Mechanistic studies of ABC transporter HmuUV-T facilitating heme uptake in Yersinia pestis

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Mechanistic Studies of ABC Transporter HmuUV-T
Facilitating Heme Uptake in Yersinia pestis

A thesis submitted to attain the degree of

DOCTOR OF SCIENCES of ETH ZURICH

(Dr. sc. ETH Zurich)

presented by

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2013
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## Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>Å</td>
<td>Angström</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>Adenosine 5′-(β,γ-imido)triphosphate</td>
</tr>
<tr>
<td>ARS</td>
<td>ATP regenerating system</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>DDM</td>
<td>Dodecyl-β-D-maltopyranoside</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECF</td>
<td>Energy-coupling factor</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EPR</td>
<td>Electron Paramagnetic Resonance Spectroscopy</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>His-tag</td>
<td>Histidine affinity tag</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal Titration Calorimetry</td>
</tr>
<tr>
<td>K_D</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>MBP</td>
<td>Maltose binding protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
</tr>
<tr>
<td>MST</td>
<td>Microscale thermophoresis</td>
</tr>
<tr>
<td>NBD</td>
<td>Nucleotide-binding domain</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>Nickel-nitrilotriacetic acid</td>
</tr>
<tr>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>Optical density measured at 600 nm</td>
</tr>
<tr>
<td>OMR</td>
<td>Outer membrane receptor</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>P&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>PPIX</td>
<td>Protoporphyrin IX</td>
</tr>
<tr>
<td>SBP</td>
<td>Substrate binding protein</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid phase extraction</td>
</tr>
<tr>
<td>TB</td>
<td>Terrific broth</td>
</tr>
<tr>
<td>TCDB</td>
<td>Transporter Classification Database</td>
</tr>
<tr>
<td>TEV</td>
<td>Tobacco etch virus</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
</tbody>
</table>
Summary

Transport across biological membranes is fundamental to any form of life and is mediated by transport proteins. ATP binding cassette (ABC) transporters constitute a large superfamily of integral membrane proteins that catalyze translocation of a wide variety of substrates at the expense of ATP hydrolysis. ABC importers are exclusively found in prokaryotic organisms and work as nutrient uptake systems. ABC exporters are ubiquitously expressed in all kingdoms of life and include multidrug efflux pumps. ABC transporters share a common structural organization comprising four core domains. Two transmembrane domains (TMDs) form the substrate translocation pathway and two cytoplasmic nucleotide-binding domains (NBDs) bind and hydrolyze ATP. Canonical ABC importers require a specific substrate binding protein (SBP) that captures the substrate and delivers it to the cognate transporter. They can be subdivided into type I and type II importers according to the architectures of the TMDs. Type II ABC importers catalyze uptake of iron compounds across the bacterial plasma membrane. Even though successful iron acquisition is a determinant of bacterial virulence, the translocation reaction is poorly understood at a molecular level.

In this thesis, the molecular mechanism of the *Yersinia pestis* type II heme importer-binding protein system HmuUV-T was investigated. To study the translocation reaction, a quantitative *in vitro* heme transport assay was established. The new assay allowed for the determination of *in vitro* rates of HmuUV-catalyzed heme transport into proteoliposomes. In a site-directed mutagenesis study the role of conserved residues in the translocation pathway of HmuUV and at the interface with the binding protein HmuT was established. A combination of transport activity measurements, ATPase activity assays and studies of the HmuUV-T complex formation was applied to achieve this.

It has been recently demonstrated that the type II ABC importers operate by a mechanism distinct from the alternating access mode that was previously thought to be shared by all ABC transporters. Whether all ABC type II importers function alike is however, unclear. To elucidate the conformational changes of HmuUV during the transport cycle, I aimed to solve a high-resolution structure of the HmuUV-T complex. Initial HmuUV-T crystals were obtained, the diffraction quality was, however, insufficient for structure determination. Nevertheless, investigation of the ATP hydrolysis kinetics in the HmuUV-T system and disulphide crosslinking studies suggest that mechanistic differences to the well-characterized vitamin B₁₂ uptake system BtuCD-F exist.
Zusammenfassung

Transmembrantransport ist grundlegend für alle Organismen. ABC Transporter sind eine große Familie von Transmembranproteinen, die Substrate ATP-abhängig durch die Membran translokalisieren. ABC Importer stellen eine Untergruppe der ABC Transporter dar, die ausschließlich in Prokaryoten vorkommen und zur Nahrungsaufnahme dienen. ABC Exporter dagegen werden in allen Lebewesen exprimiert und sind unter anderem für das herausschleusen von Medikamenten verantwortlich. Alle ABC Transporter haben einen ähnlichen Aufbau, der zusammen vier Hauptdomänen umfasst: zwei Transmembran-Domänen (TMDs) sind für die Translokalisation verantwortlich und zwei zytoplasmatische Nukleotid-bindende Domänen (NBDs) binden und hydrolysieren ATP. Kanonische ABC Importer benötigen für den Transport ein Substrat-Bindeprotein (SBP), welches für die Substratbindung und den Weitertransport zum eigentlichen Transporter verantwortlich ist. Abhängig von der jeweiligen Architektur der Transmembrandomäne, können ABC Importer in Typ I und Typ II Importer unterteilt werden. Typ II Importer sind dabei typischerweise für den Eisentransport durch die bakterielle Membran verantwortlich und obwohl die bakterielle Virulenz stark von der Eisenaufnahme abhängt, ist der molekulare Mechanismus der Eisenaufnahme bis heute kaum erforscht.


Es ist bekannt, dass sich der molekulare Transportmechanismus der Typ II ABC Importer grundsätzlich von dem abwechselnden Zugangsmodus des klassischen Modells der ABC Transporter unterscheidet. Es ist jedoch nicht bekannt, ob alle ABC Typ II Transport einen solchen Mechanismus verwenden. Um die Konformationsänderung von HmuUV während des Transportzyklus zu analysieren, wurde in der vorliegenden Arbeit versucht die Kristallstruktur von HmuUV-T zu bestimmen. Dieses Ziel konnte jedoch durch die unzureichende Diffraktionsqualität der HmuUV-T Kristalle nicht erreicht werden. Trotzdem konnten durch Untersuchung der ATP-Hydrolysekinetik von HmuUV-T, sowie der
Bestimmung der Disulfidbrückenvernetzung, signifikante mechanistische Unterschiede zum sehr gut untersuchten Vitamin B\textsubscript{12} Aufnahmesystem BtuCD-F ermittelt werden.
1. Introduction

Transport across biological membranes is fundamental to any form of life. To accommodate the diversity of molecules a cell may need to acquire from the environment or extrude, many different transporters are encoded in the genomes of organisms [1]. Altogether, more than 600 transporter protein families are currently included in the Transporter Classification Database (TCDB) [2]. According to the source of energy used by transport proteins, channels, primary and secondary transporters are distinguished. Channels catalyze the facilitated diffusion of solutes down a concentration gradient, an energy-independent process. Primary active transporters, including ATP-binding cassette (ABC) transporters, harness the energy of ATP hydrolysis to power substrate translocation. Secondary active transporters, including uniporters, antiporters, and symporters, use the energy stored in ion gradients to drive transport [3, 4].

HmuUV-T, the transporter investigated in this thesis work, is a bacterial type II ABC importer catalyzing heme uptake into the cytoplasm of the Gram-negative pathogen *Yersinia pestis*. Thus, in the first subchapter, function, architecture and molecular mechanism of ABC transporters are described. As heme is a major iron source for pathogenic bacteria residing in mammalian hosts, iron uptake systems in Gram-negative bacteria are introduced, followed by more detailed information on the iron acquisition in *Yersinia pestis*. To study the molecular mechanism of transport proteins, *in vitro* functional assays are used. Therefore, the common assay setups for investigation of the ABC transporters are briefly described. At last, open questions are formulated and aims of the thesis are stated, accompanied by a brief outline of the content of the following chapters.

1.1 ATP-binding cassette (ABC) transporters

1.1.1 Function and clinical relevance

ABC transporters constitute a large superfamily of integral membrane proteins that includes both importers and exporters. While importers are exclusively found in prokaryotic organisms, exporter-type ABC transporters are expressed ubiquitously in all kingdoms of life [1, 5]. The efforts to elucidate the structure and mechanisms of ABC transporters in detail are driven both by fundamental interest and by their considerable clinical relevance.
**Prokaryotic ABC transporters**

The main function of the ABC importers is the uptake of nutrients. They transport a wide variety of substrates ranging from carbon, energy and nitrogen sources, which are required in large amounts by the cells, to transition metal ions that are required and tolerated only in trace amounts [6]. Substrates vary greatly in size and chemical nature, including mono- and oligosaccharides, amino acids and oligopeptides, organic and inorganic ions, vitamins and iron siderophores [4]. ABC importers play an important role in the survival of pathogens within mammalian hosts by enabling the uptake of essential but often scarcely available nutrients. For example, mutations in ABC transporters associated with the uptake of metal ions, such as iron, zinc, and manganese, have been shown to affect bacterial virulence in animal infection models. Examples include causative agents of bubonic plague, cholera and salmonellosis - *Yersinia pestis*, *Vibrio cholera* and *Salmonella enterica*, respectively [7-11]. Beyond ensuring the nutrient supply, ABC importers are involved in osmoregulation by mediating the uptake of compatible solutes, and participate in the cell-to-cell communication by internalizing pheromones and other signaling molecules [6].

Prokaryotic ABC exporters are abundant and have close homologues in eukaryotes. The most extensively studied systems are involved in drug efflux, protein secretion and lipid translocation. Multidrug-resistance (MDR) efflux pumps encoded by bacteria can confer clinically relevant resistance to antibiotics. They are typically able to extrude a range of structurally unrelated drugs, as demonstrated for the LmrA transporter form *Lactococcus lactis* [12, 13]. ABC exporters are an essential component of the widespread Sec-independent type I protein secretion system in bacteria. Secreted proteins include important virulence factors such as exotoxins targeting other bacterial species or the host, for example, the alpha hemolysin HlyA [14]. ABC transporters implicated in the flipping of lipids and lipid-linked oligosaccharides take part in the lipopolysaccharide biosynthesis or deliver precursors for N-linked protein glycosylation, as demonstrated for MsbA from *Escherichia coli* and PglK from *Campylobacter jejuni*, respectively [15, 16].

**Eukaryotic ABC transporters**

Most eukaryotic ABC transporters characterized thus far mediate the translocation of substrates from the cytoplasm or the inner leaflet of the lipid bilayer to the exterior of the cell or into the lumen of organelles. In humans, ABC exporters are crucial participants in lipid, fatty acid and cholesterol translocation, antigen presentation and mitochondrial iron homeostasis. The non-canonical human ABC exporters - cystic fibrosis transmembrane
conductance regulator (CFTR) and sulfonyl urea receptors - function as ATP-sensitive ion channels. Mutations in human ABC transporters have been associated with a range of disorders including hypercholesterolemia, cystic fibrosis and diabetes [1, 17, 18]. Furthermore, within the human genome, at least three ABC exporters have been identified as multidrug resistance pumps: P-glycoprotein, multidrug resistance protein 1, and breast cancer resistance protein. These proteins extrude a broad range of lipophilic compounds, including chemotherapeutic agents, resulting in multidrug resistance of tumor cells [19, 20].

1.1.2 Molecular architecture

Despite the large, diverse population of substrates handled and the difference in the polarity of transport, ABC transporters share a common structural organization comprising four core domains. Two transmembrane domains (TMDs) form the substrate translocation pathway and two cytoplasmic ABCs (also termed nucleotide-binding domains (NBDs)) bind and hydrolyze ATP. In ABC importers, the TMDs and NBDs are separate polypeptide chains. In bacterial exporters, by contrast, a TMD is fused to a NBD, generating a ‘half-transporter’ that forms a homodimer or heterodimer to generate the complete, functional ABC transporter (Figure 1.1). Many eukaryotic ABC exporters are ‘full-transporters’ that feature all four domains in a single polypeptide chain [1, 4, 5, 21].

The NBD is the conserved domain of the ABC transporter superfamily. In contrast, the sequences and architectures of the TMDs are variable, reflecting the chemical diversity of the translocated substrates. Besides the four ubiquitous core-domains, additional elements may be found fused to the TMDs and/or NBDs of ABC transporters that mostly serve regulatory functions [1, 4, 5, 22].

Diversity in the TMD folds

According to the architectures of the TMDs, ABC transporters can be subdivided into following classes:

1) **ABC exporters** - contain 6 transmembrane (TM) helices in each TMD, or 12 for the assembled transporter. These extend well beyond the cytoplasmic boundary of the lipid bilayer, and as a consequence, the ABC domains are spaced approximately 25 Å away from the membrane. The transmembrane region is organized in two 'wings' composed of helices TM1-2 from one subunit, and TM3-6 of the other in a domain-swapped
arrangement, originally demonstrated for the Sav1866 exporter from *Staphylococcus aureus* (Figure 1.1) [1, 5, 23].

![Diagram of ABC exporters](image)

**Figure 1.1: ABC exporters.** a, Schematic of domain organization and function. b, Ribbon diagram of the of the Sav1866 structure (PDB 2HYD). Bound ADP is in ball-and-stick representation. Each NBD is fused to a TMD, creating a 'half transporter'. The stoichiometry of the assembled transporter is $(Sav1866)_2$. The two subunits are shown in green and yellow. Figure adapted from [5].

2) ABC importers:

   a) **Binding protein dependent or canonical ABC importers** - require a specific substrate binding protein (SBP) that captures the substrate with high affinity and delivers it to the cognate importer (Figure 1.2a). In gram-negative bacteria, the SBPs diffuse freely in the periplasm. Gram-positive bacteria and archea anchor their binding proteins to the outer surface of the cell membrane via an N-terminal lipid moiety or an N-terminal hydrophobic helix. Furthermore, SBPs can also be fused to the TMDs [1, 5, 24]:

   i) **Type I ABC importers or small importers** - the core topology contains 5 TM helices per half-transporter as observed in the *Escherichia coli* methionine importer MetI [25]. The additional N-terminal helix(-ces) present in other type I ABC importers - molybdate/tungstate transporter ModBC from *Archaeoglobus*
flugidus [26] and Escherichia coli maltose transporter MalFGK [27] - interact primarily with the partner TMD. However, an extensive domain swapping as observed in ABC exporters is absent (Figure 1.2b). Typical substrates for type I importers include sugars, amino acids and ions [1, 5, 18].

ii) Type II ABC importers or large importers - the fold originally found in the vitamin B₁₂ transporter BtuCD from Escherichia coli contains 10 helices in each TMD for a total of 20 membrane-spanning segments (Figure 1.2c). Helices pack together in a rather intricate topology, with TM5 and TM10 dominating the interface between the TMDs. The transported substrates are generally larger than those of type I importers, such as iron siderophores and heme [1, 5, 28].

b) Energy-coupling factor (ECF) transporters - a recently identified class of ABC importers comprising a transmembrane substrate binding protein, the S component. The two structures of full ECF transporters published so far show the 6 TM helices of

![Figure 1.2: Binding protein dependent importers. a, Schematic of domain organization and function. b, Structure of type I importer ModBC-A in ribbon representation with bound tungstate shown in spheres (PDB 2ONK). ModB subunits (TMDs) are in blue and yellow, ModC domains (NBDs) in green and purple, ModA (SBP) is in red. c, Structure of type II importer BtuCD-F in ribbon representation with bound AMP-PNP indicated in ball and stick and Mg²⁺ as pink spheres (PDB 4FI3). BtuC subunits (TMDs) colored in marine and light blue, BtuD subunits (NBDs) in light and dark green, BtuF (SBP) is shown in orange. b and c, The stoichiometries of the assembled complexes are ModB₂C₂A and BtuC₂D₂F. Figure adapted from [5, 32, 35].](image)
the S component lying almost parallel to the membrane plane bound exclusively by the 5 TM helices and 2 or 3 cytoplasmic helices of the second TMD - the T component. Only the T component contacts the both NBDs (Figure 1.3) [6, 29, 30].

NBDs – a common engine attached to specialized TMDs

All NBDs contain two subdomains - a larger RecA-like subdomain, also called the catalytic core domain and found in other ATPases, and a smaller $\alpha$-helical subdomain that is unique to ABC systems. Binding and hydrolysis of ATP are catalyzed by residues from several highly conserved motifs. The most prominent of these are the P-loop or Walker A motif, located in the RecA-like subdomain, and the ABC signature motif LSGGQ in the $\alpha$-helical subdomain. Both are crucial for nucleotide binding. Additionally, RecA-like subdomain contains: (i) the Walker B motif providing a conserved glutamate residue that acts as a catalytic base orchestrating the nucleophilic attack on ATP via a water molecule, (ii) the D-loop involved in the contact interface between the two NBDs, and (iii) the switch region that contains a highly conserved histidine side chain thought to acts as a linchpin holding together the ATP $\gamma$-phosphate, the attacking water and the catalytic glutamate for catalysis. The two subdomains are connected by two flexible loops, one of which contains a highly conserved glutamine residue and is known as the Q-loop [4, 5, 31-33].
In an intact transporter, the NBDs assemble such that these conserved motifs are exposed at a shared interface in a 'head-to-tail' fashion. The P-loop of one NBD is oriented towards the signature motive of the other (Figure 1.4a). This arrangement generates two ATP binding and hydrolysis sites located at the interface between the two NBDs. Hence, nucleotide binding and hydrolysis is a property of dimeric and not of individual ABC domains. In the absence of a nucleotide, there is a gap at the domain interface, with water being able to access the nucleotide-binding sites. When ATP is bound, the interface closes and the nucleotides are sandwiched between the conserved sequence motifs of both domains (Figure 1.4) [1, 5, 32].

1.1.3 Transport Mechanism

The ubiquitous power stroke

The key to the functioning of ABC transporters is that the relative position of the two ABC domains depends on their nucleotide state. The driver for transport is an on-off 'switch' between the two principal conformations of the NBDs: a 'closed sandwich dimer' formed by binding two ATP molecules at the dimer interface, and dissociation of the 'closed sandwich dimer' to an 'open dimer' facilitated by ATP hydrolysis and Pi/ADP release (Figure 1.4a). These nucleotide dependent conformational transitions ultimately drive the conformational changes in the TMDs resulting in substrate translocation. Thus, ATP binding, hydrolysis and

![Figure 1.4: Schematic of the ‘closing’ motion of NBDs upon binding of ATP.](image)

*Figure 1.4: Schematic of the ‘closing’ motion of NBDs upon binding of ATP. a, View from the membrane (showing the surface facing the TMDs). b, View from the side of the NBDs. NBD ‘closing’ is transmitted to the TMDs via a distance change of the coupling helices. Figure adapted from [5, 21].*
product dissociation provides the principal energy input or power stroke for transport [1, 5, 17, 34].

Effective coupling requires the transmission of the molecular motion from the NBDs to the TMDs. At this interface, architecturally conserved α-helices, which are part of the TMDs, are present in all reported ABC transporter crystal structures. These 'coupling helices' interact with grooves formed at the boundaries of the two sub-domains of the NBDs (Figure 1.4b). The region of the NBD that interacts with the TMD primarily involves the Q-loop. The conserved glutamine participates in the nucleotide binding and is thought to sense the γ-phosphate moiety of ATP. Thus, in has been proposed, that conformational changes in this region are involved in coupling of nucleotide hydrolysis to the conformational states of the TMDs [1, 5, 32].

**Proposed transport models**

Because of the strict conservation of the NBDs and the coupling helices of the TMD domains, it was believed for many years that all ABC transporters must share a unifying transport mechanism. Recently, however, convincing structural and functional evidence has led to the conclusion that, even though the conserved cytoplasmic motor domains produce a power stroke that is similar for all ABC transporters, the distinct TMDs respond differently. While the long-established alternating access model explains the function of exporters and type I importers, a peristaltic mechanism has been recently proposed for type II importers [35]. The proposed substrate transport cycle of ECF transporters mimics filling and decanting a wine glass [29]. It is, however, still highly speculative and therefore not described here.

