatBC receptor complex or even of the whole translocase.
4. The structure of liposomes dependent on the lipid composition, e.g. as given for liposomes composed of a lipid mixture similar to that of the authentic thylakoid membrane, should be further investigated, and the possible role of this unique structure for the reconstitution of thylakoid membrane proteins should be elucidated.
5. Instead of small liposomes (< 1µm), giant unilamellar vesicles (GUV) with a diameter of 10-100µm may be used for the reconstitution, which, besides the methods used in this thesis, can be analyzed also with optical microscopic techniques, such as fluorescence microscopy (Nomura *et al.*, 2008; Varnier *et al.*, 2010). The reconstituted membrane protein, which is conjugated with a protein mark such as EGFP, can be visualized directly under fluorescence microscopy.

4. Materials and Methods

4.1. Materials

4.1.1. Chemicals

The chemicals used in this work were of analytical grade and purchased from the following companies:
Sigma-Aldrich (Deisenhofen), Roth (Karlsruhe), Serva Feinbiochemica (Heidelberg), Merck (Darmstadt), Fluka (Neu-Ulm). Millipore Corporation (Bedford, MA), Sigma-Aldrich (Muenchen), Lipid Products (England), Aventi Polar lipids (Germany).

4.1.2. Marker

Art	Name	Company
DNA standard	1kB DNA ladder	Invitrogen (Karlsruhe)
	Hyperladder I	Bioline (Luckenwalde)
Protein standard	SDS-7L	Sigma-Adrich (Muenchen)
	SDS-6B	Sigma-Adrich (Muenchen)
	Page Ruler^{TM}	Fermentas (St. Leon Rot)

4.1.3. Enzymes

Biological enzymes were purchased from: Roche Diagnostics GmbH (Mannheim), MBI Fermentas (Vilnius, Lithuania), New England Biolabs (Schwalgach), Stratagene (La Jolla, CA), Biomaster (Koeln), Promega (Mannheim) and USB (Cleveland, OH). Proteases and protease inhibitors were from Sigma.

4.1.4. Medium and buffer

Composition of various mediums

LB medium	peptone	1.0% (w/v)
pH 7.0	yeast extract	0.5% (w/v)
	NaCl	1.0% (w/v)
	+/- Bacto Agar	1.5% (w/v)
SOC medium	peptone	2.0% (w/v)
pH 7.0	yeast extract	0.5% (w/v)
	NaCl	10 mM
	KCl	2.5 mM
	$MgCl_2$	10 mM
	$MgSO_4$	10 mM
	Glucose	20 mM

Buffer solution used in this work

Reconstitution buffer	NaCl	100 mM
pH 7.8	Tris	10 mM

4.1.5. Antibodies

name		origin
α-TatA	first antibody, from rabbit	own lab
α-TatB	first antibody, from rabbit	own lab
α-TatC	first antibody, from rabbit	own lab
α- rabbit IgG-HRP	second antibody	Sigma-Aldrich (Muenchen)
α- rabbit IgG-AP	second antibody	Sigma-Aldrich (Muenchen)

The Tat antibodies were purified according to the protocol of Narhi *et al.* (1997).

4.1.6. Lipids

name	origin
PC	Sigma-Aldrich
MGDG	Lipid Products
DGDG	Lipid Products
PG	Lipid Products
SQDG	Lipid Products

4.1.7. Column matrix materials (resin) and empty columns

name	origin
Column matrix materials:	
Superdex® 75 PC 3.2/3.0	*Amersham Biosciences*
MiniQ® PC 3.2/3	*Amersham Biosciences*
Ni-Sepharose 6 Fast Flow (in 20% ethanol)	*Amersham Biosciences*
Strep-Tactin Superflow (50% Suspension)	IBA
empty columns:	
plastic disposable polystyrene columns (0.5 - 2 ml bed volumn)	Thermo Scientific
plastic disposable polystyrene columns (1 - 5 ml bed volumn)	Bio-Rad

4.1.8. Tat protein sequences for the overexpression and RTS

sequence of $TatA_{His6}$:

overexpression:

MCNALFGLGVPELAVIAGVAALLFGPKKLPEIGKSIGKTVKSFQQAAKEFESELKTEPEESVAESS
QVATSNKEEEKKTEVSSSSKENVHHHHHH (*His6-tag*)

RTS:

MCNALFGLGVPELAVIAGVAALLFGPKKLPEIGKSIGKTVKSFQQAAKEFESELKTEPEESVAESS
QVATSNKEEEKKTEVSSSSKENVGGSHHHHHH (linker + *His6-tag*)

sequence of TatB$_{Strep}$:

overexpression:

MASLFGVGAPEALVIGVVALLVFGPKGLAEVARNLGKTLRTFQPTIRELQDVSRDFKSTLEREIG
LDDISTPNVYNQNRTNPVQPPPPPPPPSVPSTEAPVTANDPNDSQSPKAYTSEDYLKFTEEQLKAL
SPAESQTEDQTQTQEPPQPTTVQTPTGESQPNGTARETTAASPPRQDWSHPQFEK (*Strep*-tag®II)

RTS:

MASLFGVGAPEALVIGVVALLVFGPKGLAEVARNLGKTLRTFQPTIRELQDVSRDFKSTLEREIG
LDDISTPNVYNQNRTNPVQPPPPPPPPSVPSTEAPVTANDPNDSQSPKAYTSEDYLKFTEEQLKAL
SPAESQTEDQTQTQEPPQPTTVQTPTGESQPNGTARETTAASPPRQDGGSHHHHHH (linker +
His6-tag)

sequence of TatC$_{His6}$:

overexpression:

MCPYAVTFCNSWREAGLRYSVTQRRSKGFGPVSALNDDDSPTETTPGVGSAVEDRPPDSSEDRS
SSVYEFLYPRKEELPDDKEMTIFDHLEELRERIFVSVLAVGAAILGCFAFSKDLIVFLEAPVKTQG
VRFLQLAPGEFFFTTLKVSGYCGLLLGSPVILYEIIAFVLPGLTRAERRFLGPIVFGSSLLFYAGLAF
SYWVLTPAALNFFVNYAEGVVESLWSIDQYFEFVLVLMFSTGLSFQVPVIQLLLGQVGVVSGDQ
MLSIWRYVVVGAVVAAAVVTPSTDPVTQMLLATPLLGLYLGGAWMVKLTGRHHHHHH (*His6-
tag*)

RTS:

MCPYAVTFCNSWREAGLRYSVTQRRSKGFGPVSALNDDDSPTETTPGVGSAVEDRPPDSSEDRS
SSVYEFLYPRKEELPDDKEMTIFDHLEELRERIFVSVLAVGAAILGCFAFSKDLIVFLEAPVKTQG
VRFLQLAPGEFFFTTLKVSGYCGLLLGSPVILYEIIAFVLPGLTRAERRFLGPIVFGSSLLFYAGLAF
SYWVLTPAALNFFVNYAEGVVESLWSIDQYFEFVLVLMFSTGLSFQVPVIQLLLGQVGVVSGDQ
MLSIWRYVVVGAVVAAAVVTPSTDPVTQMLLATPLLGLYLGGAWMVKLTGRGGSHHHHHH
(linker + *His6-tag*)