*Alternating access model for exporters and type I importers*

The key feature of this model is the presence of a substrate binding site that can alternatively access either the extracellular side or the intracellular side of the membrane, corresponding to the outward- and inward-facing conformations of TMDs, respectively. The nucleotide-bound state of the NBDs is linked to the outward-facing conformation and the nucleotide-free state to the inward-facing conformation (Figures 1.1b and 1.2b). The relative binding affinities for substrate of the two conformations will largely determine the net direction of the transport. The inward-facing conformation of an exporter is expected to have a higher affinity for substrate than the outward facing conformation. The opposite will generally hold for importers. However, in the outward-facing conformation, binding of the substrate to the TM binding site might be facilitated by the binding protein sealing off an enclosed cavity and
increasing the local concentration of the substrate. Furthermore, transport is a multistep process involving communication via conformational changes, in both directions, between the NBDs and TMDs. Substrate- or SBP-dependent conformational changes in the TMDs affect the positioning of the NBDs [1, 5, 17, 36].

The alternating access model has been described in detail for the *Escherichia coli* maltose importer MalFGK₂ (Figure 1.5). In addition to the crystal structures of the maltose transporter in three functional states - inward-facing [37], pre-translocation [38] and outward-facing [27] - plenty of biochemical data is available. In the absence of nucleotides and maltose-binding protein (MBP) transporter adopts an inward-facing, resting state conformation. Upon binding of the substrate-loaded MBP, the two TM subunits rotate toward the molecular center, bringing the NBDs closer together. ATP binding to this pre-translocational state promotes a concerted conformational change involving a complete closure of the NBD dimer, flipping of the TMDs to the outward-facing state and opening of the MBP. Substrate is transferred from MBP to the TM binding site. ATP hydrolysis resets the transporter into the inward-facing state to release maltose into the cell. A high-energy post-hydrolysis state is assumed. Transporter reverts to the resting state upon dissociation of ADP/Pi and MBP [36].

Peristaltic transport mechanism for the type II importer BtuCD

The best studied type II ABC importer is the *Escherichia coli* vitamin B₁₂ transporter BtuCD. Crystal structures of BtuCD have been determined in three distinct states. First, nucleotide-free outward-facing state of transporter alone was observed (state 1 in figure 1.6) [28], followed by nucleotide-free [39, 40] and AMP-PNP-bound [35] structures of the complex with the cognate binding protein BtuF (states 4 and 2 in the figure 1.6, respectively).
Based on the structural information, biochemical data and EPR (Electron Paramagnetic Resonance Spectroscopy) studies [41, 42], a peristalsis-like $\text{B}_12$ transport mechanism has been proposed. Transport cycle is initiated by the docking of $\text{B}_12$-loaded binding protein to the outward-facing conformation of BtuCD (state 1 in figure 1.6). The high-affinity $\text{B}_12$ binding pocket of BtuF becomes distorted and gets occupied by periplasmic loops of the TMDs. Upon binding of two ATP molecules and closure of the NBD dimer, the concomitant rearrangements in the TM segments trap $\text{B}_12$ in the translocation pathway. A fully enclosed cavity is formed, also sealed from the attached BtuF (state 2 in figure 1.6). Notably, from the cytoplasmic side, the translocation pathway is closed by a distinct cytoplasmic gate as in the outward-facing conformation. The cytoplasmic loops connecting TM4 and TM5 (cytoplasmic gate I) are replaced by the loops linking TM2 and TM3 (cytoplasmic gate II). After ATP hydrolysis and release of the hydrolysis products, the previously closed NBD dimer opens up pulling the coupling helices outward. This generates an inward-facing conformation with BtuF still attached to the periplasmic side of the transporter. The substrate is squeezed out from the very low affinity cavity in the TMDs, as in peristalsis (state 3 in figure 1.6). After the release of $\text{B}_12$ into the cytoplasm, TMDs adopt an asymmetric conformation, required to prevent unspecific leakage of small molecules through the translocation pathway (state 4 in figure 1.6). Different physiological factors in the cell, such as electrochemical membrane

Figure 1.6: Schematic of proposed vitamin $\text{B}_12$ transport mechanism. The states are ordered to describe a productive translocation cycle. Three out of four states (1 (PDB 1L7V), 2 (PDB 4FI3), and 4 (PDB 2Q19)) reflect determined crystal structures. PBP, periplasmic binding protein. Figure from [35].
potential, presence of ATP and vitamin B$_{12}$, are proposed to contribute to the release of BtuF, allowing a new transport cycle to be initiated [35].

1.2 Bacteria and iron

Iron is an essential element for most living organisms, including bacteria. As a catalytic center for redox reactions in many enzymes, iron facilitates numerous fundamental cellular processes such as electron transport, peroxide reduction, and nucleotide biosynthesis. Upon entry into a mammalian host, bacterial pathogens need to acquire iron to survive. Despite its presence as the most abundant transition metal in living organisms, bacteria face an acute iron supply problem. Under aerobic conditions iron exists in its oxidized ferric (Fe$^{3+}$) form. In an aqueous environment at physiological pH, the solubility of Fe$^{3+}$ is extremely low ($\sim 10^{-18}$ M) due to instant aggregation into insoluble oxy-hydroxides. In its reduced ferrous form (Fe$^{2+}$), iron activates the Fenton reaction producing hydroxyl radicals that are deleterious for most macromolecules. Due to its insolubility and toxicity, iron is sequestered into carrier proteins such as lactoferrin, transferrin, and ferritin, or by binding to the protoporphyrin ring in hemoproteins. Iron homeostasis is so strictly regulated that there is virtually no free iron in living organisms. To overcome the low bioavailability of iron, bacteria have evolved efficient strategies for energy-dependent high-affinity uptake. Usually, the genetic equipment of pathogenic bacteria allows more than one mechanism to operate [6, 43-46].

1.2.1 Iron uptake in Gram-negative bacteria

In Gram-negative bacteria, iron uptake pathways involve an outer membrane receptor (OMR), a periplasmic binding protein, and an inner membrane ABC importer. Following iron acquisition mechanisms have been described (Figure 1.7):

1) Secretion of siderophores - low molecular weight compounds with very high affinity for Fe$^{3+}$ - and their subsequent reabsorption,

2) Direct extraction of iron from host storage proteins via specific OMRs,

3) Uptake of iron from free heme or hemoproteins. Like iron itself, heme is cytotoxic. In addition to catalyzing the formation of reactive oxygen species, the lipophilic molecule easily intercalates into membranes impairing the lipid bilayers. Therefore, is sequestered by host hemoproteins and mostly stored intracellularly. To overcome the limited access to heme, pathogenic bacteria commonly secrete exotoxins such as hemolysins, cytolsins,
and proteases that can lyse cells and release heme bound by host carrier proteins. Two mechanisms of heme acquisition that differ in their initial step are known:
a) Heme-containing proteins or free heme are bound directly to specific OMRs,
b) Extracellular hemophores bind free heme or extract heme from carrier proteins and shuttle it to hemophore-specific OMRs [6, 47].

Figure 1.7: Iron acquisition in Gram-negative bacteria.

On top of their scarcity, heme and iron-siderophore complexes exceed the molecular weight cut-off of the outer membrane porins and thus require specific high-affinity OMRs for uptake into the periplasm. OMRs comprise two domains - a 22-stranded antiparallel transmembrane β-barrel and an N-terminal globular cork domain filling the lumen of the barrel. The proton-motive force of the inner membrane provides the energy for translocation. This energy is transferred to OMRs in the outer membrane via a complex of three inner membrane proteins: TonB, ExbB and ExbD. The hydrophilic domains of TonB span the periplasm and the carboxy-terminal region directly contacts the TonB box in the plug domain of OMRs. TonB
is believed to exert a mechanic pulling force causing movement or localized unfolding of the cork domain and allowing substrate translocation into the periplasm [47-49]. Once the substrate reaches the periplasmic space, it is captured by a specific substrate-binding protein (SBP). Generally, SBPs consist of two structurally similar domains (or lobes) both containing central β-sheets surrounded by α-helices. Substrate binding site is located in a central cleft between the two lobes. Iron-, siderophore- and heme-specific binding proteins typically belong to the type III SBPs, characterized by a single α-helix connecting the two lobes. Ligand binding in the type III SBPs is not accompanied by a large domain movement, as opposed to the type I and II SBPs, where a flexible hinge region connecting the lobes allows for a substantial ligand-induced conformational change [4, 6, 50].

SBP delivers its substrate to the external surface of the cognate ABC importer located in the inner membrane. Heme and iron-siderophore transporters belong to the type II ABC importers described above. They facilitate substrate translocation into the cytoplasm, where heme is directly catabolized by heme oxygenases to release free iron or bound to cytoplasmic heme binding proteins [44]. Fe^{3+}-siderophore complexes are thermodynamically very stable. Iron release mechanisms are complex and include hydrolysis or modification of the siderophore scaffold, and/or reduction of the coordinated Fe^{3+} to Fe^{2+}. Depending on the siderophore pathway, metal extraction can also occur in the periplasm already, with only iron being imported into cytoplasm [46].

**Iron acquisition in *Yersinia pestis***

*Yersinia pestis* - a Gram-negative rod with a mammalian/flea life cycle - is the causative agent of bubonic, pneumonic and septicemic plague. *Yersinia pestis* possesses numerous iron acquisition systems that may be important for survival in its various iron-deficient niches. Two inorganic iron transport systems have been demonstrated to play an important role in the pathogenesis of plague:

1) A siderophore-dependent transport system Ybt, operating by secretion and reuptake of the siderophore yersiniabactin. The Ybt system is essential for iron acquisition during the early stages of plague. Mutants lacking the Ybt transport system are completely avirulent in mice from a subcutaneous route that mimics the flea bite [51],

2) An iron and manganese uptake system Yfe, playing a role in iron acquisition during the later stages of disease, for example, in the blood-born dissemination to the liver, spleen and other internal organs [9].
Under iron-depleted conditions the Hmu (for 'hemin utilization') transport system of *Yersinia pestis* allows the bacterium to use heme and hemoproteins as iron sources. The hemin uptake locus (*hmu*) reveals five genes, *hmuRSTUV*. The translated gene products correspond to:

- HmuR - an OMR,
- HmuS - a cytoplasmic protein proposed to participate in removal of iron from heme,
- HmuT - a periplasmic SBP,
- HmuU and HmuV - membrane-spanning and ATPase domains of an inner membrane ABC importer, respectively.

Growth studies have show that deletion of the *hmu* locus generates a *Yersinia pestis* mutant growing poorly on iron-depleted medium containing free heme or mammalian heme-protein complexes [52]. However, no effect on the virulence was found after injecting mice subcutaneously or intravenously with *Yersinia pestis* strains lacking the Hmu transport system. This suggests that the hemin uptake system might be required in the flea, or in other routes or stages of infection such as a pneumonic route of infection or an intracellular stage of infection. Alternatively, the other putative heme utilization systems or the various inorganic iron transport systems might be able to acquire sufficient iron to counteract the loss of the Hmu pathway [53].

### 1.3 *In vitro* functional characterization of ABC transporters

The *Yersinia pestis* heme acquisition system *hmu*, and several other bacterial heme uptake systems, such as *shu* from *Shigella dysenteriae* and *hut* from *Vibrio cholerae*, have been functionally characterized *in vivo* [53-56]. However, to study the molecular mechanism of the involved components in detail, experiments with purified protein samples in well-controlled conditions are indispensable. ABC transporters are primary active transporters that harness the energy of ATP hydrolysis to power substrate translocation. Thus, measurements of the ATPase activity and the transport activity are performed to characterize the protein of interest. While the ATPase activity of the purified transporter can be directly measured in the detergent solution (Figure 1.8a), reconstitution of the protein into lipid vesicles, generating proteoliposomes, is necessary to study the transport reaction. As the lipid bilayer resembles the native membrane environment of the proteins much more than the detergent micelle, also the ATPase measurements are often performed in proteoliposomes. However, due to the insertion of the transporters into the vesicles in two possible orientations - with the ATPase
domains facing the lumen ('right-side-out') or the exterior ('inside-out') - only a fraction of the ATPase sites are available for the measurement and quantitative calculation of the hydrolysis rates becomes intricate (Figure 1.8c). Furthermore, the effects of addition of compounds or binding partners interacting with the extracellular (or periplasmic) side of the transporter can only be assessed after time-consuming incorporation of the selected components into the lumen of the vesicle, for example, by subjection to freeze-thaw cycles and subsequent extrusion. These limitations can be circumvented by the recently developed nanodisc technology, which enables simultaneous access to both intra- and extracellular sides of the transporter inserted into a nanometer-scale planar liposome bilayer - the nanodisc (Figure 1.8b) [57]. The lipid bilayer in nanodiscs is encircled by an amphipathic helical belt formed by two monomers of a membrane scaffold protein and thus kept soluble in an aqueous solution.

Figure 1.8: Schematic of setups used for in vitro characterization of ABC transporters. Blue rectangles symbolize TM domains and blue circles the NBDs. ATP hydrolysis activity can be assayed for a detergent solubilized transporter (a), transporter inserted into nanodiscs (b) or reconstituted into proteoliposomes (c). b, c and d, gray circles symbolize lipid bilayer, in c and d a few individual lipid molecules are schematically drawn for illustration. c, Transporters can insert into proteoliposomes in two orientations - 'inside-out' and 'right-side-out'. d. Setup of a substrate uptake assay in proteoliposomes. For a sustained transport, ATP regenerating system (A.R.S.) is incorporated into vesicles, substrate (red star) is added to the exterior. Only the right-side-out transporters catalyze transport. ATP, adenosine triphosphate; ADP, adenosine diphosphate; P\textsubscript{i}, inorganic phosphate.
For substrate translocation assays, however, the spatial separation of the liposome lumen from the exterior is necessary. Most commonly, the accumulation of the transported substrate in the liposome lumen is measured in an uptake experiment. To avoid rapid depletion of ATP in the small internal volume of the liposome, (~1 μl mg⁻¹ lipid for 400 nm vesicles [58]), an ATP regeneration system (A.R.S.) is incorporated, typically consisting of ATP, creatine kinase, creatine phosphate and Mg²⁺. Alternatively, proteoliposomes can be pre-loaded with the substrate and the efflux reaction initiated by adding ATP and Mg²⁺ to the exterior. For osmoregulated ABC transporters such as *Lactobacillus lactis* OpuA, the directionality of the transport can be switched by changing the osmolarity of the external medium [59]. Mostly, radiolabeled or fluorescent substrates are used to follow the reaction.

1.4 Open questions

As described above, successful iron acquisition is a determinant of bacterial virulence, and heme is a major iron source for pathogenic bacteria residing in mammalian hosts. The transport reaction of iron compounds across the bacterial plasma membrane is, however, poorly understood at molecular level. The *Yersinia pestis* heme transporter HmuUV-T has been functionally characterized *in vivo* [53], and is therefore a well-suited representative of type II ABC importers catalyzing uptake of iron compounds into the cytoplasm of bacterial pathogens. Open questions concerning the molecular mechanism of type II ABC importers or the whole ABC transporter superfamily, and directly relevant for HmuUV-T, are lined out below.

For the type II ABC importer BtuCD, peristaltic transport mechanism has been proposed [35]. Whether it is representative for the whole ABC type II importer family is, however, unclear. The outward-facing conformation of the BtuCD transporter in the nucleotide-free state [28] differs from the inward-facing apo structure reported for the related *Haemophilus influenzae* transporter HI1470/1 (recently renamed MolBC and suggested to transport molybdate) (Figure 1.9) [60, 61]. After complementary studies on HI470/71, employing EPR and chemical crosslinking, transport mechanism diverging from that of the BtuCD has been proposed [62]. Furthermore, in parallel to this thesis work, the structure of the *Yersinia pestis* HmuUV transporter in a nucleotide-free state was solved in our lab by Götz BA, Woo JS and Locher KP [63]. As for BtuCD, an outward-facing conformation was observed. Nevertheless, notable differences in the translocation pathways were revealed, described in more detail in chapter 4 (Figure 1.9). These findings suggest that mechanistic differences within the type II
ABC importer family might exist. To explore this diversity, biochemical investigations and visualization of type II importers in distinct conformational states by X-ray crystallography are necessary.

The principal contribution to the specificity of canonical ABC importers are the high-affinity substrate binding pockets of the periplasmic binding proteins and the protein recognition at the binding protein–transporter interface. But does SBP specificity dictate the transport specificity exclusively, or do the TMDs contribute as well? On one hand, the vitamin B₁₂ importer BtuCD has no measurable affinity for its substrate [64], while the cognate binding protein BtuF binds B₁₂ with nanomolar affinity (~15 nM) [65]. On the other hand, the presence of a substrate binding site in the TM domain MalF of the type I maltose importer MalFGK₂ has been demonstrated by mutagenesis studies [66, 67] and visualized by X-ray crystallography [27]. Furthermore, residues uniquely conserved in type II heme importers were identified in the translocation pathway of HmuUV [63], indicating that TM subunits might contribute to the substrate specificity. To clarify this point, mutagenesis studies with comprehensive analysis of the effects of the introduced changes are needed.

Two ATP binding and hydrolysis sites are located at the interface of the NBD dimer. A central question in the field of the ABC transporters concerns the role of the two sites and the communication between them: are the sites independent of each other or do they interact?
Positive cooperativity of ATP hydrolysis has been observed in numerous ABC transporters, including BtuCD [35, 68] and MalFGK₂ [69], indicating that the two sites communicate. On the other hand, in several ABC exporters, such as CFTR and antigen peptide transporter TAP1/2, only one of the two nucleotide binding sites retains all of the highly conserved residues essential for ATP hydrolysis, suggesting that hydrolysis at one site only is sufficient for function. Additional biochemical studies focused on the kinetics of the ATPase reaction are required to elucidate the level of crosstalk between the catalytic sites. Furthermore, the stoichiometry of ATP hydrolyzed to substrate translocated is unclear. In general, ABC transporters with two functional ATPase sites are thought to hydrolyze two ATP molecules in a single reaction cycle. The stoichiometry of the glycine betaine ABC importer OpuA is indeed two ATP molecules hydrolyzed for each transported substrate [59]. In maltose transporter MalFGK₂, however, rates of 1.4 to 17 ATPs per transported sugar have been reported [70], and stoichiometry of ~100 ATP per transported B₁₂ was obtained for BtuCD [71]. To investigate the stoichiometry and whether ATP hydrolysis is necessarily tightly coupled to transport, characterization of transport kinetics, combined with measurements of ATPase activity, is necessary.

1.5 Aim and outline of the thesis

The goal of my work was to study the mechanism of the translocation of iron compounds across the bacterial plasma membrane at a molecular level. The Yersinia pestis heme importer HmuUV-T was chosen as a representative of type II ABC importers catalyzing uptake of iron compounds into the cytoplasm of bacterial pathogens. To address the above-mentioned questions I, established a quantitative in vitro heme transport assay and aimed to solve a high-resolution structure of HmuUV in complex with its binding protein HmuT. Furthermore, the kinetics of the ATP hydrolysis by the HmuUV-T system were studied.

In chapter 2, establishment of methods for quantification of heme at nanomolar concentrations is described. To study the HmuUV-catalyzed transport reaction, a sensitive heme detection method is required. However, quantitation of the hydrophobic heme moiety at low concentration is technically challenging. No established, standardized procedures are available. Therefore, two alternative approaches for determination of the amount of transported heme were explored. First, a chemiluminescence-based method for heme determination by reconstitution with horseradish peroxidase (HRP) apo-enzyme was adapted.
Second, an enzymatic method for the generation of radioactively labeled heme was developed.

In chapter 3, the development of an *in vitro* heme uptake assay in proteoliposomes is described. For an optimal setup of the multi-component system, HmuT-heme binding stoichiometry and affinity were determined and HmuUV-T complex formation was studied. For this purpose, UV/Vis spectroscopy, isothermal titration calorimetry, microscale thermophoresis and size-exclusion chromatography techniques were used. To enable the calculation of the *in vitro* transport rates, method for the determination of the accessible, transport-competent fraction of HmuUV was worked out.

In chapter 4, the newly developed assay is applied for the investigation of HmuUV-catalyzed heme translocation. *In vitro* rates of heme transport into proteoliposomes were determined and, in a site-directed mutagenesis study, the role of conserved residues in the translocation pathway of HmuUV was established. A combination of transport activity measurements, ATPase activity assays and studies of the HmuUV-T complex formation was applied to achieve this. Furthermore, HmuUV-catalyzed uptake of the self-made Fe$^{55}$-labeled heme into proteoliposomes was tested.

In chapter 5, kinetics of the ATP hydrolysis by the HmuUV-T system is analyzed. Disulfide crosslinks were introduced in conserved regions of the NBDs or TMDs. The crosslinks, initially engineered to stabilize HmuUV-T in the nucleotide-bound state for crystallization, showed interesting effects on the ATPase activity, cooperativity and sensitivity to ATP analogues.

Chapter 6 contains conclusions and outlook.

HmuUV-T crystallization trials are summarized in the appendix. Initial HmuUV-T crystals were obtained both in the nucleotide-bound and apo states. The diffraction quality was, however, insufficient for structure determination.
References

46. Schalk, I.J. and L. Guillou, Fate of ferrisiderophores after import across bacterial outer membranes: different iron release strategies are observed in the cytoplasm or periplasm depending on the siderophore pathways. Amino Acids, 2013. 44(5): p. 1267-77.


2. Heme quantitation - a prerequisite for studying the mechanism of HmuUV-T

2.1. Abstract
To study the molecular mechanism of the heme importer HmuUV-T, transport activity measurements are indispensable. To follow the transport reaction, a sensitive heme detection method is required. However, heme quantification at low concentration is technically challenging and no established, standardized procedures are available. The task is complicated by the hydrophobic character of the heme moiety that readily aggregates in aqueous solutions at physiological pH. In this thesis work, two alternative approaches for quantitation of the amount of transported heme were elaborated. First, a chemiluminescence-based method for heme determination by reconstitution with horseradish peroxidase (HRP) apo-enzyme was adapted [1, 2]. Second, an enzymatic method for generation of radioactively labeled heme was developed. Ferrochelatase enzyme was used to insert $^{55}\text{Fe}^{2+}$ into protoporphyrin IX ring. Both methods enable determination of heme at low-nanomolar concentrations in the presence of the high-affinity heme-binding protein HmuT.

2.2 Introduction
Heme concentration is most commonly measured by absorption spectroscopy either directly or after conversion to pyridine hemochrome [3, 4]. The sensitivity of these assays is a function of the sensitivity of the spectrophotometer, and too low for our purpose. Although other more sensitive colorimetric and fluorescent methods have been described [4-6], they commonly require prior extraction of heme from carrier proteins using aggressive solvents and/or solubilisation in strongly alkaline conditions. In our assay setup, heme is sequestered by the high-affinity heme-binding pockets in HmuT. Working at low concentrations, an incomplete extraction process would introduce a significant error. Furthermore, the presence of aggressive solvents might cause aggregation of HmuT and co-precipitation of heme. Elaboration of two methods, allowing quantitation of HmuT-bound heme in the physiological pH range, is described in this chapter.
2.2.1 Chemiluminescence-based method

Recently, a sensitive heme determination method by reconstitution with horseradish peroxidase (HRP) apo-enzyme was reported [1, 2]. HRP apo-enzyme spontaneously reconstitutes with its cofactor heme to form an active holo-form. HRP-catalyzed luminol oxidation leads to chemiluminescence emission. Detection of the chemiluminescence signal allows for the quantification of heme (Figure 2.1). The original assay protocol was, however, developed for determination of free heme in potassium hydroxide solution [1]. Thus, several adjustments to the original procedure were necessary to achieve compatibility with the conditions used in our transport assay. The peroxidase activity of holo-HRP is extensively used in many other biochemical applications. Most commonly, holo-HRP is conjugated to an antibody for techniques such as ELISA, Western blotting and immunohistochemistry.

![Figure 2.1: Schematic of chemiluminescence-based heme determination.](image)

Upon reconstitution with heme, holo-HRP catalyzes luminol oxidation, using hydrogen peroxide as a substrate. Luminol oxidation is detected as chemiluminescence.

2.2.2 Generation of radiolabeled heme

Radiolabeled compounds are commonly used to study transport reactions both in vitro and in vivo. Typically, measurements over a broad concentration range and under diverse conditions are possible. Radioactive heme is, however, not commercially available. Therefore, we looked for a simple labeling method.

Methods for generation of $^{14}$C-, $^{13}$C- (for NMR measurements) and $^{55}$Fe-labeled heme have been described. Biosynthetic preparation of $^{14}$C- and $^{13}$C-labeled heme is achieved by addition of labeled δ-aminolevulinic acid, the first committed precursor of heme biosynthesis, to Escherichia coli cultures [4, 7]. Yet, this method requires cumbersome chromatographic
purification of the produced heme, entailing radioactive contamination of the equipment. Alternatively, $^{55}\text{Fe}^{3+}$ has been inserted into protoporphyrin IX (PPIX) ring, generating $[^{55}\text{Fe}]$heme. However, the procedure demands strict safety measures, as $^{55}\text{FeCl}_3$ is refluxed with protoporphyrin IX in glacial acetic acid [8].

In vivo, insertion of Fe$^{2+}$ into PPIX ring, is catalyzed by ferrochelatase - the terminal enzyme in the heme biosynthesis pathway. In mammalian and yeast cells, ferrochelatase is located in the mitochondrial membrane while in bacteria it is found in the cytoplasm or associated with the cytoplasmic membrane. The soluble ferrochelatase (HemH) (~35 kDa) from Bacillus subtilis has been functionally and structurally characterized [9-13]. It was, therefore, chosen to develop a procedure for enzymatic insertion of $^{55}\text{Fe}^{2+}$ into PPIX ring in vitro (Figure 2.2).

Under aerobic conditions Fe$^{2+}$ readily oxidizes to Fe$^{3+}$, however, only divalent cations can be incorporated by ferrochelatases. Apart from Fe$^{2+}$, Zn$^{2+}$ can be inserted into PPIX ring by all characterized ferrochelatases, and is often used in assays because of the aerobic sensitivity of the true substrate [11]. Additional advantage is the strong fluorescence of Zn-PPIX, allowing to monitor the product formation easily. Thus, for initial activity tests Zn$^{2+}$ was used. For development of the final reaction protocol, means of keeping iron in the reduced ferric state had to be found. The enzyme has two metal binding sites, called inner and outer site. At the inner metal binding site, close to the protoporphyrin ring, the substrate ion (Fe$^{2+}$ or Zn$^{2+}$) is bound. The outer site binds Mg$^{2+}$, which stimulates substrate insertion (Figure 2.2a) [11].

![Figure 2.2: Structure and function of the Bacillus subtilis ferrochelatase.](image)

**Figure 2.2: Structure and function of the Bacillus subtilis ferrochelatase.**

**a.** Crystal structure in ribbon representation (PDB 1C1H). N-methyl mesoporphyrin IX in the active-site cleft and a hydrated Mg$^{2+}$ ion at the outer site are shown as sticks and spheres, respectively. Water molecules are shown as red spheres. Domain 1 is colored green, domain 2 blue, the lip-region red, the linker yellow and the $\pi$-helix orange.

**b.** Reaction catalyzed by ferrochelatase. Figure adapted from [12,13].
2.3 Experimental procedures

2.3.1 Chemiluminescence-based heme quantification

Chemiluminescence-based heme detection was adapted from a previously described method [1, 2]. It comprises two steps: reconstitution of apo-HRP with heme, and holo-HRP-catalyzed luminol oxidation. In the original protocol, the reconstitution mixture contained 2.5 nM HRP apo-enzyme in 100 mM Tris–HCl pH. 8.4, and 50 to 200 pM free heme, added as a 4x solution in 10 mM KOH. To achieve compatibility with conditions used in our transport assay, adjustments were necessary. During the optimization efforts, apo-HRP concentrations up to 4.2 nM, heme concentrations between 50 pM and 250 nM, and BSA (bovine serum albumin) as a non-specific carrier for heme were tested.

The following setup was established for determining heme concentration in the aliquots removed from the transport reaction at various time points:

1. for the reconstitution step, 25 μl of sample comprising 30 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mg/ml BSA, 50 nM HmuT, and up to 70 nM heme, were mixed with 75 μl of a solution containing 5.3 nM apo-HRP (BBI Solutions), 175 mM Tris-HCl 8.5, and 1 mg/ml BSA. The final concentrations of apo-HRP, HmuT and heme in the 100 μl reconstitution mixture were 4 nM, 12.5 nM and up to 17.5 nM, respectively. Reaction was incubated for 30 minutes at room temperature to allow for equilibration;

2. to measure peroxidase activity, 100 μl chemiluminescence detection reagent containing 10 mM luminol, 200 μM H2O2 and 152 mM Tris-HCl 8.5 was added. The 50 mM luminol stock solution was freshly prepared in 100 mM NaOH. After 32 minutes incubation at room temperature, the chemiluminescence intensity was measured in a MicroBeta2 plate counter (PerkinElmer).
2.3.2 Production of ferrochelatase, heme generation and analysis

Ferrochelatase - plasmid design and expression

*HemH* gene was amplified from *Bacillus subtilis* genomic DNA, and ligated into modified pET-19b (Novagen) vectors (Figure 2.3).

Four different expression conditions were tested for the construct shown in the figure 3.3a:

1. Induction by addition of 0.4 mM IPTG at 37°C at an OD600 of 0.3 for 1 to 6 hours,
2. Induction by addition of 0.4 mM IPTG at 37°C at an OD600 of 3.0 for 1 to 5 hours,
3. Shifting cultures from 37°C to 25°C at an OD600 of 0.8, and induction by 0.4 mM IPTG 1h after shift for 1 to 6.5 hours,
4. Shifting cultures from 37°C to 25°C at an OD600 of 0.4, autoinduction for 3 to 21 hours.

For test expression, *Escherichia coli* BL21-CodonPlus (DE3)-RIPL cells (Stratagene) were grown in 50 ml Terrific Broth (TB) medium supplemented with either 1% (w/v) glucose (for IPTG induction) or 1% (w/v) glycerol (for autoinduction) and 0.05 mg/ml ampicillin. 1 ml aliquots of the cultures were taken at various time points, cells were spun down for 1 minute at 16,100g and the supernatant was discarded. Cell pellet was weighed (typically, 4-30 mg), re-suspended in 0.5 to 1 ml of buffer containing 500 mM NaCl and 50 mM Tris-HCl pH 7.5, sonicated (Bramson Sonifier 250) and centrifuged for 10 minutes at 16,1000g. Supernatant (soluble fraction) and pellet (insoluble fraction) of 100 μg cells were analyzed using 12.5% SDS-PAGE.
Large scale expression for both constructs was done in 2 liter baffled flasks containing 800 ml medium according to the protocol 3 stated above. Cells were harvested 5 hours after induction by centrifugation at 15,810g for 10 minutes at 4°C, frozen in liquid nitrogen and stored at -80°C.

**Purification of HemH**

All steps were carried out at 4°C or on ice unless indicated otherwise. Cells were resuspended in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2.5 mM MgCl₂ at a cell mass to buffer ratio (w/v) of 1:10 and disrupted using a M-110L microfluidizer (Microfluidics) at 15,000 psi (103 MPa) external pressure (three runs through 100 µm chamber). The lysate was centrifuged at 40,000g for 30 minutes. Imidazole-HCl pH 8.0 was added to the supernatant to a final concentration of 20 mM and the supernatant was loaded onto a Ni-NTA Superflow affinity column (QIAGEN) equilibrated with 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 20 mM imidazole-HCl pH 8.0 and 2.5 mM MgCl₂. The column was washed with the same buffer, but containing 50 mM imidazole, and HemH was eluted with 300 mM imidazole. The buffer was exchanged to 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 4 mM MgCl₂ using a HiPrep 26/10 desalting column (GE Healthcare). In the initial, not optimized protocol, MgCl₂ was absent from all buffers.

For cleavage of the histidine tags with the tobacco etch virus protease (TEV, produced by F.C. Lehmann) (Figure 3.3a), DTT was added to a final concentration of 4 mM, and protease was added at a HemH to TEV mass ratio of 1:1. Sample was incubated at 4°C overnight. Reducing agent was removed by desalting into 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 4 mM MgCl₂, 25 mM Imidazole pH 8.0 (HiPrep 26/10 column, GE Healthcare). Cleaved tags together with uncleaved material and TEV protease were removed by a re-run on a Ni-NTA Superflow column. The flow-through containing untagged HemH was collected and buffer was exchanged to 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 4 mM MgCl₂ by desalting.

For the cleavage using 3C protease from human rhinovirus (HRV 3C, produced by F.C. Lehmann) (Figure 3.3b), β-mercaptoethanol was added to a final concentration of 5 mM and protease was added at a HemH to 3C molar ratio 15:1. Sample was incubated for 1.5 hours at 4°C. Imidazole pH 8.0 was added to a final concentration of 25 mM and sample was re-run on a Ni-NTA Superflow column. The flow-through was processed as after TEV cleavage.
**Ferrochelatase-catalyzed reaction**

For initial HemH activity tests, protocol adapted from [11] was used. Reaction mixture contained 1 µM PPIX (Sigma), 25 µM Zn(CH$_3$COO)$_2$ or ZnCl$_2$, 10 µg/ml (0.3 µM) HemH, 4 mM MgCl$_2$, 0.3 mg/ml Tween 20 (245 µM) and 100 mM Tris-HCl pH 7.5. Solutions were prepared as follows: i) 1 mM PPIX in DMSO, further diluted in 100 mM Tris-HCl pH 7.5, 0.3 mg/ml Tween 20, ii) Zn(CH$_3$COO)$_2$, ZnCl$_2$, MgCl$_2$ and ascorbic acid in Milli-Q H$_2$O, iii) enzyme in the desalt buffer described above. Reaction was carried out in a 1 ml quartz cuvette at room temperature with stirring. Formation of Zn-PPIX was monitored on a spectrofluorometer (QuantaMaster, Photon Technology International or F-4500, Hitachi). Emission spectra between 550 and 750 nm were recorded at an excitation wavelength of 420 nm. Slit widths were set to 5 nm.

For tests with Fe$^{2+}$, 1 mM FeCl$_3$ in 0.1 M HCl was reduced to Fe$^{2+}$ by addition of twenty-fold molar excess of ascorbic acid. Fe$^{2+}$ solution was further diluted in 100 mM Tris-HCl 7.5 and reaction carried out as described above for Zn$^{2+}$. Decrease in the educt (PPIX) concentration was followed.

To avoid waste of radioactive material, the initial 25:1 molar ratio of Fe$^{2+}$ to PPIX in the reaction had to be optimized. During the optimization efforts, 5 to 25 µM concentrations of PPIX, 0.5 to 35 µM HemH, 0.1 to 1.1 mg/ml Tween 20, and MgCl$_2$ concentrations down to 10 µM were tested. The following optimal setup was established: 25 µM PPIX, 25 µM Fe$^{2+}$, 25 µM HemH, 1 mM MgCl$_2$, 0.9 mg/ml Tween 20 (735 µM), 100 mM Tris pH 7.5.

For the labeling reaction, 23 mM stock of $^{55}$FeCl$_3$ in 0.5 M HCl (PerkinElmer, NEZ043) was used with the total activity of 13.5 MBq (0.365 mCi). $^{55}$FeCl$_3$ was diluted to 450 µM in 0.5 M HCl, reduced, and further diluted to 150 µM in 0.5 M Tris-HCl pH 8.5. Labeling was carried out using three-fold molar excess of $^{55}$Fe$^{2+}$. The total volume of 5 ml contained:

- 75 µM $^{55}$Fe$^{2+}$ (2.5 ml 150 µM in 0.5 M Tris-HCl pH 8.5, 0.18 M HCl; final pH ~ 7.0),
- 25 µM PPIX (1.25 ml 100 µM in 100 mM Tris 7.5, 3.6 mg/ml Tween 20),
- 25 µM HemH (1.25 ml 100 µM in 20 mM Tris 7.5, 150 mM NaCl, 4 mM MgCl$_2$).

Labeling reaction was carried out in a 20 ml clear glass vial with Teflon lined screw cap (Avanti Polar Lipids) for 1 hour with vigorous stirring.

**Purification of heme**

Generated heme was purified using either liquid-liquid extraction or solid phase extraction (SPE). In both procedures, heme was first separated from the ferrochelatase by addition of acidic acetone [4]. For liquid-liquid extraction, sample was mixed with an equal volume of
acidic acetone (acetone with 0.3 M HCl). After 5 min incubation, diethyl ether was added to the acidic acetone extract at a 1:5.6 volume ratio (ether : extract), and additional HCl was supplied (1:2.5 volume ratio 2M HCl : ether). After vigorous mixing, layers were allowed to separate, and the lower aqueous layer was removed using a glass pipette. Upper ether layer, containing the extracted heme, was evaporated under nitrogen stream and heme re-dissolved in DMSO.

Because of the incompatibility with the Sep Pak tC18 resin (Waters, WAT036810), used for the SPE, the amount of HCl in the acidic acetone was reduced to 15 mM. Acidic acetone extract was loaded onto the tC18 column, equilibrated with a 1:1 sample buffer (see above)/acidic acetone mixture (v/v). Column was washed with the same solution and heme was eluted with diethyl ether. Eluate was evaporated under nitrogen stream, and heme re-dissolved in DMSO.

**Analysis of generated heme**

The quality of the generated heme was analyzed using following methods: i) reconstitution with the HRP apo-enzyme, forming active holo-HRP. The same setup as described for the chemiluminescent-based heme quantitation was used, ii) UV/Vis absorption spectra were recorded between 300 and 700 nm on a Varian Cary 50 spectrophotometer. For the reference, solutions made with heme purchased from Sigma-Aldrich were used, iii) LC-UV-MS (high-performance liquid chromatography coupled to diode array UV detection and mass spectrometry) analysis was kindly carried out by Alain Blanc from the Center for Radiopharmaceutical Sciences, Paul Scherrer Institute, Villigen. LC-UV-MS analysis was performed on a Waters 2975 high-performance liquid chromatography (HPLC) module interfaced with Waters LCT Premier Electrospray Ionization - Time-of-Flight (ESI-TOF) mass spectrometer operating in the positive electrospray mode. Sample was five-fold diluted in 97.5% of 0.1% formic acid in water and 2.5% isopropanol. The compounds were separated by reverse-phase gradient HPLC on a C18 column (Interchim, UP5BP1, 150x2.1mm) with a flow rate of 0.5 ml/min. The mobile phase consisted of (A) 97.5% of 0.1% formic acid in water and 2.5% isopropanol and (B) 5.0% of 0.1% formic acid in water, 92.5% acetonitrile and 2.5% isopropanol with a following gradient: 100% A (0 - 3 min), 100% A - 100% B linear gradient (3 - 15 min), followed by 100% B for 5 min. HPLC eluate was split ‘online’ 3:1 for UV detection at 405 nm and mass spectrometry.
Radioactivity of \([^{55}\text{Fe}]\text{heme}\) was measured after mixing 50 µl of sample with 250 µl of Ultima Gold (PerkinElmer) liquid scintillation cocktail using the MicroBeta\(^2\) counter (PerkinElmer).