4.1.9. cDNA clones

clone	vector	orientation	restriction enzymes	literature
mature tatA	pBW	T7 → T3	NdeI / BamHI	Wilms (2000)

mature tatB	pBW	T7 → T3	NdeI / BamHI	Wilms (2000)
mature tatC	pBW	T7 → T3	NdeI / BamHI	Wilms (2000)
mature tatA	pIVEX1.3 WG	T7 → T3	NcoI / SmaI	this work
mature tatB	pIVEX1.3 WG	T7 →T3	NcoI / SmaI	this work
mature tatC	pIVEX1.3 WG	T7 → T3	NcoI / SmaI	this work

4.1.10. Bacterial strains and vectors

Bacterial strains:		
	E.coli DH5α	Hanahan (1983)
	E.coli BL21 (DE3)	Studier and Moffatt (1986)
Vectors:		
	pBW	Wilms (2000)
	pIVEX1.3WG	(5 PRIME GmbH, Hamburg)

4.1.11. Oligonucleotides

for RTS:

name	sequence (5' → 3')
at_tatA NcoI fo	CAGCCATGGGCTGTAATGCTTTGTTTGGTC
at_tatA SmaI re	TACCCGGGTACATTCTCCTTTGAGCTTGAAG
at_tatB NcoI fo	AATCCATGGGCGCGTCTCTGTTTGGTGTTGG
at_tatB SmaI re	TACCCGGGATCTTGCCTTGGAGGAGATGCA
at_tatC NcoI fo	CCGCCATGGGCTGTCCTTACGCTGTAACTTTC
at_tatC SmaI re	ATCCCGGGCCGACCTGTGAGCTTGACCAT

for overexpression in *E.coli*:

name	sequence (5' → 3')
At-tatB-NdeI-F	TGATTCATATGTCTCTGTTTGGTGTTGGAGC
At-tatB-BamHI-R1	ATTTTGGATCCTTTAATCTTGCCTTGGAGGAGATG
At-tatC-NdeI-F	CAAAGCATATGAGTCCTTACGCTGTAACTTTCTGC
At-tatC- BamHI - R1	CAAGTGGATCCCCGACCTGTGAGCTTGACC

4.1.12. Reaction kits

name	origin
NucleoSpin®Extract	Machery-Nagel (Dueren)
NucleoSpin®Plasmid	Machery-Nagel (Dueren)
BigDye®Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems (Darmstadt)
TOPO TA Cloning®Kit	Invitrogen (Karlsruhe)
RTS 100 WG	5 PRIME (Hamburg)

4.2. Methods

4.2.1. Heterologeous overexpression of Tat proteins in *E.coli*

4.2.1.1. Analytical overexpression (5 ml scale)

Ten colonies grown on an LB agar plate containing 50 µg/ml ampiciline (Amp) were selected using sterile toothpicks, which were then thrown into ten tubes containing 5 ml LB medium with 50 µg/ml Amp (LB_{amp} medium) and one tube as control contained only LB_{amp} medium. All tubes were shaken at 37°C, 250 rpm overnight. Then new cultures were prepared by adding 50 µl overnight culture to 5 ml LB_{amp} medium, and the cultures were grown at 37°C, 250 rpm until OD_{600nm} of the cultures reached 0.5. 1 ml aliquot was taken out from each tube (minus fraction). The remaining cultures in the tubes were induced by addition of rhamnose in a final concentration of 0.2% (w/v) and incubated for further 3 h. Then 1 ml aliquot was taken out from each tube (plus fractions). All minus fractions and plus fractions were precipitated at 13000 rpm (tabletop centrifuge) for 1 min, the resultant pellets were resuspended in fourfold SDS Laemmli sample buffer, and analyzed in a 15% SDS-PAA gel. For TatA, the gel after electrophoresis was stained using the Zn-imidazole staining method. Since the amounts of overexpressed TatB and TatC are too low to yield distinct protein bands which can be distinguished from the bulk *E.coli* proteins by Zn-imidazole staining, the gels were applied further to Western analysis. Using Zn-imidazole staining method (TatA) or Western analysis (TatB and TatC), the cultures expressing Tat proteins were selected and used for the following preparative overexpression.

4.2.1.2. Preparative overexpression (500 ml scale)

500 ml LB medium was pre-warmed at 37°C and 0.5 ml 50 mg/ml Amp was added. A 2.5 ml aliquot from overnight culture was added into this LB_{amp} medium. The culture grew at 37°C, 250 rpm until OD_{600nm} reached 0.5, then the expression was induced with addition of rhamnose in a final concentration of 0.2% (w/v). After grewing further for 3

h, bacteria were spun down at 4000 rpm (Sorvall, ST-H750) for 20 min. The supernatant was discarded and the pellet was resuspended in 1xPBS.

4.2.1.3. French Press and fractionation through centrifugation

French press is used to mechanically disrupt cell walls by passing them through a narrow valve under high pressure. The press uses an external pump to drive a piston within a large metal cylinder containing liquid sample like cell culture. The sample is under high pressure and squeezed passing a needle valve. High pressure together with shear force destroys cell walls.

The bacterial pellet resuspended in 1xPBS was transferred into the metal cylinder of French Press and passed the valve at 1000 psi (69 bar). In order to get a complete disruption of cell walls, two cycles of French Press were carried out. Inclusion bodies (IBs) and cell debris were spun down at 10000 rpm, 4°C for 30 min (Sorvall, SL-50T), the resulting supernatant was further separated into the cytosol (supernatant)- and membrane-fraction (pellet) by ultra centrifugation at 28000 rpm for 1 h (Beckman SW28). Through these centrifugation steps the cell suspension was separated into three fractions: cytosol-, membrane-, and IBs-fraction.

4.2.2. Molecular biological methods

The following basic molecular methods were performed according to Sambrook *et al.* (1989): PCR, DNA restriction, ligation, agarose gel electrophoresis. Plasmid preparation, DNA extraction and DNA sequencing were done as described in the instruction manuals of the kits.

4.2.2.1. Preparation of electro-competent *E.coli* cells

Solutions: (ice cold)

LB medium (without antibiotics)	500 ml
sterile dd. H_2O	750 ml
10% (v/v) glycerol	16.5 ml

Procedure:

A DH5α bacterial stock was touched with a sterile toothpick, which was thrown into a tube containing 5 ml LB medium. The culture was grown at 37°C, 250 rpm overnight. The next day 500 ml LB medium was inoculated with 2.5 ml overnight culture, then the cells grew at 37 °C, 250 rpm and the OD_{600nm} was measured every 20 min till the value reached 0.4- 0.5. The cell culture was cooled in cold room for 10 min. The culture was divided equally into two flasks. The cells were sedimented through centrifugation at 4 °C, 6000 rpm (SLC-250T) for 15 min, and the cell-pellets were resuspended in 2 x 250 ml prechilled dd. H_2O, which were spun down at 4 °C, 6000 rpm (SLC-250T) for 15 min, and the pellets were resuspended with 2 x 125 ml prechilled dd. H_2O. The latter centrifugation step was repeated and the pellets were resuspended in 2 x 7.8 ml pre-chilled dd. H_2O containing 10% glycerol. The two 7.8 ml cell cultures were transferred to one 50 ml conical tube, then was spun down at 4°C, 6000 rpm (SL-50T), and the cell pellet was resuspended in 1 ml prechilled dd. H_2O containing 10% glycerol. Finally the prepared competent cells were dispensed in 25 prechilled eppendorf tubes of each 40 µl aliquot, then the tubes were snap frozen in liquid N_2 and stored at -80°C.