### 2.4 Results and Discussion

#### 2.4.1 Chemiluminescence-based assay

The original protocol [1, 2], that was used as the starting point for the establishment of the chemiluminescence-based heme quantitation method, was designed for determination of free heme in 10 mM KOH solution. Our transport assay has to be carried out at a lower pH, and heme has to be quantified in the presence of the high-affinity heme-binding protein HmuT.

In an aqueous solution at near-neutral pH, heme readily aggregates and sticks to the plastic walls of the reaction tubes and pipette tips. Therefore, application of BSA as a non-specific carrier for heme in aqueous solutions was tested (Figure 2.4). It was evident that, in the absence of a carrier, heme is aggregating during the preparation of the standard solutions and is not available for the subsequent reconstitution with the apo-HRP. In the presence of 1 mg/ml (15 µM) BSA, even low nanomolar concentrations of heme could be detected. Hence, BSA was used in all aqueous heme-containing solutions.

![Figure 2.4: Effect of BSA as an unspecific carrier for heme in aqueous solution.](image)

As shown in the figure 2.4, the chemiluminescence signal is not linear at low nanomolar heme concentrations. Therefore, different heme and apo-HRP concentrations were tested to find a linear range. Experiments were done in the presence of HmuT. Given the high affinity of HmuT for its substrate, a complete transfer of heme to apo-HRP cannot be assumed.
Rather, HmuT and apo-HRP compete for the available heme. This is demonstrated by the shift of the calibration curve in the absence of HmuT (Figure 2.5, inset). It was found that the chemiluminescence signal generated by 4 nM HRP in the presence of 12.5 nM HmuT is linear at 10-17.5 nM heme concentrations (Figure 2.5).

The linear detection range is very narrow, and, at higher heme concentrations, saturation is readily reached. This reflects the saturation of apo-HRP binding sites with heme. In the transport assay, heme concentrations up to 420 nM were used. Hence, dilution was required to bring the concentration down to the linear range of the calibration curve. First, aliquots taken from the transport reaction were diluted two- to six-fold in an ice-cold 'stop' buffer. The second four-fold dilution step occurred while mixing of the samples with the apo-HRP solution. Thus, eight- to twenty-four-fold dilution was carried out allowing to measure initial heme concentrations between 80 and 420 nM.

The chemiluminescence based heme quantification method enables determination of heme concentration at pH compatible with functional assays and in the presence of high-affinity heme-binding protein. No prior extraction of heme is needed. Limiting factors are the narrow linear detection range, and the requirement for a very controlled environment for the enzymatic luminol oxidation reaction. This imposes constrains on the sample composition. Furthermore, as fluctuations in the chemiluminescence signal between independent experiments are observed, each measurement requires a separate calibration.
2.4.2 Enzymatic radiolabeling of heme

**Ferrochelatase - expression and purification**

Production of a functional ferrochelatase enzyme was the first critical step in the generation of radiolabeled heme. The soluble, cytoplasmic ferrochelatase HemH from *Bacillus subtilis* is well characterized, and protocols for expression and purification have been published [10, 11, 14]. However, the reported procedures either involve purification of HemH from inclusion bodies, using 7M urea to solubilize the aggregated protein followed by dialysis for renaturation [11], or ammonium sulfate precipitation of the enzyme as a first purification step preceding ion exchange chromatography [10, 14]. In these reports, the more convenient and gentle Ni$^{2+}$-affinity purification was avoided due to the affinity of the histidine-tag for metal ions, which are also substrates and co-factors for HemH.

We established an expression protocol, where the aggregation of HemH and formation of inclusion bodies is reduced by expressing the protein at 25°C instead of 37°C, as described in [10, 11, 14]. Furthermore, a method for Ni$^{2+}$-affinity purification in the presence of Mg$^{2+}$, followed by a protease cleavage of the N-terminal deca-histidine tag was elaborated. A pure HemH sample, remaining monodisperse over several weeks at 4°C was obtained (Figure 2.6). The final protein yield after the purification and cleavage of the histidine tag was ~13 mg HemH/ g cells. Efficient cleavage of the histidine tag was achieved, using the 3C protease and a construct, where a six amino acid linker is introduced between the cleavage site and the HemH gene (Figure 2.3b). The presence of Mg$^{2+}$ in all purification buffers and the removal of the His$_{10}$-tag proved to be crucial for the preparation of ferrochelatase with a reproducible, consistent activity. The activity of His$_{10}$-HemH, purified and stored in the absence of Mg$^{2+}$, varied greatly between the preparations and decreased rapidly, when kept at 4°C or frozen at -80 °C. With the optimized procedure, the activity remained constant over at least two months of storage at 4 °C, -20 °C or -80 °C. The ferrochelatase enzyme has a binding site for Mg$^{2+}$ (Figure 2.2a). Mg$^{2+}$ stimulates the incorporation of the substrate ion (Zn$^{2+}$ or Fe$^{2+}$) into PPIX ring [11]. Evidently, it is also necessary to maintain the functionality of the enzyme during storage.
Ferrochelatase-catalyzed reaction

The produced HemH enzyme was used to insert $^{55}\text{Fe}^{2+}$ into PPIX ring, generating $[^{55}\text{Fe}]$heme (Figure 2.2b). To establish the procedure, tests with cold Fe$^{2+}$ and Zn$^{2+}$ were carried out. Zn$^{2+}$ is not susceptible to oxidation by air oxygen and, in contrast to heme, Zn-PPIX gives a strong fluorescence signal, making it easy to follow the product formation. The functionality of newly produced enzyme batches was tested, using 25-fold molar excess of Zn$^{2+}$ over PPIX ($25 \mu\text{M Zn}^{2+}, 1 \mu\text{M PPIX}, 0.3 \mu\text{M HemH}, 0.3 \text{mg/ml Tween 20}, 4 \text{mM Mg}^{2+}$). At these conditions, reaction proceeds rapidly, and a full conversion of PPIX to Zn-PPIX takes place within 5 minutes (Figure 2.7a).

For experiments with the true substrate Fe$^{2+}$, Fe$^{3+}$ was successfully reduced using ascorbic acid and the initial 1:25 molar ratio of PPIX to Fe$^{2+}$ was brought to 1:1 by increasing the PPIX concentration. This was necessary to avoid massive waste of $55\text{Fe}^{2+}$ in the labeling reaction. However, several issues arose during the optimization efforts. To avoid aggregation of PPIX, a concomitant increase in the Tween 20 concentration was necessary. This, however, had a negative effect on the activity of the ferrochelatase. Furthermore, with the

Figure 2.6: Purification of the Bacillus subtilis ferrochelatase HemH. a. Purification procedure was analyzed using 12.5% SDS-PAGE. Cells were cracked, and centrifuged to remove insoluble material (IS). Supernatant (S) was taken for Ni$^{2+}$-affinity purification. Samples from flow-through (FT), wash (W) and eluate (ELU) fractions are shown. To visualize eventual minor contaminants, high amount of HemH (~8 μg) was loaded in the lane ELU II. D, desalted HemH. M, protein molecular weight marker. b, Cleavage of the His$_{10}$-tag using 3C protease. Complete cleavage of the desalted HemH (D) is achieved after 1.5 h incubation with 3C protease at a molar ratio of 20:1 at 4ºC. c, Monodispersity and aggregation behavior of the purified enzyme were analyzed by SEC (Superdex 200 10/300 GL column). Gel filtration chromatogram of HemH desalt sample (6 mg/ml) kept at 4ºC for five weeks is shown. i, injection, v, void.
increasing concentrations of PPIX, the amount of MgCl₂ had to be reduced; otherwise, precipitation of PPIX into insoluble flakes was observed. As described above, Mg²⁺ is indispensable for the functionality of the enzyme and could not be left out. After testing numerous conditions (see Experimental procedures), following setup for the generation of heme was established: 25 µM PPIX, 25 µM Fe²⁺, 25 µM HemH, 1 mM MgCl₂ and 0.9 mg/ml Tween 20 (735 µM). An enzyme concentration, equal to that of the educts, and a prolonged incubation period (~1 h), was required to facilitate a near-complete conversion of PPIX to heme (Figure 2.7b).

![Figure 2.7: Ferrochelatase-catalyzed insertion of divalent ions into PPIX ring.](image)

To follow the insertion of Zn²⁺ (a) or Fe²⁺ (b) into PPIX, fluorescence emission spectra were recorded at various time points (indicated) during the reaction. PPIX displays an emission maximum at 632 nm; Zn-PPIX peak is observed at 590 nm. Excitation wavelength was set to 420 nm. a, Reaction with 25-fold molar excess of Zn²⁺ over PPIX (25 µM Zn²⁺, 1 µM PPIX) proceeds rapidly, appearance of the strongly fluorescent product Zn-PPIX is evident. 5 min and 10 min traces overlap. b, Reaction with equimolar amounts of Fe²⁺ and PPIX (25 µM Fe²⁺, 25 µM PPIX) is slower, and the generated heme shows no fluorescence. The minor peak at ~590 nm might indicate the presence of trace amounts of contaminating divalent metal ions. The considerable difference in the fluorescence counts is due to measurement on two different instruments.
Analysis of generated heme

Both for the cold and [\(^{55}\)Fe]heme, the ability to reconstitute with the HRP apo-enzyme was tested. Holo-HRP-catalyzed luminol oxidation was detected by measuring chemiluminescence signal, as in the chemiluminescence-based heme quantification method described above. Thus, apart from proofing the functionality of the generated heme, this method also allows for the determination of heme concentrations in nanomolar range. This is convenient, as very small amounts of the radioactive material are needed to perform the assay. To avoid contamination of the equipment, two further methods were applied to cold heme produced using a procedure identical to \(^{55}\)Fe-labeling: UV/Vis absorption spectra were measured, and LC-UV-MS (liquid chromatography coupled to UV detection and mass spectrometry) analysis was kindly carried out by Alain Blanc from the Center for Radiopharmaceutical Sciences, Paul Scherrer Institute, Villigen.

Absorption spectra of the generated heme were identical to the reference spectra. The educt PPIX also absorbs in the same range of the UV/Vis spectrum, the absorption maximum is, however, slightly shifted towards longer wavelength and additional distinct features are observed (Figure 2.8a). Mass spectrometry confirmed the presence of heme in the reaction mixture after 1.5 hours of incubation, and in the final purified product (Figure 2.8b). Apart from the peak corresponding to heme alone at m/z of 616, adducts with acetonitrile and DMSO were observed. Acetonitrile was used as the mobile phase in the reverse-phase HPLC preceding the mass spectrometry analysis. DMSO peak was absent, when a sample taken from the reaction mixture prior to purification was loaded. The final purified product is redissolved in DMSO, and, evidently, some solvent molecules remain associated with heme during the analysis.

According to the concentration measurements, using the chemiluminescence-based method and absorption spectrometry, the final yields of the generated heme were 50-70%, depending on preparation. As an almost complete conversion of PPIX to heme was observed, losses might be attributed to the association of the generated heme with the enzyme. As described above, enzyme concentration, equal to that of the educts, was used. When solid phase extraction (SPE) was applied for the purification of heme, no material was retained on the hydrophobic column material, unless heme was previously separated from the ferrochelatase by addition of acidic acetone. This indicates that the product remains associated with the enzyme after reaction. As the separation by acidic acetone might be incomplete, losses occur. Although the yields for both purification methods, liquid-liquid extraction and SPE, were similar, SPE was chosen for the radioactive labeling protocol. In comparison to the liquid-
liquid extraction, it is more convenient and poses less contamination risk (no extensive mixing of the phases or shaking is necessary).

Due to the long storage period and the very low specific activity of the $^{55}$FeCl$_3$ stock (700 MBq or 18.9 mCi/mg $^{55}$Fe, as of reaction date), the labeling reaction was carried out with a three-fold molar excess of $^{55}$Fe$^{2+}$ over PPIX. The yield, according to chemiluminescence-based measurement and calculated for the initial concentration of PPIX, was 70%. Counts, significantly higher than the background, could be determined for $[^{55}$Fe]heme concentrations down to ~10 nM, which provides a detection limit low enough for the use in the transport of heme.

Figure 2.8: Analysis of generated heme. a, UV/Vis absorption spectra of ten-fold diluted self-made heme, 6 µM heme standard solution and 6 µM PPIX standard solution in DMSO. Heme displays absorption maximum at 404 nm, the educt PPIX at 407 nm. b, ESI-TOF mass spectrum of purified self-made heme in DMSO. The major peaks correspond to heme (m/z 616.15), heme-acetonitrile (ACN) adduct (m/z 657.18) and heme-DMSO adduct (m/z 694.17). Acetonitrile was used as the mobile phase in the reverse-phase HPLC used to separate the compounds prior to mass spectroscopy analysis.
assay. Thus, the ferrochelatase-catalyzed insertion of $^{55}\text{Fe}$ into PPIX ring serves as a simple, convenient method for radiolabeling of heme for functional assays. The amount of equipment required for the labeling reaction and purification of the product is minimal, hence radioactive contamination of the lab instrumentation is avoided. Apart from $^{55}\text{Fe}$, $^{59}\text{Fe}$ could be used for labeling. $^{55}\text{Fe}$ was chosen because of its long half-life of 2.7 years, which allows the usage of the prepared radioactive stock over a long period of time. However, the $\gamma$-radiation of $^{59}\text{Fe}$ might provide higher sensitivity compared to the principal emission of X-rays and Auger electrons by $^{55}\text{Fe}$. The yield of the reaction might be further increased by optimizing the purification procedure.

References

3. Development of an *in vitro* heme transport assay

3.1. Abstract

The *Yersinia pestis* heme acquisition system *hmu* has been functionally characterized *in vivo* [1]. However, to study the molecular mechanism of the involved components in detail, experiments with purified protein samples in well-controlled conditions are indispensable. Here I describe the development of an *in vitro* heme transport assay, used to quantitatively measure heme uptake into proteoliposomes by the *Yersinia pestis* type II ABC importer HmuUV. Heme is a lipophilic molecule. Therefore, it had to be confined to the binding pockets of the substrate-delivery protein HmuT. For an optimal setup, HmuT-heme binding stoichiometry and affinity were determined using a combination of UV/Vis spectroscopy, isothermal titration calorimetry and microscale thermophoresis techniques. To calculate the *in vitro* transport rates, a method for the quantitation of the accessible, transport-competent fraction of HmuUV was developed. The method relays on the formation of a stable high-affinity HmuUV-T complex. Hence, the HmuUV-T complex formation was studied. Furthermore, the controls used in the transport assay were validated.

3.2 Introduction

3.2.1 Planned assay setup and open questions

The following procedure was intended to measure *in vitro* heme transport rates: purified, detergent-solubilized HmuUV is incorporated into lipid vesicles, generating proteoliposomes. To enable sustained transport, ARS (ATP regenerating system) is supplied to the interior of the vesicles. Substrate is delivered to HmuUV by heme-loaded HmuT, added to the outside. Uptake is measured by determining the decrease of the external heme concentration. To demonstrate the specificity of the reaction, various controls were designed: i) absence of ATP in ARS, ii) inability of HmuUV to hydrolyze ATP (HmuUV<sup>E173Q</sup> mutant) and iii) inability of binding protein to dock to the transporter (HmuT<sup>E77A E206A</sup> double mutant). However, several issues had to be addressed before reliable, reproducible measurements were possible, such as preventing unspecific partitioning of the hydrophobic heme moiety into the lipid bilayer, determination of the transporter orientation in the proteoliposomes, validation of controls and quantitation of transported heme (Figure 3.1).
3.2.2 Orientation of membrane-reconstituted proteins

ABC transporters can insert into the proteoliposomes in two possible orientations - with the ATPase domains facing the lumen ('right-side-out') or the exterior ('inside-out') of the vesicle. In our assay setup, only the 'right-side-out' transporters catalyze heme uptake (Figure 3.1).

To calculate the in vitro transport rates, quantification of the accessible, transport-competent fraction of the protein is crucial. Different methods have been reported to achieve this. Commonly, proteoliposomes are incubated with proteolytic enzymes (e.g., trypsin or chymotrypsin), and the sidedness of proteins is determined from gel electrophoresis patterns before and after the treatment [2, 3]. Alternatively, the orientation of membrane-reconstituted proteins can be determined from the accessibility of cysteines for thiol-specific reagents [4]. Furthermore, functional tests can be used, such as comparison of the ATPase activities of sealed and permeabilized proteoliposomes [5]. For the ABC type II importer BtuCD, the number of accessible transporters was quantitated by measuring the amount of radiolabeled substrate associated with the proteoliposomes in the presence of the binding protein and absence of ATP [6]. Most of the before-mentioned methods are, however, labor-intensive and/or indirect. Therefore, we were looking to establish a simple, straight-forward method for the determination of the accessible, transport-competent fraction of HmuUV.
3.2.3 Heme in solution & heme-binding protein HmuT

Heme is a hydrophobic molecule with low molecular weight (617 Da) that easily intercalates into lipid bilayers [7]. In aqueous solutions, heme readily forms dimers and stacked aggregates [8]. Hence, presence of free heme in the transport assay had to be avoided, and care taken while preparing all aqueous heme-containing solutions. To make sure that no free heme unspecifically enters the liposomes from outside, thus compromising the measurement, it had to be sequestered by HmuT.

Crystal structure of heme-bound HmuT was solved in our lab by D. Mattle, J.S. Woo, B.A. Götz and K.P. Locher. Unexpectedly, it revealed two stacked heme molecules in the central binding cleft (Figure 3.2) [9]. For the first time, two substrate molecules were observed in the binding pocket of a SBP. Whether this is physiological and what the binding affinities are remained, however, unclear.

3.2.4 Negative controls

HmuT surface glutamates

The glutamate residues Glu77 and Glu206 on the surface of HmuT (Figure 2.2) are conserved among binding proteins delivering substrates to metal-chelate type ABC importers [10]. The structure of the vitamin B\textsubscript{12} importer BtuCD in complex with its binding protein BtuF, revealed that the conserved glutamates of BtuF interact with patches of conserved arginine residues of the transporter. These interactions are crucial for docking, as the BtuCD-F complex could not be formed if the glutamates of BtuF were mutated to alanines [11]. Also, mutations of the analogous glutamates in the \textit{Staphylococcus aureus} ferrichrome binding protein FhuD2 resulted in decreased uptake of this siderophore [12]. Thus, the conserved surface glutamates of HmuT were also expected to play a crucial role in the docking to
HmuUV. A double alanine mutant HmuT$^{E77A\ E206A}$ was designed as a negative control for substrate delivery in the transport assay. However, the impaired docking ability of the HmuT mutant and unchanged heme binding properties had to be experimentally demonstrated.