4.2.2.2. Transformation of *E.coli* cells through electroporation

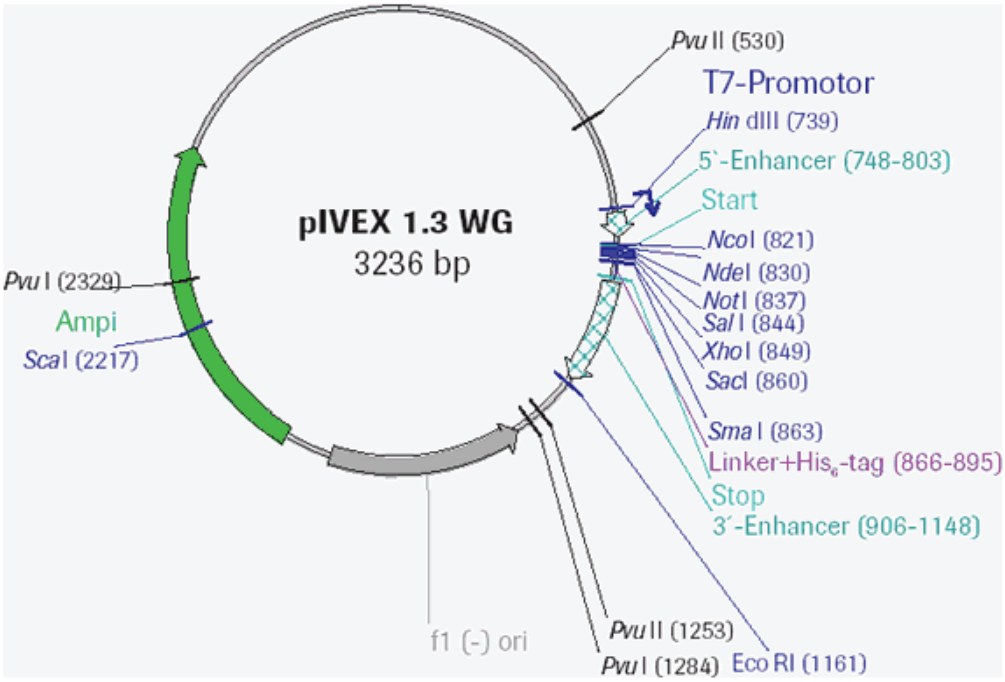
The electroporator "*E.coli* pulser" (Bio Rad) was set at 1.8 kV for DH5α cells. The transformation cuvettes (Biozym, Oldendorf) were placed on ice. The electro-competent cells were thawed on ice. 1-2 µl plasmid DNA was mixed with 40 µl electro-competent cells in the prechilled cuvette. The cuvette holder was slided into position and cells were zapped. After the transformation 1 ml SOC medium was added immediately to the cuvette and transformed cells were transferred to a 1.5 ml Eppendorf tube and incubated at 37°C with shaking (250 rpm) for 1 h. 100 µl transformed cells were placed onto a LB_{amp} agar plate. The remaining 900 µl were spun down in a tabletop centrifuge for 20 s and the pellet was resuspended in 200 µl SOC medium, which was all placed onto another LB_{amp} agar plate. The plates were incubated at 37°C until the colonies appeared.

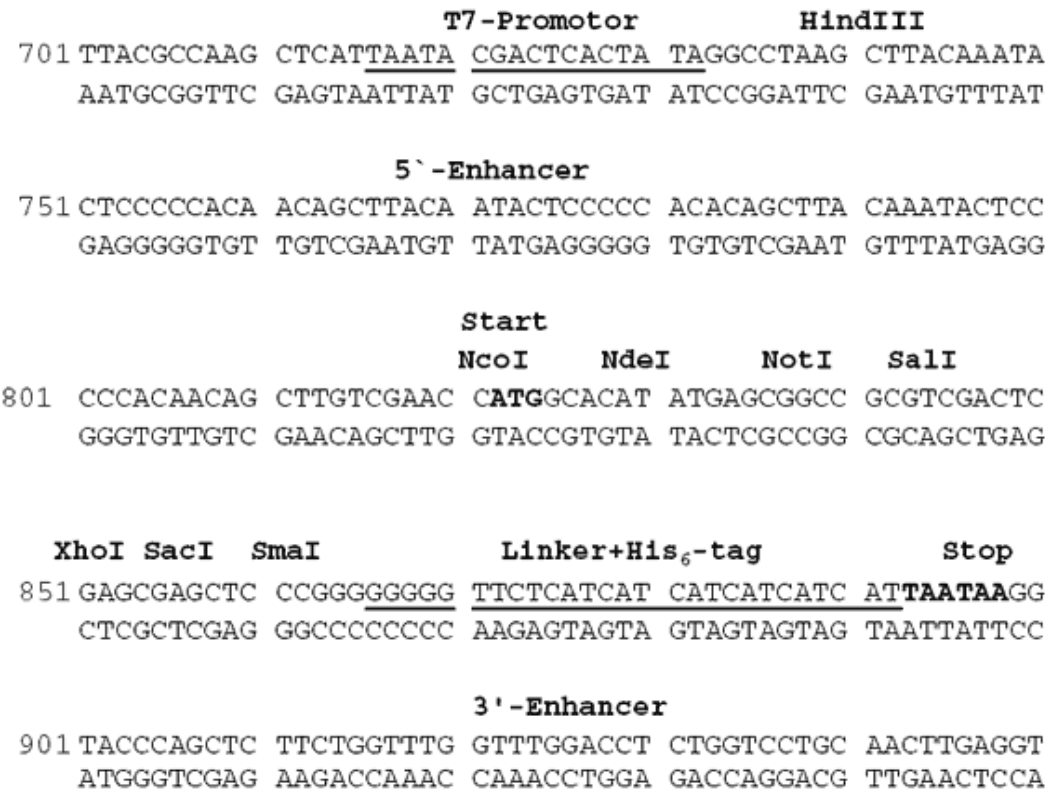
4.2.2.3. Cloning of tat genes into pIVEX1.3 WG (Wheat Germ) vector for RTS

The Wheat Germ RTS (rapid translation system) is a preparative scalable eukaryotic cell-free translation system. pIVEX WG vectors are designed for high-level expression

of eukaryotic proteins in the RTS Wheat Germ continuous exchange cell-free (CECF) system. The vectors contain all regulatory elements necessary for *in vitro* expression based on a combination of T7 RNA polymerase and wheat embryo lysate. Cloning into RTS pIVEX Wheat Germ vectors allows optimal protein expression in RTS 100 Wheat Germ CECF kits.

Map and sequence of vector **pIVEX1.3WG:**





Procedure of cloning:

The cloning of the mature tat cDNA into the pIVEX1.3WG vector is based on pBAT vectors containing the tat precursor cDNA prepared formerly in our laboratory. Using the precursor tatpBAT as template, the mature tat cDNAs were amplified by PCR using a primer pair containing NcoI and SmaI restriction sites. The PCR products were purified through the *NucleoSpin® extraction* kit according to the manufacturer instructions. The DNA was digested with NcoI and SmaI. In parallel pIVEX vector was also digested with the same restriction enzymes and dephosphorylated afterwards using CIP. The digested DNA and vector were loaded to an 1% (w/v) agarose gel. After electrophoresis DNA and vector were extracted from gel pieces with *NucleoSpin® Extract* kit according to the manufacturer introductions. Purified PCR products were ligated into pIVEX1.3WG vector. Finally pIVEX1.3WG vector with desired mature tat gene was transformed into *E.coli* cells through electroporation (details see section 4.2.2.2.).

4.2.2.4. Colony PCR

After transformation and growing on LB_{amp} agar plates, cells containing the plasmids with desired DNA were identified by colony PCR, which is a fast method to screen bacterial colonies. The selected colonies were touched with toothpicks, which were then dipped into PCR tubes containing all components except the DNA template. After PCR the amplified DNA was separated in a 1% (w/v) agarose gel, and colonies carrying plasmids with desired DNA were selected.

4.2.2.5. Sequencing

The sequencing reaction was carried out according to the procedure described in the BigDye® Terminator v3.1 Cycle Sequencing Kits. The sequencing was done in the Institute of Genetics (MLU - Halle).

4.2.2.6. Cell free translation of Tat proteins using RTS

The lyophilized reagents were resuspended in the reconstitution buffer supplied by the manufacturer and placed on ice before use. 50 µl reaction solution and 1 ml feeding solution were prepared in two Eppendorf tubes as described in the table below, then gently vortexed and set carefully into the two chambers of the RTS reaction device, finally the chambers were sealed and placed into a thermomixer.

Reaction solution (50 µl):

reaction mix	15 µl
amino acid mixture (without Met)	4 µl
methionine	1 µl
WG lysate	15 µl
tatpIVEX1.3WG plasmid	2 µg
H_2O	added to 50 µl

Feeding solution (1 ml):

feeding mix	900 µl

amino acid mixture (without Met)	80 µl
methionine	20 µl

The Wheat Germ RTS reaction ran at 24°C, 900 rpm for 24 h. After the reaction, the 50 µl translation reaction was carefully transferred into a new Eppendorf tube. Translated Tat proteins could be used for reconstitution experiments. In addition to the standard RTS protocol described here, liposomes and detergents could also be added into the translation reaction to optimize the translation of Tat proteins or to achieve better reconstitution results like cotranslational reconstitution in the presence of liposomes (see section 4.2.7.4)

4.2.3. Biochemical methods

4.2.3.1 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Solutions:

4x Laemmli sample buffer	0.25 M Tris/HCl, pH 6.8 8 % (w/v) SDS 40 % (w/v) glycerine 20 % (w/v) β-mercaptoethanol 0,016 % (w/v) bromine phenol blue
30% acrylamide solution	29.2 % (w/v) acrylamide 0.8 % (w/v) methylen bisacrylamide
SDS running buffer:	25 mM Tris 192 mM glycine 0.1 % SDS

In this work two kinds of gels were used: mini-gel (100 x 80 x 1 mm) and midi gel (180 x 190 x 1.5 mm).

Mini gel (100 x 80 x 1 mm):

stock solutions	running gel (15% AA)	stacking gel (5% AA)
H_2O	4 ml	3.6 ml
2 M Tris/HCl, pH 8.8	3 ml	-

1 M Tris/HCl, pH 6.8	-	0.625 µl
30 % acrylamide	7.5 ml	0.835 µl
10 % SDS	150 µl	50 µl
10 % (w/v) APS	50 µl	50 µl
TEMED	7.5 µl	5 µl
final volume	15 ml	5 ml

Midi gel (180 x 190 x 1.5 mm):

stock solutions	running gel (15% AA)	stacking gel (5% AA)
H_2O	14.8 ml	6.95 ml
2 M Tris/HCl, pH 8.8	9.4 ml	-
1 M Tris/HCl, pH 6.8	-	1.25 ml
30 % acrylamide	25 ml	1.67 ml
10 % SDS	500 µl	100 µl
10 % (w/v) APS	300 µl	80 µl
TEMED	30 µl	8 µl
final volume	50 ml	10 ml

"semi dry" Western blot

After the electrophoresis, the proteins were transferred to a PVDF membrane (Immobilon-P, *Millipore*) using the semi-dry blot equipment (*OWL Separation Systems*) according to the manufacturer instructions.

"enhanced chemiluminescence" (ECL) development

solutions:

10x PBS	750 mM NaCl 30 mM KCl 45 mM Na_2HPO_4 5 mM KH_2PO_4
washing solution I	1x PBS 0.1 % (v/v) tween 20 5 % (w/v) skim milk powder

washing solution II	1x PBS 0.1 % (v/v) Tween 20
ECL reagent I	2.5 mM luminol 400 µM *p*-coumarin acid 100 mM Tris/HCl pH 8.5
ECL reagent II	5.3 mM H_2O_2 100 mM Tris/HCl pH 8.5

Procedure:

The membrane was blocked in washing solution I at RT for 2 h, followed by decoration with the primary antibodies diluted in washing solution I (1:1000) at RT for 2 h (or overnight in cold room). Then the membrane was washed in washing solution I for 4 x 5 min. Now the membrane was incubated in horseradish peroxidase (HRP) conjugated secondary antibodies diluted in washing solution I (1:10000) for 2 h at RT. Then the membrane was washed in washing solution II for 4 x 5 min. The HRP development solution was freshly prepared by mixing equal volumes of ECL reagent I and ECL reagent II. (The following procedure was carried out in dark room.) After 1 min incubation in the HRP development solution, the membrane was covered in a transparent film and exposed to X-ray film in an ECL cassette. The exposure time varied between 5 s and 30 min depending on the protein amount on the film. Finally the X-ray film was developed in the ECL-developing- and the ECL-fixing solutions (Amersham). The film was dried and scanned for documentation.