**Conserved catalytic glutamate residue in the NBDs**

The conserved Walker B motif in the NBDs of the ABC transporters contains a catalytic glutamate residue. It acts as a general base to polarize the attacking water for ATP hydrolysis [13]. A glutamine mutation at this position mimics the protonated form of glutamate and has been demonstrated to dramatically reduce the ATPase activity in several ABC transporters [14, 15]. The glutamate-to-glutamine substitution in the Walker-B motif has also been used to trap ABC transporters in the nucleotide-bound state for crystallization, for example in the AMP-PNP-bound BtuCD-F [16] and the ATP-bound malose importer MBP-MalFGK$_2$ [17] structures. The corresponding mutation in the NBDs of the heme importer (HmuUV$^{E173Q}$) was chosen to demonstrate the requirement for ATP hydrolysis during the transport reaction. The effect on the ATPase activity of the transporter had to be determined.
3.3 Experimental procedures

3.3.1 HmuUV and HmuT - plasmid design, expression and purification

Plasmid design and protein expression

The *Yersinia pestis* hmuU, hmuV and hmuT genes were amplified from pHMU7 plasmid [1], which was kindly provided by professor R.D. Perry from the University of Kentucky, and ligated into modified pET-19b (Novagen) vectors by B.A. Götz. For plasmid maps see figures 4.2a and 4.4c in [18]. Where required or indicated, point mutations were introduced using the QuikChange site-directed mutagenesis method (Stratagene). HmuU and HmuV were co-expressed from a single plasmid, with the HmuU subunit containing an amino-terminal decahistidine tag. The binding protein HmuT was expressed with a carboxy-terminal hexahistidine tag. Both constructs were expressed in *Escherichia coli* BL21-Gold (DE3) cells (Stratagene) grown in Terrific Broth (TB) medium supplemented with 1% (w/v) glucose and 0.05 mg/ml ampicillin. HmuUV and HmuT were expressed in 5 and 2 liter baffled flasks, respectively. Expression of HmuUV was induced at 37°C at an optical cell density (OD$_{600}$) of 3.0 by the addition of 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1 hour. For HmuT expression, the temperature of the cultures was shifted from 37°C to 25°C when the OD$_{600}$ reached 0.5, and the expression was induced with 0.4 mM IPTG ~1h after shifting at an OD$_{600}$ of ~1.6 for 4 hours. All cells were harvested by centrifugation (at 15,810g for 10 minutes at 4°C) and stored at -80°C.

Purification of HmuUV

All steps were carried out at 4°C or on ice unless indicated otherwise. Cells were resuspended in 50 mM Tris-HCl pH 7.5, 500 mM NaCl at a cell mass to buffer ratio (w/v) of 1:10. Cells were disrupted using a M-110L microfluidizer (Microfluidics) at 15,000 psi (103 MPa) external pressure (two runs through 200 µm and one run through 100 µM chamber). Uncracked cells were removed by centrifugation for 12 min at 4000g. From the supernatant, the membrane fraction was isolated by ultracentrifugation at 100,000g for 30 minutes. The resulting membrane pellets were homogenized in 50 mM Tris-HCl pH 7.5, 500 mM NaCl at a 1:1 cell mass to buffer ratio (w/v) and stored at -80°C. Membranes were solubilized in 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 15% (w/v) glycerol and 1% (w/v) n-dodecyl-β-D-maltopyranoside (DDM, Anatrace) for 1 hour. This suspension was sonicated using a rod sonicator (Sonifier 250, Branson) at output 4.5 and 50% duty cycle for 1 minute every 20
minutes. Insoluble debris was removed by centrifugation at 40,000g for 30 minutes. Imidazole-HCl pH 8.0 was added to the supernatant to a final concentration of 20 mM and the supernatant was loaded onto a Ni-NTA Superflow affinity column (QIAGEN) equilibrated with 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 20 mM imidazole-HCl pH 8.0, 15% (w/v) glycerol and 0.01% (w/v) DDM. The column was washed with the same buffer, but containing 50 mM imidazole, and HmuUV was eluted with 200 mM imidazole. The buffer was exchanged to 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5 mM EDTA-NaOH pH 8.0, 1 mM dithiothreitol (DTT) and 0.01% (w/v) DDM using a HiPrep 26/10 desalting column (GE Healthcare).

**Purification of HmuT**

All steps were carried out at 4°C or on ice unless indicated otherwise. Cells were resuspended in 50 mM Tris-HCl pH 7.5, 150 mM NaCl at a cell mass to buffer ratio (w/v) of 1:10 and disrupted using a M-110L microfluidizer (Microfluidics) at 15,000 psi (103 MPa) external pressure (three runs through 100 µm chamber). The lysate was centrifuged at 40,000g for 30 minutes. Imidazole-HCl pH 8.0 was added to the supernatant to a final concentration of 20 mM and the supernatant was loaded onto a Ni-NTA Superflow affinity column (QIAGEN) equilibrated with 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 20 mM imidazole-HCl pH 8.0. The column was washed with the equilibration buffer and HmuT was eluted with 300 mM imidazole. The buffer was exchanged to 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA-NaOH pH 8.0 using a HiPrep 26/10 desalting column (GE Healthcare).

**3.3.2 Preparation and analysis of proteoliposomes**

**Membrane reconstitution of HmuUV**

Lipid preparation and protein reconstitution were performed as described previously [6, 19], with minor modifications. Egg L-α-phosphatidylcholine and *E. coli* polar lipid extract (both from Avanti Polar Lipids at 20 mg/ml in chloroform) were mixed at a ratio (v/v) of 1:3. The solvent was removed by rotary evaporation and lyophilization. Dried lipids were resuspended in 5 mM Tris-HCl 7.5, 150 mM NaCl at room temperature at a concentration of 20 mg/ml and sonicated in an ultrasonic bath. The lipid suspension was subjected to three rounds of freezing in liquid nitrogen and thawing at room temperature and was stored at -80°C until use.

For reconstitution, lipids were slowly thawed at room temperature and diluted to a concentration of 4.4 mg/ml in 10 mM Tris-HCl 8.0, 150 mM NaCl, 0.2% (v/v) TritonX-100. The mixture was equilibrated at room temperature with gentle agitation for 1 hour. Purified
HmuUV in 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5 mM EDTA-NaOH pH 8.0, 1 mM DTT, 0.01% (w/v) DDM, and 0.2% (v/v) TritonX-100 was added at a lipid to protein ratio of 50:1 (w/w) and the suspension was incubated for another hour at room temperature. To remove detergent, 40 mg/ml polystyrene beads (BioBeads SM2, BioRad) were added to the protein-lipid mixture in a series of steps: 30 minutes at room temperature, 60 minutes at 4 °C, overnight at 4 °C, and 2 hours at 4 °C. The polystyrene beads were removed and the proteoliposomes were collected by ultracentrifugation at 4°C 186,000g for 25 minutes. The proteoliposomes were washed once and resuspended at a lipid concentration of 20 mg/ml in 10 mM Tris-HCl pH 8.0, 150 mM NaCl and were either used directly or flash-frozen in liquid nitrogen and stored at -80°C until use.

Determination of the right-side-out fraction of HmuUV in the proteoliposomes

To quantitate the total amount of HmuUV in the proteoliposomes, samples were analyzed using 12.5% SDS-PAGE. The intensities of the HmuV bands were determined using the KODAK Molecular Imaging software and compared to reference amounts of purified HmuUV protein analyzed on the same gel. The protein concentration of the reference samples was determined by measuring the absorbance of purified HmuUV at 280 nm.

The fraction of right-side-out reconstituted transporter was quantified using a fluorescently labeled HmuT derivative. A HmuT mutant (Q147C) was labeled with the Alexa Fluor 488 C5 maleimide dye (Invitrogen). 80 μM HmuTQ147C in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA-NaOH pH 8.0, 0.8 mM TCEP was mixed with 0.8 mM dye and incubated overnight at 4ºC. Reaction was stopped with 15 mM β-mercaptoethanol, and excess dye removed by size exclusion chromatography (SEC) (Superdex 200 10/300 GL, GE Healthcare; buffer composition 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA-NaOH pH 8.0). The labeling efficiency was quantified by determining the conjugated protein concentration using the Bio-Rad DC protein assay and measuring the absorbance of the dye at 494 nm.

An excess of fluorescently labeled HmuT was added to HmuUV-containing proteoliposomes containing the ARS but lacking ATP (final concentrations: 1.25 mg/ml lipid, 0.8 μM HmuTQ147C-Alexa488). Samples were incubated at room temperature for 15 minutes in the dark. Proteoliposomes were then pelleted by ultracentrifugation at 4°C 186,000g for 15 minutes, washed once and resuspended in the initial volume. 1% TritonX-100 (v/v) was added to dissolve the liposomes and the fluorescence intensity was measured using a Synergy HT (BioTek) plate reader (excitation wavelength, 485 nm; emission wavelength, 516 nm). Each measurement was corrected using control liposomes devoid of transporter. Calibration
was performed both with HmuUV proteoliposomes and empty liposomes. Experiments were carried out in 30 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM MgCl2, 1 mg/ml BSA.

3.3.3 HmuT-heme binding assays

Spectroscopic titration assay
Purified HmuT was desalted into 150 mM NaCl and 20 mM Tris–HCl pH 7.5 using a HiPrep 26/10 desalting column (GE Healthcare) and diluted in the same buffer to a concentration of 5 μM as determined by amido black assay. A 0.5 mM monomeric hemin stock solution was prepared as described earlier [20] by initially dissolving 3.3 mg hemin chloride (Sigma) in 500 μL of 0.1 M NaOH to which 500 μL of 1 M Tris pH 8.0 was added. This solution was diluted with 20 mM Tris pH 7.5 and 150 mM NaCl to a total volume of 10 mL. Successive aliquots of 0.5 mM hemin (1–20 μM, in 1 μM steps) were added into a sample cuvette containing 5 μM HmuT and a reference cuvette containing buffer alone. Samples were equilibrated for 5 min after addition of each heme aliquot, and UV/Vis spectra between 300 and 700 nm were recorded with a Varian Cary 50 spectrophotometer at 20 °C.

Isothermal titration calorimetry (ITC)
A 0.5 mM monomeric hemin stock solution was prepared as described above for the spectroscopic binding assay. A 20 μM HmuT solution was prepared in the same buffer as hemin. Both the protein and the heme solutions were centrifuged and degassed for 5 min prior to the measurements. ITC measurements were carried out at 30 °C by means of a MicroCalorimetry system (MicroCal) by titrating hemin into the 20 μM HmuT solution. Heme dilution enthalpies were determined in separate experiments and subtracted. Data were analyzed with the Origin software package (OriginLab), and the thermodynamic parameters were determined by fitting the observed curves of the integrated heat per mole of added titrant.

Microscale thermophoresis (MST)
Concentration of HmuTQ147C-Alexa488 was kept constant at 5 nM. Concentration of hemin was varied between 1000 nM and 0.03 nM in 1:1 dilution steps. Experiment was carried out in the MST buffer provided by NanoTemper Technologies GmbH (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 10 mM MgCl2, 0.05 % Tween-20). Samples were spun down at 16,100g for 5 min and loaded into MST NT.115 standard glass capillaries. The measurement was performed using the Monolith NT.115 instrument (NanoTemper Technologies GmbH) at
10°C. LED power was set to 80%, MST power to 20%. Data were analyzed using the NT Analysis Software (NanoTemper Technologies GmbH).

Two models were used for data fitting:

1) $K_D$ fit (law of mass action):

$$F_{\text{NORM}}(c_T) = F_{\text{NORM,F}} + \frac{F_{\text{NORM,B}} - F_{\text{NORM,F}}}{2} \left( c_F + c_T + K_D - \sqrt{(c_F + c_T + K_D)^2 - 4c_Fc_T} \right)$$

2) Hill equation:

$$F_{\text{NORM}}(c_T) = F_{\text{NORM,F}} + \frac{F_{\text{NORM,B}} - F_{\text{NORM,F}}}{1 + \left( \frac{E_{C50}}{c_T} \right)^n}$$

$F_{\text{NORM}}$ - normalized fluorescence, measured for the respective titrant concentration $c_T$,

$F_{\text{NORM,F}}$ - normalized fluorescence of the unbound (free) state of fluorescently labeled molecules,

$F_{\text{NORM,B}}$ - normalized fluorescence of the bound state of fluorescently labeled molecules,

$c_F$ - concentration of the fluorescently labeled molecules (constant),

$c_T$ - concentration of the titrant,

$K_D$ - equilibrium dissociation constant,

$E_{C50}$ - half maximal effective concentration (concentration at which half of the fluorescently labeled molecules are bound),

$n$ - Hill coefficient.

### 3.3.4 HmuUV-T complex formation

**Size-exclusion chromatography**

Transporter was incubated with two-fold molar excess of wild-type HmuT or HmuT$_{E77A \;E206A}$ for 15 minutes at room temperature. The mixture was subjected to size exclusion chromatography (Superdex 200 10/300 GL, GE Healthcare). Two peaks were observed: The first corresponded to HmuUV or the HmuUV-T complex and the second contained excess HmuT. The tip of the first peak was collected and analyzed by 16% Tricine SDS-PAGE [21].

**Microscale thermophoresis (MST)**

Concentration of HmuT$_{Q147C-\text{Alexa488}}$ or HmuT$_{E77A \;Q147C \;E206A-\text{Alexa488}}$ was kept constant at 10 nM. For measurements with HmuT$_{Q147C-\text{Alexa488}}$, concentration of HmuUV was varied between 1000 nM and 0.03 nM. For measurements with HmuT$_{E77A \;Q147C \;E206A-\text{Alexa488}}$, concentration of HmuUV was varied between 10 μM and 0.3 nM. Experiment was
carried out in 10 mM Tris-HCl pH 8.0, 150 mM NaCl and 0.01% (w/v) DDM. Samples were spun down at 16,100g for 5 min and loaded into MST NT.115 standard glass capillaries. The measurement was performed using the Monolith NT.115 instrument (NanoTemper Technologies GmbH) at 10°C. LED power was set to 70%, MST power to 40%. Data were analyzed using the NT Analysis Software (NanoTemper Technologies GmbH. Equation for the determination of $K_D$ (see above) was used for fitting.

### 3.3.5 ATPase assays

ATP hydrolysis of wild-type HmuUV and HmuUVE$_{173}^{Q}$ in detergent solution was measured at room temperature. Reactions contained either 0.6 μM purified HmuUV or 6 μM HmuUVE$_{173}^{C}$ in 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5 mM EDTA-NaOH pH 8.0, 0.01% (w/v) DDM, 1 mM DTT, 10 mM MgCl$_2$ and excess HmuT (1.2 μM to 24 μM HmuT). 2 mM ATP was added to initiate the reaction. 50 μl aliquots were removed at various time points and mixed with 50 μl 12% SDS. Inorganic phosphate was assayed by the modified molybdate method described earlier [22]. ATPase rates during the initial 25 minutes were determined for wild-type HmuUV and during 1 hour for HmuUVE$_{173}^{Q}$. Linear regression was used to fit the data.
3.4 Results and Discussion

3.4.1 Membrane reconstitution of HmuUV and analysis of proteoliposomes

In order to measure the \textit{in vitro} heme uptake rates, HmuUV was reconstituted in proteoliposomes. Protocol for expression of HmuUV in \textit{Escherichia coli}, and a purification procedure yielding sufficient amounts of pure, monodisperse transporter was previously established by B. A. Götz [18]. HmuUV-containing proteoliposomes were formed by mixing purified transporter with detergent-destabilized liposomes, followed by application of BioBeads for detergent removal. The reconstitution efficiency of the transporter was 50-75\% depending on preparation, corresponding to incorporation of 0.01-0.015 mg HmuUV per milligram of phospholipid (Figure 3.3). This is similar to the reported reconstitution efficiency of ~50\%, resulting in 0.01 mg protein/ mg phospholipid, for the homologous vitamin B\textsubscript{12} importer BtuCD [6]. As expected, a fraction (25-50\%) of the purified transporter doesn’t insert into the liposomes, and is removed by the subsequent centrifugation and washing steps. Interestingly, considerably higher reconstitution efficiencies were achieved if HmuUV was reconstituted into previously un-extruded liposomes, indicating that large lipid vesicles with low surface curvature facilitate the insertion of the transporter. Reconstitution into vesicles extruded through a 400 nm polycarbonate membrane, resulted in an approximately six-fold reduced reconstitution efficiency, which is unexpected because extrusion prior to reconstitution is a standard procedure [19].

![Figure 3.3: Reconstitution efficiency of the HmuUV transporter. 12.5% SDS-PAGE for quantitation of the total amount of the HmuUV in the proteoliposomes. Results from three independent purifications and reconstitutions are shown, indicated as 1, 2, and 3. The intensities of the HmuV bands were determined and compared to reference amounts of purified HmuUV protein (REF) analyzed on the same gel. LIPO, proteoliposomes, M, protein molecular weight marker.](image-url)
The quantification of the total amount of HmuUV in the proteoliposomes is, however, not sufficient for the calculation of the in vitro transport rates. The relative proportion of the 'right-side-in' to 'inside-out' transporters has to be determined. In our assay setup, only the 'right-side-out' transporters catalyze heme uptake (Figure 3.1). For the quantification of the accessible, transport-competent fraction of the protein a fluorescently labeled HmuT derivative HmuT_{Q147C}-Alexa488 was used. 'Inside-out' HmuUV is unable to form high-affinity complex with HmuT. In contrast, HmuT bound to the 'right-side-out' transporters remains associated with the proteoliposomes through the centrifugation and washing steps (Figure 3.4). It has been reported for BtuCD that, in the presence of ATP, the affinity for the cognate binding protein BtuF is decreased (K_D \approx 10^{-13} \text{ M} vs. K_D \approx 10^{-11} \text{ M}) [23]. Therefore, proteoliposomes containing ARS but lacking ATP were used. Apart from the absence of ATP, the same procedure as for the transport assays was carried out - liposomes were subjected to three freeze-thaw cycles and extruded through a 400 nm membrane. 22 \pm 4 \% of HmuUV were found reconstituted in the 'right-side-out' orientation. This is consistent with the presumption that the transporters preferably insert into the liposomes with the soluble NBD domains facing outside. Directly after reconstitution, the fraction of the 'right-side-out' HmuUV might have been even lower, as the orientation is assumed to become increasingly randomized with each freeze-thaw cycle. According to [19], five cycles of freeze-thaw are needed to fully scramble the orientation of the transporter complex in the membrane. The observed ratio of the accessible transporter is higher than the 7 \pm 2\% reporter for BtuCD [6]. The difference might be attributed to the use of a different detergent in the protein preparation. While, in our case, DDM-solubilized HmuUV was combined with Triton X-100-destabilized liposomes, Triton X-100-solubilized BtuCD was used in [6]. It has been demonstrated for the Candida albicans drug resistance protein 1 (Cdr1p), that the reconstitution of DDM-solubilized protein results in proteoliposomes where Cdr1p is predominantly inserted 'right-side-out' (75-80\%), while TritonX-100-solubilized Cdr1p yields vesicles with transporter in a predominantly 'inside-out' (~70\%) orientation [5]. Thus, the choice of detergent might affect the vectorial reconstitution ratio. Furthermore, a different method was used to determine the number of accessible transporters in the BtuCD-containing proteoliposomes - the amount of radiolabeled substrate associated with the vesicles in the presence of the binding protein and absence of ATP was measured [6].
All in all, usage of a fluorescently labeled binding protein represents a novel, simple way of determining the ratio of 'right-side-in' vs. 'inside-out' reconstituted transporters. It might be suitable for other binding protein-dependent ABC type II importers that form stable high-affinity complexes with their SBPs.

### 3.4.2 HmuT-heme binding: stoichiometry and affinity

To avoid unspecific partitioning of heme into the lipid bilayer during the transport reaction, the right ratio of HmuT to heme had to be found. The two stacked heme molecules, observed in the holo-HmuT structure by D. Mattle, J.S. Woo, B.A. Götz and K.P. Locher [9], suggest a binding ratio of two hemes per HmuT. It was, however, speculated that this might be a crystallographic artifact and the second heme might not bind with physiologically relevant affinity. To assess whether HmuT binds two heme molecules in solution and what the binding affinities are, three distinct methods were used: UV/Vis spectroscopy, ITC and MTS.
Heme was titrated into HmuT solution and UV/Vis spectra were recorded between 300 and 700 nm. Heme, but not the protein, has absorption peaks in this range. Two distinct absorption maxima for the two binding events were observed, one at ∼400 nm and another at ∼375 nm. This reflects the two strong and distinct interactions of the HmuT side chains Y70 and H167 with the Fe atoms of the heme moieties (Figure 3.5). Because of the distinct spectral properties of the two protein-bound hemes and the low absorbance of hemin, a quantitative evaluation of these titrations to obtain $K_D$ values was difficult, although the results suggested that both dissociation constants are in the submicromolar range. Identical spectrum was measured for the HmuTE77A E206A double mutant, indicating that its heme-binding properties are not affected by the introduced changes.