Alkaline phosphatase (AP) development

Solutions:

10x PBS	750 mM NaCl 30 mM KCl 45 mM Na_2HPO_4 5 mM KH_2PO_4
washing solution I	1x PBS 0.1 % (v/v) tween 20 5 % (w/v) skim milk powder

washing solution II	1x PBS 0.1 % (v/v) Tween 20
washing solution III	0.1 M diethanolamine, pH 9.6 1 mM $MgCl_2$
Staining solution (0.125 ml per cm^2 membrane)	30 µL BCIP (stock solution: 50 mg/ml) 60 µL NBT (stock solution: 50 mg/ml) add dd. H_2O to 30 ml

Procedure:

The membrane was blocked in washing solution I at RT for 2 h, followed by an incubation with the primary antibodies diluted in washing solution I (1:1000) at RT for 2 h (or overnight in cold room). Then the membrane was washed in washing solution I for 4 x 10 min. Now the membrane was incubated in AP conjugated secondary antibodies diluted in washing solution I (1:30000) for 2 h at RT. Then the membrane was washed in washing solution II for in 2 x 10 min, followed by 2 x 10 min in washing solution III. Finally the membrane was incubated in staining solution for AP development supplied with BCIP and NBT until satisfactory signals were achieved. The development was terminated by rinsing the membrane in dd. H_2O. The membrane was scanned or dried for documentation.

4.2.3.2 Protein content estimation after Western analysis

After AP development (see section 4.2.3.1.) the PVDF membrane with protein bands was scanned and the image was saved as a TIFF file, which was then transformed to a black/white TIFF image with the program *PhotoShop*. The band intensities of the black/white TIFF image were quantified with the software *2D-Image master* (*Amersham Biosciences*).

4.2.3.3 Coomassie colloidal staining

This protein staining method was performed according to the protocol of Neuhoff *et al.* (1985).

Solutions:

solution A	2% *ortho* phosphoric acid 10% ammonium sulfate
solution B	5% Coomassie Brillant Blue G250
staining solution	98% solution A + 2% solution B (stirred at RT overnight) add 1 vol. methanol to 4 vol. staining solution before use
fixation solution (200 ml)	40% methanol 10% acetic acid

Procedure:

After the electrophoresis the SDS-PAA gel was incubated in the fixation solution at RT for 1 h, followed by incubation in the staining solution at RT overnight. The gel was destained by washing with water for several times. If the gel was overstained, it could be better destained with 20% methanol.

4.2.3.4 Zn-imidazole staining

Solutions:

Solution A	0.2 M imidazole 0.1% (w/v) SDS
Solution B	0.2 M $ZnSO_4$ in H_2O

Procedure:

This Zn-imidazole staining method was performed according to the protocol of Castellanos-Serra *et al.* (1999). The gel was rinsed in distilled H_2O for 30 s, then incubated in solution A for 20 min with gentle shaking. The gel was transferred to solution B and developed for 1-5 min until protein spots on the gel were well resolved over a black background. Finally the gel was rinsed again in distilled H_2O. The gel could be scanned and the protein spots could be cut out from the gel for protein elution.

4.2.3.5 TCA/DOC precipitation

Solution:

TCA/DOC solution	4 mg deoxycholic acid (DOC) 1 g trichloroacetic acid (TCA) add dd. H_2O to 1 ml
100% acetone (-20°C)	

Procedure:

1/10 volume of TCA/DOC solution was added to 1 volume protein solution, then the mixture was shortly vortexed and incubated on ice for 15 min. The precipitated proteins were spun down at 13000 rpm (tabletop centrifuge), 4°C for 10 min, and the supernatant was carefully taken out and discarded. The pellet was washed with acetone without mixing, then spun down at 13000 rpm, 4°C, 10 min again. The supernatant was discarded and the pellet was dried in SpeedVac for 15 min. The dried pellet was resuspended in twofold Laemmli sample buffer and loaded onto a 15% SDS-PAA gel.

4.2.4. Chromatography

4.2.4.1 Strep-Tactin affinity chromatography

buffers:

washing buffer pH 8.0	100 mM Tris-HCl 150 mM NaCl 1mM EDTA
elution buffer	2.5 mM desthiobiotin in washing buffer
regeneration buffer	1mM HABA in washing buffer

Procedure (performed in cold room):

- Degassing: All solutions were degassed.
- Assembly and pretreatment of the column: The empty plastic disposable column (bed volumn 0.5-2 ml, *Thermo scientific*) was assembled as following. The bottom cap was closed, the top cap was opened, and the column was flushed

with H_2O. In order to remove air from the end piece of the column the bottom cap was shortly opend and closed again. Then a porous polyethylene disc was pressed to the bottom end of the column. The *Strep-Tactin Superflow* (50% medium in 20% EtOH) was resuspended and poured into the column along a glass rod held against the column wall. The bottom cap was opened to let the medium sediment by gravity. After a bed height of 1 cm was reached (column volumes = 0.5 ml), the bottom cap was closed and the bed was covered with 2 cm H_2O. Then a second porous polyethylene disc was forced to the top of the gel bed without compressing the matrix.

- Washing: The column was washed with 5 CV H_2O to remove the EtOH from the column.
- Equilibration: The column was equilibrated with washing buffer for 1 h at a flow rate of 70 - 100 µl per min.
- Sample preparation: 30 ml cytosolic fraction of $TatB_{Strep}$ in PBS was ultra centrifuged at 150 000 g, 4°C for 1 h.
- Binding: The supernatant was loaded to the column with a flow rate of 70 - 100 µl per minute and the flow through was collected.
- Washing: Unspecificly bound proteins were removed with washing buffer for 1 h at a flow rate of 70 - 100 µl per min.
- Elution: The strep-tagged TatB proteins specifically bound to the column were eluted with the elution buffer. 5 x 1 ml elution fractions were collected.
- Regeneration: The used column was regenerated with 3 x 5 CV regeneration buffer. After equilibration with the washing buffer, the column was overlaid with 2 cm washing buffer and stored in refrigerator.

Aliquots from the flow through, the washing- and the elution-fractions were taken out and analysed by SDS-PAA gel electrophoresis.

4.2.4.2 Ni-NTA affinity chromatography

Buffer solutions:

binding buffer	20 mM HEPES
pH 7.5	0.5M NaCl
	20 mM Imidazole

washing buffer I pH 7.5	20 mM HEPES 0.5 M NaCl 20 mM imidazole 6 M GuaHCl
washing buffer II pH 7.5	20 mM HEPES 0.5 M NaCl 20 mM imidazole 8 M urea
elution buffer pH 7.5	20mM HEPES 0.5M NaCl 500mM imidazole 8 M urea

Procedure:

- Degassing: All solutions were degassed.
- Assembly and pretreatment of the column: The empty plastic disposable column (bed volumn 1-5 ml) was assembled as following. The bottom cap was closed, the top cap was opened, and the column was flushed with H_2O. In order to remove air from the end piece of the column the bottom cap was shortly opend and closed again. A porous polyethylene disc was already pre-installed at the bottom end of this kind of column. The *Ni-Sepharose 6 Fast Flow* (in 20% EtOH) was resuspended and poured into the column along a glass rod held against the column wall. The bottom cap was opened to let the medium sediment by gravity. After a bed height of 1 cm was reached (CV = 2 ml), the bottom cap was closed, the bed was covered with 2 cm H_2O and a porous polyethylene disc was forced to the top of the gel bed without compressing the matrix.
- Washing: The column was washed with 5 CV H_2O to remove the EtOH from the column.
- Equilibration: The column was equilibrated with 10 CV binding buffer and 10 CV washing buffer I.
- Sample preparation: The $TatC_{His6}$ obatined from the IBs fraction and dissolved in washing buffer I was ultra centrifuged at 150 000 g, 4 °C for 1h.

- Binding: The supernatant was loaded to the column and the flow through was collected.
- Washing: Unspecificly bound proteins were removed with 5 CV washing buffer I.
- Buffer exchange: The column was washed with 5 CV washing buffer II.
- Elution: $TatC_{His6}$ was eluted with 4 CV elution buffer. 4 x 2 ml elution fractions were collected.

Aliquots from the flow through, the washing- and the elution-fractions were taken out and analyzed by SDS-PAA gel electrophoresis.

4.2.4.3 RP-HPLC (*reversed phase* - HPLC)

Purification of $TatB_{Strep}$

After the purification through affinity chromatography, most of the $TatB_{Strep}$ proteins eluted in the fractions E2 and E3. Besides the $TatB_{Strep}$, there were still lots of other proteins in these fractions, which could be largely removed by RP-HPLC. The RP-HPLC was performed under the following conditions:

column	EC 250/10 Nucleosil 500-7 C3 PPN (Macherey-Nagel)
sample	2 ml (the sample was centrifuged at 13000 rpm, 4°C for 10 min to remove protein aggregates.)
eluent A	H_2O + 0.05% trifluoroacetic acid
eluent B	acetonitrile + 0.05% trifluoroacetic acid
flow rate	3 ml/min
gradient (in B%)	1-10 min with 10%, 10-11 min to 40%, 11-70 min to 70%, 70-71 min to 90%, 71-90 min with 90%
detection	220 nm

Purification of $TatC_{His6}$

To remove the contaminants in the elution fractions after the Ni-NTA affinity chromatography, the $TatC_{His6}$ containing sample was further purified with RP-HPLC. Since TatC is much more hydrophobic than TatB, a three-eluent system (instead of two-eluent) was used in the RP-HPLC procedure, which was carried out under the following conditions:

column	EC 125/4 Nucleosil 500-5 C3 PPN
sample	2 ml (the sample was centrifuged at 13000 rpm, 4°C for 10 min to remove protein aggregates.)
eluent A	H_2O + 0.05% trifluoroacetic acid
eluent B	acetonitrile + 0.05% trifluoroacetic acid
eluent C	100% *n*-propanol + 0.05% trifluoroacetic acid
flow rate	1 ml/min (CH_3CN/H_2O), 0.5 ml/min (*n*-propanol)
gradient (in B%)	0-6 min with 10% B, 6-7 min with 10-30% B, 7-37 min with 30-60% B, 37-45 min with 60-90%, 45-50 min with 90% B, 50-55 min with 90-0% B, 0-100% C, 55-70 min 100% C, 70-71 min 0-10% B 100-0% C, 71-90 min 10% B
detection	280 nm

The $TatB_{Strep}$ (or $TatC_{His6}$) containing fractions were dried through spin vacuum. The pellets were resuspended in 10 mM NaH_2PO_4 (pH 7.2) / 0.01% SDS. After 1 h vortexing at RT the protein solution was stored at -20°C ($TatB_{Strep}$) or 4°C ($TatC_{His6}$). All fractions were analyzed by SDS-PAA gel electrophoresis.

4.2.5. 2D-SDS-PAGE

Two-dimensional SDS-PAGE was used to further purify $TatB_{Strep}$ after Strep-Tactin affinity chromatography. An essential advantage of the 2D-SDS-PAGE is that hydrophobic proteins can be well separated from water-soluble proteins. Since the acrylamide concentration, urea content, and the trailing ion used for SDS-gels modify electrophoretic mobilities in a protein-dependent way, we coupled two SDS-gels to a 2D- SDS-PAGE: 10% acrylamide/Bis with 6 M urea Tris-tricine gel for 1-D SDS-PAGE (gel dimensions: 180 x 190 x 1mm), and 15% acrylamide/Bis Tris-glycin for 2-D SDS-PAGE (gel dimensions: 180 x 190 x 1.5 mm).

After the 1-D SDS PAGE, the whole protein lane was cut out and placed on the 2-D SDS PAGE. After electrophoresis and following Zn-imidazole staining, protein spots in 2-D SDS-gels appeared dispersed around a diagonal. The dispersing effect is explained in part by protein-dependent variation of electrophoretic mobilities presumably caused by altered SDS-binding, and by anomalous migration of highly hydrophobic proteins in gels with different acrylamide concentration (Rais *et al.*, 2004). Highly hydrophobic proteins migrate faster than water-soluble and moderately hydrophobic proteins with similar mass in low acrylamide gels, but approach normal electrophoretic mobility in high acrylamide gels.

4.2.6. Liposomes

4.2.6.1 Liposome preparation

Liposome preparation using EtOH injection method

The egg yolk phosphatidyl choline (PC, Sigma) was dissolved in organic solvent ($CHCl_3$:CH_3OH=2:1, v/v) with a final concentration of 100 mg/ml. 10 µl aliquot (equals approximately to 1 mg) was transferred into a glass flask and dried under N_2 atmosphere until a thin lipid film formed on the flask wall. The lipid film was resuspended in 80 µl ethanol (absolut). The lipid suspension was then slowly injected into 4 ml HEPES buffer (10 mM pH 8.0). The final ethanol concentration should not exceed 2 % (v/v), since a higher concentration of ethanol may cause protein precipitation in subsequent reconstitution steps. Liposomes (MLV) were formed during this EtOH injection process

and were harvested through centrifugation at 100 000 g, 4°C for 30 min. The liposome pellet was resuspended in reconstitution buffer.

Rapid reversed phase evaporation method for preparation of galactosyl liposomes

MGDG is the predominant lipid in native thylakoid membranes and prefers to form inverted micelles in aqueous environment, rather than bilayer vesicles. Using the previous hydration of a thin lipid film to transfer MGDG-containing lipid mixtures into an aqueous environment, the MGDG tends to form large clumps that stick strongly to the glass wall of the vessel. Therefore, it makes the final lipid concentration difficult to be estimated. With the following rapid RPEV method MGDG-containing lipid mixtures can be easily and efficiently transferred to an aqueous environment without loss on the glass wall of the vessel, because more bilayer structures were formed, compared to the hydration method. The method used in this work was modified according to Sprague *et al.* 1984.