To determine dissociation constants, ITC experiments were carried out. The results revealed two discrete heme-binding steps, the first with a subnanomolar affinity ($K_D$ of ∼0.29 nM) and the second with a lower but still rather high affinity ($K_D$ of ∼29 nM) (Figure 3.6). For a single binding event, ITC can typically determine dissociation constants between ∼ 20 nM and 20
μM [24], suggesting that a subnanomolar $K_D$ is usually outside of the experimental reach of the method. However, because there is a second heme-binding event in HmuT that is coupled to the first and has a $K_D$ within the useful range of ITC, the observed data can be successfully fit assuming a two-step, sequential binding process, which yields quantitative information about the first binding event (higher affinity) as well. The reliability of curve fitting was evaluated by J.S. Woo who performed simulations of the binding data using various combinations of dissociation constants. Data could also be reasonably fit with other combinations of dissociation constants, as long as (i) two sequential binding events are assumed, (ii) the higher $K_D$ (second heme binding, lower affinity) has a value of no larger than 30 nM, and (iii) the two dissociation constants differ by a factor of 100. This suggests that the affinity of YpHmuT for heme could, in principle, be even higher than suggested by fitting of the ITC data [9].

Heme binding by HmuT could also be detected during the test runs of the MST equipment. By fitting the preliminary data with the function for $K_D$ determination, a value of $\sim$ 50 nM was obtained (Figure 3.7a). This is in the same range as the affinity for the second binding event measured by ITC. The subnanomolar $K_D$ of the first binding event is outside of the experimental reach of the method. Interestingly, the data were better represented by the Hill equation (Figure 3.7b). The Hill coefficient suggested positive cooperativity. Even though preliminary, the results obtained using the MST method confirm the high-affinity heme binding to HmuT.

<table>
<thead>
<tr>
<th>Curve fitting model: Two sequential binding sites</th>
<th>$K_D$ (nM)</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$-T\Delta S$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st binding</td>
<td>0.29 ± 0.088</td>
<td>-2.1 ± 0.025</td>
<td>-11</td>
</tr>
<tr>
<td>2nd binding</td>
<td>29 ± 7.7</td>
<td>-1.0 ± 0.028</td>
<td>-9.4</td>
</tr>
</tbody>
</table>

Figure 3.6: ITC study of heme binding to HmuT. a, Hemin (0.5 mM) was added to a solution of 20 μM HmuT in steps and at 30 °C to a final molar ratio of $\sim$ 3.2. b, The data were analyzed using Origin software and revealed two distinct binding steps. c, The $K_D$ values were computed from the curve fittings, and the thermodynamic parameters are indicated. Figure from [9].
The ITC and spectroscopy studies confirmed the binding ratio of two hemes per HmuT. The affinities for both binding events lay in the physiologically relevant range and are similar to those of other binding proteins. For example, a $K_D$ of $\sim 15$ nM has been reported for the interaction of the vitamin B$_{12}$ binding protein BtuF with its substrate [25]. For the transport assay, a ratio of 1.4 hemes per HmuT was chosen. The binding pocket of HmuT was undersaturated to minimize free heme in solution. In the standard setup, 420 nM heme and 300 nM HmuT were used.

### 3.4.3 Studies of HmuUV-T complex formation

The above described method for the quantification of the accessible, transport competent fraction of the transporter in the proteoliposomes relays on a high-affinity complex formation with the binding protein. $K_D$ values, obtained by surface plasmon resonance, for the interaction of two ABC type II importers with their cognate binding proteins have been reported [23]. In the absence of nucleotide and substrate, vitamin B$_{12}$ importer BtuCD binds BtuF with a $1.16 \times 10^{-13}$ M affinity, while for the putative molybdate transporter (Hi1470/1) - binding protein interaction a $K_D$ of $5.72 \times 10^{-9}$ M was measured. Even though the values differ significantly, high binding affinity is observed in both cases. It was first demonstrated by B.A. Götz that HmuUV and HmuT remain associated during a SEC run and elute as a single peak [18]. This indicates formation of a stable high-affinity complex. The opposite was observed for the interaction with the binding protein mutant HmuT$^{E_{77}A \ E_{206}A}$, used as a

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**Figure 3.7**: A preliminary MST study of heme binding to HmuT. Hemin was titrated to a constant amount (5 nM) of HmuT-Alexa488. a and b represent the same data, fitted using the equation for $K_D$ determination (a) and Hill equation (b). In b, the Hill coefficient ($h$) was fixed to 2. If fitted, $h$ of $\sim 3$ was obtained. $F_{\text{NORM}}$, normalized fluorescence; EC50, concentration at which half of the HmuT-Alexa488 molecules have bound heme(s). Error bars, standard deviation; n = 2.
negative control in the transport assays - HmuT\textsuperscript{E77A E206A} eluted as a separate peak (Figure 3.8).

In a preliminary MTS experiment, the affinity of the HmuUV-T complex formation was measured for the first time. A $K_D$ of $\sim 6 \times 10^{-9}$ M was obtained (Figure 3.9a). This is in a good agreement with the value reported for the Hi1470/1-Hi1472 complex, and four orders of magnitude higher than that of BtuCD-F.

The $K_D$ for the transporter interaction with the HmuT double alanine mutant could not be determined in the applied HmuUV concentration range. It could, however, be estimated that the value is above $10^{-2}$ M (Figure 3.9b). The results are consistent with the qualitative observations from the SEC analysis. They support the usage of the wild-type HmuT as a sensor for available HmuUV, and HmuT\textsuperscript{E77A E206A} as a negative control for substrate delivery in the transport assay.
3.4.4 ATP hydrolysis by the catalytic glutamate mutant

HmuUV^{E173Q} was designed as a negative control in the transport assay to demonstrate the requirement for ATP hydrolysis during the transport reaction. The corresponding mutation in the Walker-B motif of the BtuCD transporter reduced the hydrolysis rate more than 1000-fold [14]. For HmuUV^{E173Q}, only a ~120-fold decrease was observed (~200 nmol ATP min^{-1} mg^{-1} transporter for the wild-type vs. ~1.7 nmol ATP min^{-1} mg^{-1} for the mutant). While the wild-type HmuUV hydrolyzes an ATP molecule every ~2.2 seconds, HmuUV^{E173Q} requires ~4.5 minutes. Thus, the effect of the introduced mutation was less dramatic than expected but still great enough for the usage of the HmuUV^{E173Q} as a control in the transport assays.

References


4. HmuUV-catalyzed heme transport

4.1 Abstract

Successful iron acquisition is a determinant of bacterial virulence [1]. The translocation of iron compounds across the bacterial plasma membrane is, however, poorly understood at a molecular level. This is particularly true in the case of heme, a substrate whose hydrophobicity and technically challenging quantification at low concentration has thus far prevented determination of in vitro transport rates. The newly established transport assay was used to determine in vitro rates of HmuUV-catalyzed heme transport into proteoliposomes and to establish the role of conserved residues in the translocation pathway of HmuUV. This study has been published in [2]. Furthermore, HmuUV-catalyzed uptake of the self-made Fe\textsuperscript{55}-labeled heme into proteoliposomes was tested.

4.2 Introduction

4.2.1 Architecture of HmuUV

In parallel to this thesis work, the X-ray structure of the HmuUV transporter at 3.0 Å resolution in a nucleotide-free state was solved in our lab by B.A. Götz, J.S. Woo and K.P. Locher (Figure 4.1) [2]. As expected, the structure revealed that HmuUV belongs to the type II ABC importers that include all known bacterial heme and siderophore transporters [3] as well as the structurally and functionally characterized vitamin B\textsubscript{12} transporter BtuCD–F [4-6]. The fold of the transmembrane HmuU subunit features ten TM helices for a total of 20 TM segments in the assembled transporter. Consistent with the absence of nucleotide in the

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Figure 4.1: Structure of Y. pestis HmuUV in ribbon representation (PDB 4G1U). Membrane-spanning HmuU subunits are shown in light and dark green, and nucleotide-binding HmuV subunits in red and orange. The separately determined structure of the cognate binding protein HmuT (PDB 3MD9) is depicted in light gray in an orientation that illustrates the likely docking to HmuUV. HmuT contains two stacked heme molecules in the binding pocket and two conserved glutamate residues (Glu77 and Glu206) that are essential for docking. Figure adapted from [2].
crystallization setup, a gap separating the Walker-A and ABC signature motifs at the interface of the HmuV subunits was observed.

4.2.2 Heme translocation pathway

At the interface of the TMDs, a cavity mainly formed by transmembrane helix 5 (TM5) and helix 5a (H5a) was visualized that is accessible from the periplasm but closed to the cytoplasm. That is, the transporter adopts an outward-facing conformation (Figures 4.2 and 4.3a). Given the homology to BtuCD, the observed cavity in HmuUV was assigned the function of a heme translocation pathway. A hemin molecule could be modeled into the cavity without producing steric clashes. However, the surface of the presumed translocation pathway bears no resemblance to reported binding pockets of heme-dependent enzymes, and neither for HmuUV nor for the HmuUV-T complex affinity for heme in detergent solution could be detected [7]. This is similar to observations made with the B₁₂ transporter BtuCD [8], and suggests the absence of a membrane-localized substrate-binding pocket in type II ABC importers. Both HmuUV and BtuCD adopt outward-facing conformations in the nucleotide-free apo state [6]. The protein sequences of the HmuU and BtuC subunits are 37% identical (57% similar), whereas those of HmuV and BtuD are 28% identical (45% similar). Despite the similarity in sequence, architecture and overall conformation, a superposition of HmuUV and BtuCD revealed differences in the translocation pathways (Figure 4.2). Most notably, the size of the periplasmic entrance to the translocation pathway is smaller in HmuUV. Using structure-based sequence alignments, conserved spacer residues in the TM helix 10 of HmuUV (Tyr320) and TM5 of BtuCD (Trp162) were
identified, that are responsible for different crossing angles of TM helices and thus for the smaller entrance to the translocation pathway of HmuUV relative to BtuCD. This adaptation probably reflects the relative sizes of the substrates (cobalamin, 1,355 Da, heme 617 Da).

### 4.2.3 Conserved residues in the translocation pathway

The principal contribution to the specificity of ABC importers are the high-affinity substrate binding pockets of the periplasmic binding proteins and the protein recognition at the binding protein–transporter interface. Nevertheless, a detailed sequence alignment of type II ABC importers of various iron-chelate substrates, performed by J.S. Woo, revealed many conserved HmuU residues facing the translocation pathway (Figure 4.3). Of those, Gly164 and Arg176 are uniquely conserved in heme transporters, whereas others are also present in transporters of chemically distinct substrates such as ferrichrome. The conservation of the cavity-lining residues suggests that the transmembrane subunits might contribute to the substrate specificity and it might not be exclusively dictated by the binding protein HmuT.

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**Figure 4.3: Sequence conservation in the gating helices.**

**a.** Close-up view of HmuU helices TM5, TM5’, H5a and H5a’ (prime indicates the second subunit of the HmuU dimer), with modeled hemin shown both as blue sticks and transparent surface for size comparison. HmuU side chains facing the translocation pathway are shown as sticks and labeled.

**b.** Sequence alignment covering TM5 and H5a. Sequences are of functionally characterized ABC transporters, with substrates indicated on the left. The chosen iron-chelate transporters have diverse substrates and reflect a phylogenetically dispersed sample. Arrows mark residues facing the translocation pathway of HmuUV, with red arrows depicting Gly164 and Arg176. Yp, *Y. pestis*; Bd, *S. dysenteriae*; Bp, *Bordetella pertussis*; Pa, *Pseudomonas aeruginosa*; Sm, *S. meliloti*; Vc, *Vibrio cholerae*; Pd, *Photobacterium damselae*; Ec, *E. coli*; Cj, *Campylobacter jejuni* and Va, *Vibrio anguillarum*. Figure from [2].
4.3. Experimental Procedures

Methods for expression and purification of both HmuUV and HmuT, as well as membrane reconstitution of HmuUV and analysis of the generated proteoliposomes are described in chapter 2.

4.3.1 In vitro heme transport assay

To facilitate heme uptake, an ATP-regenerating system (ARS) was incorporated into the lumen of HmuUV-containing proteoliposomes by three cycles of freezing in liquid nitrogen and thawing at room temperature in a water bath. The ARS consisted of 2.4 mg/ml creatine kinase (Roche), 24 mM Na₂-creatine phosphate (Roche), 5 mM ATP (Sigma), 10 mM MgCl₂ in 20 mM Tris-HCl 8.0, 150 mM NaCl and 1 mg/ml bovine serum albumin (BSA). The proteoliposomes were then extruded through a 400 nm polycarbonate membrane using a Mini-Extruder (Avanti Polar Lipids) and pelleted by ultracentrifugation at 4°C 186,000g for 15 minutes. They were washed once and resuspended at a lipid concentration of 10 mg/ml in a pre-cooled 'outside buffer' containing 30 mM Tris-HCl 8.0, 150 mM NaCl, 10 mM MgCl₂, 1 mg/ml BSA. The suspension was kept on ice to limit the depletion of ATP by the basal ATPase rate of HmuUV before the transport assay. For transport, proteoliposomes were diluted in outside buffer to a lipid concentration of 2.5 mg/ml and incubated at 37°C for 5 minutes before hemin-bound binding protein HmuT was added to initiate the transport reaction. The final concentrations in the assay mixture were: 2 mg/ml lipid, 40-50 nM right-side-out HmuUV, 300 nM HmuT, 420 nM hemin. 40 µl aliquots were removed at various time points and diluted into 200 µl of ice-cold outside buffer to stop the reaction. The proteoliposomes in the aliquots were pelleted by ultracentrifugation at 4°C 131,900g for 15 minutes and the supernatant was collected for determination of the heme concentration (see Quantification of heme).

To ensure that specific heme uptake was measured, several controls were performed: (i) no ATP was present; (ii) Wild-type HmuUV was replaced by the mutant HmuUV<sup>E173Q</sup> that has a impaired ATPase activity; (iii) Wild-type HmuT was replaced by the double mutant HmuT<sup>E77A/E206A</sup> that is unable to dock to HmuUV, but has unaltered heme-binding characteristics; (iv) A combination of (ii) and (iii).

To increase the consistency of the observed transport rates, each of the mutant transporters containing amino acid changes in HmuU was reconstituted into liposomes and assayed for the transport activity in parallel with wild-type transporter. The hemin uptake rate of the mutant
was compared to that of the simultaneously processed wild-type transporter to account for differences in liposome preparations. Control measurements with HmuT$^{E77A/E206A}$ were run in parallel in all cases. Rates were calculated in the linear range of the transport reaction (18 minutes for wild-type HmuUV and HmuU mutants G164A, R176K, R176Q; 27 minutes for HmuU mutants G164Y and R176A and all control reactions). All experiments were done at least in triplicate.

Calibration was performed for each transport assay with both wild-type HmuT and HmuT$^{E77A/E206A}$. HmuT and heme were added to pre-warmed wild-type HmuUV proteoliposomes containing the ARS but lacking ATP, yielding final concentrations of 2 mg/ml lipid, 300 nM HmuT and 420, 360, 300, 240 or 180 nM heme. A 40 µl aliquot was taken out immediately after mixing and processed as described above.

To test the HmuUV-catalyzed uptake of the self-made $[^{55}\text{Fe}]$heme, an experiment with the wild-type transporter and the HmuUV$^{E173Q}$ mutant as a control was carried out. Uptake was measured over 18 minutes, experiment was performed in triplicate. The radiolabeled heme was mixed with cold heme at a molar ratio of 1:2. That is, 140 nM $[^{55}\text{Fe}]$heme and 280 nM cold heme were used, for a total of 420 nM in the assay mixture. Assay was performed as described above, except a 1:2 (molar ratio) mixture of hot and cold heme in the outside buffer was directly used for calibration without mixing with the proteoliposomes. Total heme concentration in the calibration samples was 70, 56, 42, 28, 14 and 0 nM. The highest concentration corresponds to the assay mixture with 420 nM heme that has been six-fold diluted in ice-cold outside buffer to stop the reaction.

### 4.3.2 Quantitation of heme

For all experiments with the HmuU mutants, the simultaneously processed wild-type transporter and the controls, the chemiluminescence-based heme quantitation method was used, as described in chapter 2.

After the uptake experiment with $[^{55}\text{Fe}]$heme, 50 µl of each sample were mixed with 250 µl of Ultima Gold (PerkinElmer) liquid scintillation cocktail and counted using the MicroBeta$^2$ counter (PerkinElmer). Integration time was set to 20 minutes.

### 4.3.3 ATPase assays

ATP hydrolysis of HmuUV in detergent solution was measured at room temperature. Reactions contained 0.6 µM purified HmuUV, 10 mM Tris-Cl pH 8.0, 150 mM NaCl, 0.5
mM EDTA-NaOH pH 8.0, 0.01% (w/v) DDM, 1 mM DTT, 10 mM MgCl₂ and either no HmuT or 1.2 µM HmuT. 2 mM ATP was added to initiate the reaction. 50 µl aliquots were removed at various time points and mixed with 50 µl 12% SDS. Inorganic phosphate was assayed by the modified molybdate method described earlier [9]. The same set-up was used to measure the ATPase activity of HmuUV as a function of the HmuT concentration. HmuT was present at 0, 0.1, 0.2, 0.3, 0.6, 1.2, 2.4 or 8 µM. ATPase rates during the initial 25 minutes were determined, plotted vs. the applied HmuT concentrations and the data was fitted using the Michaelis-Menten model for enzyme kinetics (GraphPad 5.0d software).

For measuring the ATPase activity of HmuUV over a range of HmuT concentrations in proteoliposomes, reactions contained 3 mg/ml lipids, 0.22 µM inside-out HmuUV (quantified as described in chapter 2), 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM MgCl₂. HmuT was present at 0, 0.3, 0.5, 1, 2 or 10 µM, and was incorporated in the liposome lumen by three freeze-thaw cycles. Prior to assays, the proteoliposomes were extruded through a 400 nm polycarbonate membrane and pre-incubated at 37°C for 5 minutes before 2 mM ATP was added to initiate the reaction. ATPase rates during the initial 25 minutes were determined. Data was processed as described above.

4.3.4 HmuUV-T complex formation studies

Wild-type and mutant HmuUV were incubated with two-fold molar excess of the binding protein HmuT for 15 minutes at room temperature. The mixture was subjected to size exclusion chromatography (Superdex 200 10/300 GL, GE Healthcare). Two peaks were observed: The first corresponded to HmuUV or the HmuUV-T complex and the second contained excess HmuT. The tip of the first peak was collected and analyzed by 16% Tricine SDS-PAGE [10]. Gels were imaged using a KODAK Gel Logic 200 Imaging system and the relative intensities of the HmuT and HmuV bands were determined using the ImageJ software. The relative intensities (HmuT:HmuV band) for the HmuU mutants were normalized to the wild-type complex analyzed on the same gel. As a negative control, the HmuT mutant HmuT^{E77A/E206A} was used, because it was unable to bind to HmuUV.
4.4 Results and Discussion

4.4.1 In vitro rate of HmuUV-catalyzed heme transport

The novel assay setup, built up as described in the two preceding chapters, allowed for the determination of the in vitro heme transport rates. Generally, the chemiluminescence-based heme quantification method was used (Figure 4.4a). Previously, in vitro translocation of heme by the homologous heme transporter ShuUV from Shigella dysenteriae has been reported [11], however, only the total amount of heme released from the interior of proteoliposomes could be quantified.

For the reconstituted wild-type HmuUV at 37°C, an in vitro heme transport rate of 1.0 ± 0.1 nmol heme min⁻¹ mg⁻¹ transporter was determined. The transport was strictly dependent on ATP hydrolysis in the liposome lumen as well as successful substrate delivery to the transporter by the cognate binding protein (Figure 4.4b). The mutant HmuUV containing a glutamine in the Walker-B motif of HmuV (HmuV^{E173Q}) was transport-deficient, as was wild-type HmuUV if the binding protein double mutant HmuT^{E77A E206A} was used. As described

![Figure 4.4: In vitro heme transport assay. a, Schematic of the two-step assay. Proteoliposomes contain HmuUV (TMDs in light and dark green; NBDs in red and orange) in two possible orientations. Only right-side-out transporters catalyze heme uptake. An ATP regenerating system (ARS) is required for sustained transport, and heme is delivered to HmuUV by HmuT (light gray). The amount of transported heme is quantified using the chemiluminescence-based method. b, HmuUV-catalyzed hemin transport rates determined at 37°C. The specificity of the reaction was demonstrated by various controls: absence of ATP; inability of HmuUV to hydrolyze ATP (HmuV mutant E173Q) and inability of binding protein to dock to the transporter (HmuT^{E77A E206A} double mutant). WT, wild type. Error bars, standard deviation; n = 3.](image-url)
before, HmuTd77T E206A binds heme with unchanged affinity but does not form high-affinity complex with the transporter. The in vitro transport rate of heme by HmuUV is lower than that observed for the related cobalamin transporter BtuCD (4.3 nmol vitamin B₁₂ min⁻¹ mg⁻¹ BtuCD [12]). The difference could be attributed to the lower concentrations of HmuT and hemin in the assay (0.3 µM HmuT and 0.42 µM hemin compared to 1 µM BtuF and 5 µM B₁₂), which was required owing to the narrow linear detection range of the HRP-based heme-quantification method.

Under the applied assay conditions, HmuUV takes ~7 minutes to translocate one heme moiety. Gram-negative bacteria require ~10⁵-10⁶ iron ions per bacterial cell per generation [13, 14]. Taking into account Yersinia pestis doubling time of ~1.5 hours [15, 16], ~8000 HmuUV transporters would have to be expressed in the inner membrane of the pathogen to satisfy this requirement which is a rather high copy number. Therefore, the in vivo transport rate is most probably higher than measured in our in vitro setup. On the other hand, other iron uptake systems have been identified in Yersinia pestis [17, 18] and an alternative, hemophore-dependent heme uptake system might operate [19]. Hence, the hmu system does not have to satisfy the iron requirement of the cell alone.

4.4.2 Translocation of ⁵⁵Fe-labeled heme by HmuUV

To test if the 'self-made' [⁵⁵Fe]heme can be used to follow HmuUV-catalyzed heme uptake into the proteoliposomes, an experiment with the wild-type transporter and the HmuUVE173Q mutant as a control was carried out. A proteoliposome batch, previously assayed under the same conditions using the chemiluminescence-based quantification method was used (Figure 4.5). The initial hemin uptake rates over the first 9 minutes of the reaction determined using the two distinct methods were in a good agreement: 1.18 ± 0.08 nmol heme min⁻¹ mg⁻¹ wild-type transporter for the radioactive measurement and 1.23 ± 0.18 nmol heme min⁻¹ mg⁻¹ wild-type HmuUV for the chemiluminescent quantification (Figure 4.5b). Usually also the 18 minutes time-point was fitted with a linear regression (R² = 0.99 for the example shown in figure 4.5a, wild-type HmuUV, chemiluminescent method) and used for the calculation of the transport rates. However, in the experiment where [⁵⁵Fe]heme was used the reaction slowed down after the first 9 minutes (Figure 4.5a). To determine, whether this is related to the characteristics of the generated radiolabeled heme, further experiments are needed.

In principle, the produced [⁵⁵Fe]heme is suitable for monitoring the HmuUV-catalyzed heme uptake in vitro. In contrast to the chemiluminescent method, the radioactivity-based heme quantitation features a wide linear detection range. This allows for more flexibility in the
assay design - a wide range of heme concentrations can be employed. Furthermore, radiolabeled heme might enable in vivo measurements, for example, using _Escherichia coli_ spheroplasts containing overexpressed HmuUV. This might serve as a fast, convenient method for screening the effects of introduced mutations on the transport activity without the need for purification and reconstitution of the protein. As the $^{55}$Fe-labeled heme was synthetized shortly before submission of this thesis work, these possibilities hasn't been explored yet.

Figure 4.5: HmuUV-catalyzed translocation of $^{55}$Fe-labeled heme. Heme uptake into HmuUV-containing proteoliposomes at 37°C was followed using either the chemiluminescence-based heme quantitation method (LUMI) or $^{55}$Fe-heme. HmuUV$^{E173Q}$ was used as a negative control in both cases. a, Aliquots were removed at various time points, and the decrease in the external heme concentration was measured. Data is plotted as the amount of heme transported into the liposomes per mg transporter over time. The connecting lines do not represent a regression function. b, Initial rate of the transport reaction. Uptake rates during the first 9 minutes of the transport reaction are shown. WT, wild-type. Error bars, standard deviation, n = 3.
4.4.3 Role of conserved residues in the translocation pathway

To establish the role of the uniquely conserved residues in the heme translocation pathway of HmuU (Figure 4.3), mutagenesis studies were carried out. The effect of mutations of Gly164 and Arg176 on (i) ATP-hydrolysis rate and its stimulation by HmuT binding, (ii) the ability of the mutant HmuUV to form a complex with HmuT and (iii) *in vitro* heme transport activity was investigated.

The basal ATP hydrolysis rate of wild-type HmuUV is stimulated in the presence of the binding protein HmuT. As demonstrated in the figure 4.7, a two-fold molar excess of HmuT over HmuUV is sufficient to achieve maximal stimulation. Therefore, the same conditions, with a two-fold molar excess of the binding protein over the purified transporter in detergent, were used to test the HmuU mutants for the stimulation of the ATPase activity by HmuT (Figure 4.9a).

![Figure 4.7: HmuT dependence of HmuUV-catalyzed ATPase activity in detergent at room temperature. ATPase rates were determined over a range of HmuT concentrations (n = 3). The same conditions, with a two-fold molar excess of the binding protein, were used to test the HmuU mutants for the stimulation of the ATPase activity by HmuT. In order to fit the data with the Michaelis-Menten model, the basal ATPase rate (in the absence of HmuT) was subtracted from the data points. $K_{\text{m,app}}$, apparent Michaelis constant (μM); $v_{\text{max}}$, maximum enzyme velocity (nmol ATP min$^{-1}$ mg$^{-1}$ HmuUV). Error bars standard deviation.](image)

As described in chapter 2, the wild-type HmuUV-T complex is stable in detergent solution and elutes as a single peak on size-exclusion chromatography (Figure 2.8). To test the ability of the HmuUV mutants to form a high-affinity complex with HmuT, the amount of HmuT that eluted with HmuUV on size-exclusion chromatography was quantified. The relevant peak fraction was collected and analyzed by Tricine SDS-PAGE [10] (Figure 4.9c). The observed HmuT:HmuV ratio was normalized to wild-type HmuUV-T, analyzed on the same gel (Figure 4.9d).
For the \textit{in vitro} transport assays, each of the mutant transporters containing amino acid changes in HmuU was reconstituted into liposomes and assayed for the transport activity in parallel with wild-type transporter. The hemin uptake rate of the mutant was compared to that of the simultaneously processed wild-type transporter. Control measurements with HmuT\textsuperscript{E77A E206A} were run in parallel in all cases. A typical readout of such a transport experiment is shown in the figure 4.8. To exclude that the observed differences are due to misfolding and aggregation of the HmuU mutants, monodispersity of the purified protein in detergent was analyzed by SEC and basal ATPase activity (in the absence of HmuT) was measured in all cases (Figure 4.9a).

Figure 4.8: Representative heme transport experiment in proteoliposomes at 37\textdegree C. Wild-type HmuUV (WT) and HmuUR\textsubscript{176A}V were reconstituted into liposomes and assayed for transport activity in parallel. Measurements with the binding protein double mutant HmuT\textsuperscript{E77A E206A} were used as a negative control. Aliquots were removed at various time points, and the decrease in the external heme concentration was measured. To calculate the heme transport rates, the data were fitted with a linear regression. Error bars, standard deviation, \( n = 3 \).

The Arg176 was mutated to alanine, glutamine and lysine. Truncation of Arg176 to alanine (HmuU\textsuperscript{R176A}V) almost completely abolished transport activity. Complex formation with HmuT was reduced by ~75\%, as was the stimulation of the ATPase rate. Only slightly higher ATPase stimulation, HmuUV-T complex formation and transport rates were observed when Arg176 was mutated to a glutamine rather than an alanine. Notably, when a positive charge was present in the side chain (R176K mutation), much of the transport activity and the affinity for HmuT was retained, even though the ATPase stimulation by HmuT was only ~1.7-fold (Figures 4.8 and 4.9). These results suggest that Arg176 has a role in generating the specific recognition interface with HmuT. The positive charge at this position is crucial for the formation of a tight HmuUV-T complex and efficient substrate delivery for transport. This is consistent with the location of this residue at the periplasmic entrance to the translocation pathway (Figure 4.3). The inspection of HmuT structure did not reveal an obvious interaction partner for Arg176. Therefore, direct structure determination of the HmuUV-T complex is required to clarify this point.
The second conserved HmuU residue Gly164 marks the narrowing point in the entrance funnel of the translocation pathway (Figure 4.3). Gly164 was exchanged for alanine and tyrosine, aiming to introduce a small and a larger change in size. Whereas the HmuUG164AV mutant showed no considerable decrease in the ATPase rate, ATPase stimulation or interaction with HmuT, its transport activity was reduced by ~30%. This suggests that the two introduced methyl groups, one from each alanine residue, caused a steric hindrance for heme in the translocation pathway. Mutation of Gly164 to a tyrosine resulted in an almost complete loss of transport. Simultaneously, the basal ATPase rate of the G164Y mutant was increased two-fold, suggesting that the introduced bulky residue in the translocation pathway altered the
HmuUV dynamics or conformation, thus increasing the ATPase activity. Complex formation with HmuT was only slightly decreased, but the ATPase rate in the presence of HmuT did not exceed that of the wild-type HmuUV–T complex. This indicated that the G164Y mutant resulted in almost complete uncoupling of the ATP hydrolysis and transport activities by plugging the entrance to the translocation pathway (Figure 4.9). The results indicate that Gly164 might have a role in screening for substrate size.

The mutagenesis studies suggest that the translocation pathway of HmuUV has been optimized for efficient heme uptake, as its interior is under strong evolutionary pressure. For best efficiency, a tight fit for the transported substrate, but not a high-affinity binding pocket, seems beneficial. Thus, the TM domains introduce strict limitations on the size of the transported substrate in the absence of a specific substrate binding site in the translocation pathway. This might also reflect a mechanism developed to avoid unspecific co-transport of small solutes. Notably, the Sinorhizobium meliloti homolog of HmuUV has been reported to transport three distinct substrates: hemin, ferrichrome and ferrioxamine [20]. This suggests that some type II importers may have evolved broader specificity rather than high transport efficiency.

4.4.4 Transport stoichiometry

To investigate the stoichiometry of ATP hydrolyzed to substrate translocated, ATPase activity measurements in proteoliposomes at 37°C were carried out. As demonstrated in the figure 4.10, ATP hydrolysis rate of membrane reconstituted wild-type HmuUV is stimulated in the presence of the binding protein HmuT, consistent with the observations made with purified protein in detergent (Figure 4.7). A maximum velocity of ~300 nmol ATP min⁻¹ mg⁻¹ HmuUV was reached. Hence, an apparent stoichiometry of ~300 ATP per transported heme was calculated. This is unlikely to be physiologically relevant because of the vast amount of energy that the bacterial cell would have to invest to import heme. The 1:300 stoichiometry of heme vs. ATP could be attributed to the high basal ATPase rate in vitro, a feature observed in many purified ABC transporters. Although basal ATPase activity has been found to be an in vivo feature of the Saccharomyces cerevisiae transporter Pdr5 [21], the basal ATPase rate of HmuUV in the bacterial cell is most probably much lower than observed in vitro. The various parameters that distinguish the in vitro setup from the in vivo situation, such as membrane potential and chemical gradients, will play a role. In general, ABC transporters with two functional ATPase sites are thought to hydrolyze two ATP molecules in a single reaction cycle. However, it might be possible that, in the type II ABC transporters catalyzing uptake of
essential but scarcely available compounds, ATP hydrolysis is not necessarily tightly coupled to transport. Rather, ATP is constantly hydrolyzed at a low rate to keep the protein in a transport competent state. In vitro, also the type II vitamin B$_{12}$ importer BtuCD consumes ~100 ATP per transported substrate [12].

As described in chapter 2, HmuT binds two heme molecules in its central binding cleft with 30 and 0.3 nM affinities (Figure 2.6). This raises the possibility that HmuUV may simultaneously transport two heme molecules per reaction cycle. While this is difficult to address experimentally, our measurements suggest that also the heme moiety, bound by HmuT with ~0.3 nM affinity can be translocated. In the applied setup, HmuT, loaded with 1.4 molar equivalents of heme, was used to initiate the transport reaction (420 nM heme and 300 nM HmuT). The chemiluminescence-based heme quantification method allows for reliable determination of heme concentrations down to 10 nM (Figure 3.5). This corresponds to 240 nM heme in the assay setup, as the samples are diluted six-fold to stop the reaction followed by a further four-fold dilution with the apo-HRP solution. Thus, the lowest measurable heme concentration in the assay corresponds to 0.8 hemes per HmuT (240 nM heme and 300 nM HmuT). Heme concentrations down to this ratio have been measured in the transport assays, indicating that both hemes can be translocated. However, to investigate this in detail, an initial 1:1 molar ratio of heme to HmuT has to be used, preferably combined with the radioactivity-based heme quantitation that features a wide linear detection range and allows for more flexibility in the choice of the heme concentration range used in the assay.

![Figure 4.10: HmuT dependence of HmuUV-catalyzed ATPase activity in proteoliposomes at 37°C. ATPase rates were determined at varying HmuT concentrations in the liposome lumen (n=3). In order to fit the data with the Michaelis-Menten model, the basal ATPase rate (in the absence of HmuT) was subtracted from the data points. K$_{\text{m,app}}$, apparent Michaelis constant; $v_{\text{max}}$, maximum enzyme velocity. Error bars standard deviation.](image)
References

5. Kinetics of ATP hydrolysis and effects of disulphide crosslinking

5.1 Abstract

In ABC transporters, two ATP binding and hydrolysis sites are located at the interface of the NBD dimer. The degree of interdependence between the two sites is, however, unclear. To address this question, I examined the dependence of ATP hydrolysis on ATP concentration in the HmuUV-T system. Experiments with detergent-solubilized and membrane-reconstituted transporter were carried out. Furthermore, engineered disulfide bonds were introduced in conserved regions of the NBDs or TMDs. The effects of the crosslinks on the ATPase activity, cooperativity and sensitivity to ATP analogues were investigated.

5.2 Introduction

5.2.1 Role of two catalytic sites

In an intact ABC transporter, the NBDs assemble such that the conserved motifs, containing residues essential for ATP binding and hydrolysis, are exposed at a shared dimer interface in a 'head-to-tail' fashion. This arrangement generates two catalytic sites (Figure 1.4 and 5.1b). Positive cooperativity of ATP hydrolysis has been observed in several ABC transporters, including the vitamin B₁₂ importer BtuCD [1, 2] and the maltose importer MalFGK₂ [3]. This indicates that the two sites interact, and both might have to be occupied before the NBDs can close and hydrolyze ATP. On the other hand, in some ABC exporters, such as cystic fibrosis transmembrane conductance regulator CFTR and antigen peptide transporter TAP1/2, only one of the two nucleotide binding sites retains all of the highly conserved residues essential for ATP hydrolysis, suggesting that hydrolysis at one site only is sufficient for function [4].
Figure 5.1: Engineered disulphide bonds covalently linking the NBD subunits. a, Fragment of the protein sequence alignment of the NBD subunits BtuD and HmuV. The D-loop residues mutated to cysteine and used for crosslinking both in BtuD and HmuV are indicated by black arrow. The gray arrow indicates the position of the A177, crosslinked in HmuV only. Conserved sequence motifs are highlighted and labeled. b and c, The NBD dimers BtuD$_2$ (b) and HmuV$_2$ (c) viewed from the interface with the TMD domains. The two NBD subunits are colored in dark and light gray. Residues used for crosslinking are labeled in violet and residue numbers are indicated. b, The NBD subunits from the AMP-PNP-bound BtuCD-F (PDB 4FI3). The engineered NBD-linking disulphide bond is shown as yellow sticks. The bound AMP-PNP, Mg$^{2+}$ and sequence motifs essential for hydrolysis are labeled. The same color code is used in all panels. The catalytic Walker B glutamate (in salmon) is exchanged to glutamine in BtuD and the D-loop aspartate is not visible, therefore labeled in c. c, The NBD subunits from the apo HmuUV (PDB 4G1U). The dashed lines indicate the distances between the C$\alpha$ atoms of opposing S176 (black line, 5.7 Å) and A177 (violet line, 10.1 Å) residues. Prime indicates residues in the second subunit of the NBD dimer.
5.2.2 Application of engineered disulphide bonds

In transporter proteins, disulphide crosslinking has been used both for functional studies to investigate the proximity of selected residues in different conformational states [5, 6] and for structural analysis to trap labile conformations [1, 7]. In particular, a covalent disulphide bond between the two NBDs was used to trap the BtuCD-F complex in the AMP-PNP-bound state for crystallization [1]. The Asn162 residue in the conserved D-loop of both BtuD subunits was replaced with cysteine and, upon Cu²⁺ oxidation, a crosslink was formed (Figure 5.1a,b). The choice of the residue was based on previously reported high-resolution structures of nucleotide-bound NBDs, where the equivalent residues were found in close contact [4, 8-10]. The introduced crosslink reduced the ATP hydrolysis rate ~20-fold but the positive cooperativity of the ATPase reaction, K₀.₅ for ATP and sensitivity to the non-hydrolysable ATP analogue AMP-PNP remained unaltered. As the HmuV and BtuD subunits are 28% identical and 45% similar, it was investigated if the same approach might be applied to stabilize HmuUV-T in a nucleotide bound conformation for structural studies. The HmuV residue Ser176, corresponding to Asn162 in BtuD, and the neighboring Ala177 residue were chosen for crosslinking tests (Figure 5.1a,c). Functional analysis of HmuUV-T variants containing engineered disulfide bridges revealed unexpected effects, distinct from those observed in BtuCD-F.

5.2.3 Two cytoplasmic gates in ABC type II transporters

In the AMP-PNP bound BtuCD-F structure, a new conformation of the membrane-spanning BtuC subunits was observed. The central translocation pathway is sealed from both sides, and an enclosed cavity is formed halfway across the membrane. Notably, from the cytoplasmic side, the translocation pathway is closed by a distinct cytoplasmic gate as was previously observed for the outward-facing apo BtuCD [11]. The cytoplasmic loops connecting TM4 and TM5 (cytoplasmic gate I) have moved out and are replaced by the loops linking TM2 and TM3 (the newly recognized cytoplasmic gate II) (Figure 5.2a) [1]. The relevance of the observed conformation of the transmembrane helices was confirmed by disulphide crosslinking in solution in the absence of the NBD-stabilizing disulphide bond that was introduced for structural analysis. Sequence alignments indicated that the cytoplasmic gate II may be conserved in type II ABC importers. The residues Asn83 and Leu85 (BtuC numbering) were identified as the most conserved ones in the loop connecting TM2 and TM3.
Indeed, closure of the cytoplasmic gate II upon nucleotide binding has recently been demonstrated also for the ABC type II importer MolBC (formerly known as HI1470/71). EPR spectroscopy and disulphide crosslinking of the corresponding Asn89 and Leu91 residues in MolB was employed [6]. In the outward-facing apo HmuUV structure, the equivalent Asn82 and Leu84 residues are 22 Å and 19.2 Å apart, respectively (distances between Cα atoms) (Figure 5.2b). It was tested by disulphide crosslinking, whether the closed conformation of the presumed cytoplasmic gate II can also be detected in HmuUV. Effects of the introduced crosslinks on the ATPase reaction were studied.