Galactosyl lipids (*Lipids product*) dissolved in organic solvent ($CHCl_3$:CH_3OH = 2:1, v/v) were mixed to 10 mg/ml at a ratio (51% MGDG, 26% DGDG, 4% PC, 7% SL, 9% PG) roughly mimicing the natural lipid composition of the thylakoid membrane. The lipid mixture was dried under N_2 atmosphere until a thin lipid film formed on the flask wall. The flask was transferred on ice and 1 ml Freon 11 was added to the lipid film, followed by addition of 2 ml distilled H_2O. The ratio of Freon to water was 1:2 (v/v) in all experiments. The mixture was vortexed vigorously until an emulsion was formed. The flask was taken from ice and warmed at RT to remove Freon 11 from the emulsion.

Preparation of PC liposomes with hydration method

The egg yolk phosphatidylcholine (Sigma) was dissolved in organic solvent ($CHCl_3$:CH_3OH= 2:1, v/v) with a concentration of 100 mg/ml. 200 µl of the lipid solution (= 20 mg) were taken out and transferred to a round-bottomed flask. The solvent was evaporated in a rotary evaporator until a thin dry lipid film was formed on the flask, which was then pumped in vacuum overnight to remove the residual organic solvent. The dried lipid film was hydrated with 1 ml of reconstitution buffer and vortexed strongly till the lipid film was completely dissolved in the reconstitution buffer. To obtain unilamellar liposomes the hydrated lipid solution was extruded through two stacked polycarbonate membranes with a pore diameter of 200 nm for 19

times. The extruded liposomes can be stored at 4 °C. In this work the PC liposomes were diluted with reconstitution buffer to the working concentration of 4 mg/ml and always prepared freshly.

4.2.6.2 Liposome size determination through Zetasizer

Zetasizer is an instrument measuring the size of particles using the light scattering technique. In this work a Zetasizer 3000 HSA (Malvern) was used to measure the size of liposomes. Since optimal liposome concentration was approximately 0.001- 0.002 mg/ml, 1-2 µl aliquots from the 20 mg/ml liposome stock solution were diluted with the reconstitution buffer to 1 ml, transferred into a plastic vessel and analyzed with the Zetasizer 3000 HSA. The measurement was carried out at 25°C with a measure angle of 90° and a wavelength of 633 nm. The size distribution of liposomes was also measured by the Zetasizer 3000 HSA.

4.2.6.3 Liposome study under TEM (transmission electron microscopy)

The optimal concentration of liposomes for the TEM was about 1 mg/ml. A 5 µl aliquot was applied carefully onto a TEM-grid (Formvar/Carbon, Plano), and left it on for 5 min. Then the superfluous sample was drawn off from the edge of the grid with a filter paper and the grid was dried in a fume hood for 5 min. The grid was then negatively stained with 2 µl 1 % uranyl acetate solution for 30 seconds, which afterwards was drawn off from the edge of the grid with a filter paper. The grid was dried in a fume hood for 5 min. TEM analysis was carried out by L. Zhang at Max Planck Institute of Microstructure Physics.

4.2.7. Reconstitution

4.2.7.1 Spontaneous reconstitution

1 mg/ml galactosyl liposomes with a ratio of 51% MGDG, 26% DGDG, 4% PC, 7% SL, and 9% PG, which roughly mimics the natural lipid composition of the thylakoid membranes, were prepared according to the above described rapid RPEV method. Purified TatA, TatB and TatC were tested both separately and in combination as TatBC and TatABC for the insertion.

1 µg from each protein was added to 1 ml solution of the galactosyl liposomes, and the solution was shortly vortexed and incubated on ice for 30 min. Protein- and lipid-aggregates were removed by a centrifugation at 10000 g for 10 min. The centrifugation steps are shown in Figure 2.7. The supernatant of this low speed centrifugation contained uninserted free Tat proteins, empty liposomes, and the desired proteoliposomes. Transferring this supernatant to a new reaction tube, the proteoliposomes were pelletized by an ultra centrifugation of 100,000 g at 4 °C for 30 min. The proteoliposome pellet was then washed three times with each 100 µl reconstitution buffer by repeating resuspension and pelletizing. The final proteoliposome pellet was resuspended in 100 µl reconstitution buffer.

4.2.7.2 Detergent-mediated reconstitution I: detergent removal with dialysis method

Solutions:

Galactosyl lipids for liposome preparation

class	MGDG	DGDG	PC	SL	PG
stock (mg/ml)	10	10	100	10	10
volumn	510 µl	260 µl	4 µl	70 µl	90 µl

20% (w/v) OGP stock solution:	200 mg OGP add distilled H_2O to 1 ml.
detergent-protein solution:	 1 µg purified Tat proteins 50 µl 20% (w/v) OGP add reconstitution buffer to 1 ml
dialysis buffer solution:	2 x 1 L reconstitution buffer

Procedure:

Five lipids (total weight: 1 mg) in the ratio described in the above table were mixed together in a round bottomed flask. The lipid mixture was dried to a thin lipid film through rotational evaporation, and then the flask was set in vacuum overnight to remove residual organic solvent. The prepared detergent-protein solution was added

onto the thin lipid film, and the mixture was gently vortexed untill the lipid film was completely dissolved in the solution. Then the detergent-protein-lipid solution was transferred into dialysis tubing with a cut off of 3.5 kDa molecular weight. The tubing was sealed and put in a beaker containing 1 L reconstitution buffer. The dialysis was performed at 4 °C overnight, next day the buffer was replaced by new 1 L reconstitution buffer, and the dialysis was continued for at least four hours. After dialysis the protein-lipid solution was taken out of dialysis tubing and transferred to a 1.5 ml Eppendorf tube, which was centrifuged at 10 000 g, 4°C for 10 min to remove aggregates. The supernatant was applied to extruder and passed through two layers polycarbonate membranes with a pore diameter of 200 nm for 19 cycles to get unilamellar liposomes. Then the proteoliposomes were harvested by an ultra centrifugation at 100.000 g, 4 °C for 30 min, and the supernatant was kept for later SDS-PAGE analysis. The proteoliposome pellet was washed three times by repeating resuspension and ultra centrifugation steps. The washed proteoliposome pellet was resuspended in 100 µl reconstitution buffer. The supernantant, washing fractions, and the resuspended proteoliposome solution were analyzed with SDS-PAGE for the Tat proteins. The control experiments without liposomes were performed in an identical way.

4.2.7.3 Detergent-mediated reconstitution II: detergent-removal with Bio-Beads

Pretreatment of Bio-Beads SM-2*:*

40 ml methanol was added to 6 g Bio-Beads SM-2 (Bio-Rad). The mixture was stirred for 15 min and the beads were collected in a plastic chromatography column with a fine filter (Bio Rad) and washed with further 100 ml of methanol. The beads were not allowed to run dry by washing immediately with 600 ml of dd. H_2O. The moist beads were stored under dd. H_2O in the column until required (Holloway, 1973).

Procedure of reconstitution:

- *liposome preparation*: 1 ml 4 mg/ml PC liposomes with a diameter of 200 nm were prepared according to the standard protocol (hydration method, see section 4.2.6.1.).
- *solubilization of liposomes*: 20 µl 10% (2 mg) Triton X-100 was added to the 4 mg/ml PC liposome solution, followed by 1 h incubation at RT under

continuously stirring until the solubilization reaches an equilibrium state, at which the OD_{540nm} value of the solution becomes stable.

- *protein addition*: About 1 µg Tat proteins dissolved in reconstitution buffer with detergent (10 mM Tris, pH 7.8, 100 mM NaCl, 2% (w/v) Triton X-100) were added to the partially solubilized liposomes and incubated at RT for 30 min under continuously stirring.
- (The overexpressed $TatA_{His6}$ protein was dissolved in reconstitution buffer already containing 2% (w/v) Triton X-100, and added directly into the 4 mg/ml PC liposome solution. In this case, the two steps: *solubilization of liposomes* and *protein addition* were carried out simultaneously.)
- *detergent removal by Bio-Beads SM-2*: 80 mg Bio Beads prewashed with MeOH and dd. H_2O were added to the lipid-detergent-protein suspension and incubated for 2 h at RT on rotator, then the supernatant was incubated with a second portion of 80 mg Bio Beads and incubated at 4°C for 2 h to remove residual Triton X-100.
- *reformation of proteoliposomes*: Proteoliposomes were reformed during removal of Triton X-100, and the supernatant was transferred to a new 1.5 ml Eppendorf tube.
- *purification of proteoliposomes*: Purification of proteoliposomes was performed using discontinuous sucrose density centrifugation (for proteoliposomes of overexpressed $TatA_{His6}$) and by sucrose gradient "floating" technique (for proteoliposomes of RTS $TatC_{His6}$). Detailed procedures of those two techniques are described in the section 4.2.8.1 and 4.2.8.2. The purified proteoliposomes were analyzed subsequently with SDS-PAGE.

4.2.7.4 Cotranslational reconstitution

Solutions:

detergent stock	5 % (w/v) Brij35 in dd. H_2O
PC liposomes stock	20 mg/ml (freshly prepared)
tatBpIVEX1.3WG	482 ng/µl
tatCpIVEX1.3WG	942 ng/µl

Cell-free translation using rapid translation system (RTS)

Reaction solution		
(50 µl)	reaction mix	15 µl
	amino acids	4 µl
	methionine	1 µl
	Wheat Germ lysate	15 µl
	tat plasmid	2 µg
	PC-liposomes*	10 µl 20 mg/ml
	Brij35*	1 µl 5%
	sterile H_2O	add to 50 µl
Feeding solution	amino acids	80 µl
(1 ml)	methionine	20 µl
	Brij35*	20 µl 5%
	feeding mix	add to 900 µl

*: components that were added to RTS reaction only when desired.

Procedure:

- *liposome preparation*:

  20 mg/ml PC liposomes with a diameter of 200 nm (PC200) were prepared according to the standard protocol (hydration method, see section 4.2.6.1.).

- *cotranslational reconstitution through modified RTS*:

  The translation mixture and feeding mixture were prepared as described in the above table, and detergent Brij35 and PC liposomes were added to reconstitution experiments when desired (details were noted in the results).

  for $TatB_{Strep}$: cell-free translation was performed in the presence of 4 mg/ml PC200 liposomes, while no liposomes were added to the translation system in the control translation.

  for $TatC_{His6}$: *in vitro* translated $TatC_{His6}$ mostly precipated, so Brij35 was added to the translation reaction to make $TatC_{His6}$ soluble. In the cotranslational reconstitution experiment two concentrations of Brij35 were tested: 0.1% and 0.04%. 4 mg/ml PC200 liposomes

were used for reconstitution, while no liposomes were added to the translation system in the control translation.

The Wheat Germ RTS reaction ran at 24°C, 900 rpm for 24 h. After the reaction, the reaction mixture (50 µL) was gently mixed, then transferred to a new Eppendorf tube. A 45 µl aliquot was taken out and the proteoliposomes were purified with the sucrose gradient technique, which is described in the sections 4.2.8.1 and 4.2.8.2. The remaining 5 µl aliquot of RTS reaction mixture was stored at -20°C and used for the translation efficiency test.

4.2.8. Sucrose gradient ultra centrifugation

4.2.8.1 Sucrose density gradient ultra centrifugation

10 ml 60% (w/v) sucrose stock solution in reconstitution buffer (10 mM Tris, 100 mM NaCl, pH 7.8) was prepared and stored at -20°C before use. Four different concentrations of the stepwise sucrose gradient (10% - 40%), each of 1 ml, were prepared using reconstitution buffer and the 60% sucrose stock solution as described in following table (Two portions of each gradient solution were prepared for sample and control).

	10%	20%	30%	40%
buffer	834 µl	667 µl	500 µl	333 µl
60% sucrose	166 µl	333 µl	500 µl	667 µl
total volume	1 ml	1 ml	1 ml	1 ml

1 ml of 40% sucrose solution was added to an ultracentrifugation tube (Beckmann, 5 ml), then 1 ml of 30% sucrose solution was carefully overlaid on the 40% solution, similarly, 1 ml 20% and 1 ml 10% sucrose solutions were overlaid on each other. 200 µl resuspended proteoliposomes or reference solution were carefully overlaid onto the top of the discontinuous sucrose gradient. The ultra centrifugation tubes were set carefully into a MLS-50 rotor (Beckman) and centrifuged in at 100.000 g, 4 °C for 16 h. After centrifugation, 21 x 200 µl fractions were collected from top to bottom of the sucrose gradient, and the pellet in the ultra centrifugation tube was resuspended with 200 µl of

SDS Laemmli sample buffer. Aliquots from all fractions and the resuspended pellet were then analysed by SDS-PAGE.

4.2.8.2 "Floating" technique

After the cell free translation (RTS) of Tat proteins with or without liposomes (control), 45 µl aliquots from the two 50 µl reaction mixtures were mixed with 100 µl 60% sucrose stock solution to produce two suspensions containing 40% sucrose, which were set carefully bubble-free in the bottom of an ultra centrifugation tube. Then 1.45 ml 30 % sucrose gradient (each 725 µl for sample and control) was prepared, 725 µl from it was carefully overlaid on the 40% gradient. Finally 400 µl reconstitution buffer was put above the 30 % sucrose gradient. The sucrose gradients were prepared as described in the following table.

	0%	30%	40%
buffer	400 µl	725 µl	45 µl
60% sucrose	-	725 µl	100 µl
total volume	400 µl	1.45 ml	145 µl

The ultra centrifugation tubes were set carefully into a MLS-50 rotor (Beckman) and centrifuged in at 150.000 g, 4 °C for 2 h. After centrifugation, 10 x 200 µl fractions were collected from top to bottom of the sucrose gradient, and the pellet in the ultra centrifugation tube was resuspended with 200 µl of SDS Laemmli sample buffer. Aliquots from all fractions and the resuspended pellet were then analysed by SDS-PAGE.

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Erklärung

Ich erkläre an Eides statt, dass ich mich mit der vorliegenden Arbeit mit den Title „**Overexpression, purification and reconstitution of Tat subunits of *Arabidopsis thaliana* into liposomes.**“ erstmals um die Erlangung des Doktorgrades bewerbe, die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Le Li

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