Figure 5.2: Arrangements of cytoplasmic gates in the AMP-PNP-bound BtuCD-F (a, b) and apo HmuUV (c, d). a, c Schematic of the arrangements of the TMD domains. Green triangles symbolize the cytoplasmic gate II. b, d View on the transmembrane domains from the cytoplasmic side of the membrane (indicated by a pink half-circle in a and c). NBDs are removed. TM subunits are indicated as light grey ribbons, with coupling helices and gating helices TM5, cytoplasmic gate I and cytoplasmic gate II regions in light blue and dark green for HmuU (d). The dashed line line indicates the distance between the Cα atoms of opposing L85 (b) and L84 (d) residues. Prime indicates helices or residues in the second subunit of the TMD dimer. (BtuCD-F, PDB 4FI3; HmuUV, PDB 4G1U). Panel b from [1].
5.3 Experimental Procedures

5.3.1 Plasmid design, expression and purification

The *Yersinia pestis* *hmuU*, *hmuV* and *hmuT* genes were transferred from plasmids described in [12] to modified pET-19b (Novagen) vectors encoding 3C protease cleavage sites (Figure 5.3). Point mutations were introduced using the QuikChange site-directed mutagenesis method (Stratagene). Expression of HmuT and HmuUV was carried out as described in chapter 3.

Purification of the HmuT was performed as described in chapter 3 with subsequent cleavage of the hexa-histidine tag. β-mercaptoethanol was added to the desalted protein to a final concentration of 5 mM and 3C protease from human rhinovirus (HRV 3C, produced by F.C. Lehmann) was added at a HmuT to 3C molar ratio 15:1. Sample was incubated for 1.5 hours at 4ºC. Imidazole pH 8.0 was added to a final concentration of 25 mM and sample was re-run on a Ni-NTA Superflow affinity column (QIAGEN). The flow-through containing untagged HmuT was collected and buffer was exchanged to 20 mM Tris-HCl pH 7.5, 150 mM NaCl by desalting (HiPrep 26/10 column, GE Healthcare).

Purification of HmuUV was performed as described in chapter 3 with minor modifications. All procedures before oxidative crosslinking were performed in the presence of 5 mM β-mercaptoethanol. For the HmuUV*_{C131S S176C C229S}* and HmuUV*_{C131S A177C C229S}* constructs, the eluate from the Ni$^{2+}$-affinity purification was supplemented with ~5x excess of tag-less
HmuT and directly desalted into non-reducing buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.01% (w/v) DDM) using a HiPrep 26/10 desalting column (GE Healthcare).

For the HmuUL84CVC131S C229S, HmuUC67A L84C C205IVC68V C131S C229S and HmuUN82CVC131S C229S constructs, 3C cleavage of the deca-histidine tag was performed prior to crosslinking. Transporter was desalted into 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.01% (w/v) DDM, 5 mM β-mercaptoethanol and 3C protease was added at a HmuUV to 3C molar ratio 1:6. Sample was incubated for 2 hours at 4°C. Imidazole pH 8.0 was added to a final concentration of 25 mM and sample was re-run on a Ni-NTA Superflow affinity column (QIAGEN). The flow-through containing untagged HmuUV was collected and the reducing agent was removed by desalting.

5.3.3 Crosslinking

For the HmuUV_{C131S S176C C229S} and HmuUV_{C131S A177C C229S} constructs, 0.5 mM CuCl₂ was added to the desalted protein already containing excess HmuT. Samples were incubated at room temperature for 1 hour, the reaction was stopped by adding 0.6 mM EDTA and the Cu-EDTA removed by desalting into 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.01% (w/v) DDM (HiPrep 26/10 desalting column, GE Healthcare). The excess binding protein was removed by size-exclusion chromatography (Superdex 200 10/300 GL, GE Healthcare). The fraction containing the HmuUV-T complex was collected and used for functional assays.

For the HmuUL84CVC131S C229S, HmuUC67A L84C C205IVC68V C131S C229S and HmuUN82CVC131S C229S constructs, ~15x molar excess of untagged HmuT, 2 mM ATP and 4 mM CuCl₂ were added to the transporter immediately after desalting into non-reducing buffer. Samples were incubated for 30 minutes at room temperature, the reaction was stopped by adding 5 mM EDTA and the protein further processed as described above for the other constructs.

In the aliquots taken for SDS-PAGE analysis, reaction was quenched by 3 mM N-ethylmaleimide and non-reducing sample buffer was added. A shift of the protein band on SDS-PAGE was used as a measure of disulphide bond formation.

5.3.3 ATPase assays

ATP hydrolysis of HmuUV-T in detergent solution was measured at room temperature. Reactions contained 0.3 µM purified HmuUV-T (except of 1.2 µM for HmuUV_{C131S A177C C229S}T in non-reducing conditions), 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.01% (w/v) DDM, 5 mM MgCl₂ and either no DTT or 10 mM DTT. 2 mM ATP was added to initiate the
reaction. 50 µl aliquots were removed at various time points and mixed with 50 µl 12% SDS. Inorganic phosphate was assayed by the modified molybdate method described earlier [13]. Statistical tests were performed using GraphPad Prism 5 software (one-way analysis of variance (ANOVA) followed by Tukey’s test).

The same set-up was used to measure the ATPase activity of HmuUV-T as a function of the ATP concentration. 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2 or 5 mM ATP was added to initiate the reaction. ATPase rates during the initial 30 minutes were determined.

For measuring the ATPase activity of HmuUV over a range of ATP concentrations in proteoliposomes, reactions contained 2.2 mg/ml lipids, 0.15 µM inside-out HmuUV (quantified as described in chapter 3), 20 mM Tris-HCl pH 8.0 and 150 mM NaCl. HmuT was present at 10 µM, and was incorporated in the liposome lumen by three freeze-thaw cycles. Prior to assays, the proteoliposomes were extruded through a 400 nm polycarbonate membrane and 2 mM ATP and 5 mM MgCl2 were added to initiate the reaction. ATPase rates over 1 hour at room temperature were determined.

Data was analyzed using either the Michaelis-Menten model assuming no cooperative effects during hydrolysis:

\[
V = \frac{V_{\text{max}} [S]}{K_m + [S]}
\]

or using the Hill equation:

\[
V = \frac{V_{\text{max}} [S]^n}{[K_{0.5}]^n + [S]^n}
\]

where V - velocity, \(V_{\text{max}}\) - maximum enzyme velocity, [S] - substrate concentration (ATP concentration in the particular case), \(K_m\) - Michaelis constant, \(K_{0.5}\) - the concentration of substrate where \(V = \frac{1}{2}V_{\text{max}}\), n - Hill coefficient. The GraphPad 5.0d software was used.

For the determination of half-maximal inhibitory nucleotide concentrations for AMP-PNP and ADP-AlF4, single time points were taken within the linear range of hydrolysis reactions and in the presence of increasing nucleotide concentrations, which ranged from 1 µM to 3.3 mM for AMP-PNP and 2 µM to 6.7 mM for ADP-AlF4. Experiments were performed with HmuUV-T in detergent solution at room temperature either in non-reducing conditions or in the presence of 10 mM DTT. Data were fitted with an inhibitory dose-response curve as follows:

\[
V_{\%} = Bottom\ Plateau + \frac{Top\ Plateau - Bottom\ Plateau}{1 + 10^{(log(t) - log(ICSO))}}
\]
where $V_\%$ - enzyme velocity expressed as % of control, $[I]$ - concentration of the inhibitor, $\log IC_{50}$ - the log of the concentration of inhibitor that results in $V_\%$ half-way between bottom and top plateaus.
5.4 Results and Discussion

5.4.1 Cooperativity of the ATP hydrolysis sites

To study the crosstalk between the two nucleotide-binding and hydrolysis sites of the HmuV dimer, the effects of ATP concentration on the reaction rate were studied. Measurements were performed with HmuUV-T complex solubilized in dodecyl maltoside (DDM) and with membrane-reconstituted HmuUV in the presence of excess HmuT in the proteoliposome lumen. As demonstrated in the figure 5.4, the obtained data are well represented both by Michaelis-Menten and Hill models, the later showing marginally higher R squared values. An approximately three-fold higher maximal velocity is reached in the detergent solution. This might reflect somewhat reduced conformational flexibility of the transporter in the lipid environment at room temperature. As described in the chapter 4, ATPase rates of ~300 nmol ATP min\(^{-1}\) mg\(^{-1}\) reconstituted transporter are reached at 37°C (Figure 4.10). The obtained Hill coefficients indicate that there might be some positive cooperativity between the two catalytic sites in the detergent solution, while the value for the membrane-reconstituted HmuUV is slightly below 1. In contrast, pronounced positive cooperativity in ATP hydrolysis has been observed for the BtuCD-F complex in detergent solution [1] and for the liposome-reconstituted BtuCD [2]. These results indicate that the two catalytic sites in BtuD are more interdependent than in HmuV.

However, studies on the kinetics of the ATPase reaction are often complicated by the effects of the differences in sample preparation. In contrast to the above mentioned explicit positive cooperativity observed for BtuCD-F in octaethylene glycol monododecyl ether (C\(_{12}\)E\(_8\)) solution [1], no cooperativity was detected for BtuCD alone solubilized in \(n\)-dodecyl-\(N,N\)-dimethylamine-\(N\)-oxide (LDAO) [2]. Furthermore, decrease in the degree of positive cooperativity with increasing pH (pH 5 to 8) was observed for DDM-solubilized but not for membrane reconstituted maltose importer MalFGK\(_2\) [3]. While choice of the detergent is limited by the aggregation behavior of HmuUV, measurements with varying pH for the DDM-solubilized sample and at 37°C for the membrane-reconstituted transporter are necessary to further investigate the reaction kinetics.
5.4.2 Native cysteines in HmuUV

The assembled wild-type HmuUV contains a total of 20 cysteine residues, five in each TM domain and five in each NBD. For disulphide crosslinking studies and oftentimes also for structural analysis, a cysteine-less background is optimal. However, it was demonstrated by B.A. Götz that exchange of all native cysteine residues to serines or alanines causes severe misfolding and aggregation of the protein. Therefore, attempts were made to stepwise exchange the native cysteines, starting from the least conserved ones, for residues, chosen by protein sequence alignments and investigation of the HmuUV crystal structure. Half of the cysteine residues, two in each TMD and three in each NBD, were successfully mutated without inducing negative effects on the aggregation behavior and function of the protein. In fact, the removal of cysteines from the NBD domains causes increase in the ATPase rate, especially when assayed under reducing conditions (Figure 5.5a). This indicates that oxidative cysteine modifications, particularly in the NBD subunits, interfere with the ATP

**Figure 5.4: Kinetics of ATP hydrolysis by HmuUV.** ATPase activity of detergent solubilized (a) and membrane-reconstituted (b) HmuUV was measured as a function of ATP concentration at room temperature. Curves were generated by fitting the data to the Michaelis-Menten equation (dashed line) and to the Hill equation (solid line). Insets represent magnification of the data up to 0.5 mM ATP. The determined parameters are summarized in c, $V_{max}$, maximal enzyme velocity (nmol ATP min$^{-1}$ mg$^{-1}$ HmuUV); $K_{0.5}$, ATP concentration at half-maximal enzyme velocity (mM); $h$, Hill coefficient; $R^2$, coefficient of determination (R squared); $K_m$, Michaelis constant (mM). Values are shown as mean ± standard deviation; n = 3.

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>$V_{max}$ (nmol ATP min$^{-1}$ mg$^{-1}$ HmuUV)</th>
<th>$K_{0.5}$ (mM)</th>
<th>$h$</th>
<th>$R^2$</th>
<th>$V_{max}$ (nmol ATP min$^{-1}$ mg$^{-1}$ HmuUV)</th>
<th>$K_m$ (mM)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detergent solution</td>
<td>209 ± 2.76</td>
<td>0.18 ± 0.01</td>
<td>1.18 ± 0.05</td>
<td>0.9959</td>
<td>218 ± 2.95</td>
<td>0.21 ± 0.01</td>
<td>0.9925</td>
</tr>
<tr>
<td>Proteoliposomes</td>
<td>74 ± 3.1</td>
<td>0.29 ± 0.04</td>
<td>0.81 ± 0.05</td>
<td>0.9948</td>
<td>66 ± 1.2</td>
<td>0.20 ± 0.01</td>
<td>0.9901</td>
</tr>
</tbody>
</table>
hydrolysis. Therefore, the combination of reduced number of cysteine residues and presence of reducing agent is optimal. The number of cysteines did not seem to affect the sensitivity to ATP analogues or $K_{0.5}$ for ATP (Figure 5.5b-e). A slight increase in the Hill coefficient was observed, however, better statistics are needed to prove this point. The abbreviations Cys16, Cys14 and Cys10 used in the following subchapters and figure legends indicate the number of remaining cysteine residues in the full transporter. The introduced mutations are detailed in figure 5.5e. Both Cys16 and Cys10 backgrounds were used for crosslinking studies.

Figure 5.5: Functional characterization of HmuUV mutants with reduced numbers of cysteine residues. Measurements were performed with detergent-solubilized wild-type (WT) HmuUV-T complex and cysteine-reduced HmuUV-T variants. Cys16, Cys14 and Cys10 indicate the number of cysteine residues remaining in the full transporter. Introduced mutations are detailed in e. a, ATPase activity. Measurements were done at least in triplicate. NS, not significant, ****P ≤ 0.0001 b, Inhibition of ATPase activity by AMP-PNP. c, Inhibition of ATPase activity by ADP-AlF₄. d, Determination of $K_{0.5}$ for ATP. Data fitted using the Hill equation. Inset represents magnification of the data up to 0.5 mM ATP. e, Table summarizing the parameters obtained from b, c and d. $K_{0.5}$, ATP concentration at half-maximal enzyme velocity; h, Hill coefficient; IC50, half-maximal inhibitory concentration; ND, no data; n, number of experiments.
5.4.3 Engineered disulphide bonds linking the NBDs

Two residues in the conserved D-loop of the NBD subunits were chosen for exchange to cysteines and subsequent oxidative crosslinking - Ser176 and Ala177. Ser176 corresponds to the Asn162 used to stabilize the nucleotide-bound conformation of BtuCD-F for structural studies (Figures 5.1 and 5.6) [1]. S176C and A177C mutations were introduced on the Cys16 background, resulting in HmuUV$_{C131S}$ S176C C229S (S176C$_{Cys16}$) and HmuUV$_{C131S}$ A177C C229S (A177C$_{Cys16}$) constructs, respectively.

In the apo HmuUV crystal structure, the Cα atoms of the juxtaposed Ser176 and Ala177 residues are 5.7 Å and 10.1 Å apart, respectively (Figures 5.1c and 5.6). An almost complete spontaneous crosslinking of the HmuV domains containing the S176C mutation was observed even in the presence of β-mercaptoethanol (Figure 5.7a). This reflects the close proximity of the residues and indicates limited accessibility of the site to the reducing agent. Despite of the larger distance, some spontaneous crosslinking of the HmuV domains was also observed for the A177C mutant (Figure 5.7b). Upon oxidation by Cu$^{2+}$, a virtually complete crosslinking was achieved for both constructs. No addition of nucleotide was necessary. Notably, no unspecific crosslinking of the sixteen remaining native cysteines was detected by SDS-PAGE analysis. The effects of the NBD-linking disulphide bridges on (i) ATPase activity, (ii) sensitivity to ATP analogues and (iii) response to varying ATP concentrations were investigated.

![Figure 5.6: Close-up view of the D-loop regions of apo HmuUV (blue) and apo BtuCD (gray).](image)

The superimposition was generated by aligning the HmuV (PDB 4G1U) and BtuD (PDB 1L7V) subunits using the molecular graphics program PyMOL (RMSD = 3.3 Å). HmuV residues S176 and A177, and BtuD residue N162 were used for crosslinking. The dashed line indicates the distance between the Cα atoms of opposing N162 residues. The conserved D-loop aspartates and catalytic glutamates are labeled. Prime indicates residues in the second subunit of the NBD dimer.

In the apo HmuUV crystal structure, the Cα atoms of the juxtaposed Ser176 and Ala177 residues are 5.7 Å and 10.1 Å apart, respectively (Figures 5.1c and 5.6). An almost complete spontaneous crosslinking of the HmuV domains containing the S176C mutation was observed even in the presence of β-mercaptoethanol (Figure 5.7a). This reflects the close proximity of the residues and indicates limited accessibility of the site to the reducing agent. Despite of the larger distance, some spontaneous crosslinking of the HmuV domains was also observed for the A177C mutant (Figure 5.7b). Upon oxidation by Cu$^{2+}$, a virtually complete crosslinking was achieved for both constructs. No addition of nucleotide was necessary. Notably, no unspecific crosslinking of the sixteen remaining native cysteines was detected by SDS-PAGE analysis. The effects of the NBD-linking disulphide bridges on (i) ATPase activity, (ii) sensitivity to ATP analogues and (iii) response to varying ATP concentrations were investigated.
The presence of the S176C-S176C' link between the HmuV subunits did not significantly affect the ATP hydrolysis. However, upon reduction of the disulphide bond with 10 mM DTT, ATPase activity ~2.5-fold lower than that of the background control was measured (Figure 5.8a). Thus, a free cysteine at this position (or a cysteine adduct with DTT) is not well tolerated. This is distinct from the effect of the corresponding N162C mutation in BtuD, where the ATPase rate was reduced ~20-fold in the presence of the NBD-linking bond. Upon reduction, the activity recovered to almost a half of that of the control [1]. In the apo BtuCD structure [11], the opposing Asn162 residues are located 9.2 Å apart (Cα to Cα distance) (Figure 5.6). A disulphide bond restricts the maximum distance to ~6.5 Å, so that the conformational flexibility of the region might be restrained, affecting the ATP hydrolysis. The distance between the Ser176 residues in the apo HmuUV structure is 5.7 Å only. Thus, a disulphide bridge might not significantly restrict the intrinsic flexibility of the D-loop region.

**S176C - S176C' crosslink**

The presence of the S176C-S176C' link between the HmuV subunits did not significantly affect the ATP hydrolysis. However, upon reduction of the disulphide bond with 10 mM DTT, ATPase activity ~2.5-fold lower than that of the background control was measured (Figure 5.8a). Thus, a free cysteine at this position (or a cysteine adduct with DTT) is not well tolerated. This is distinct from the effect of the corresponding N162C mutation in BtuD, where the ATPase rate was reduced ~20-fold in the presence of the NBD-linking bond. Upon reduction, the activity recovered to almost a half of that of the control [1]. In the apo BtuCD structure [11], the opposing Asn162 residues are located 9.2 Å apart (Cα to Cα distance) (Figure 5.6). A disulphide bond restricts the maximum distance to ~6.5 Å, so that the conformational flexibility of the region might be restrained, affecting the ATP hydrolysis. The distance between the Ser176 residues in the apo HmuUV structure is 5.7 Å only. Thus, a disulphide bridge might not significantly restrict the intrinsic flexibility of the D-loop region.
In contrast to the largely unchanged ATPase activity, the sensitivity to the non-hydrolysable ATP analogue AMP-PNP and to the ADP plus $\gamma$-phosphate mimic aluminum fluoride (ADP-AlF$_4$) was severely decreased (Figure 5.8b,c,e). This makes the S176C mutant an unfavorable candidate for the co-crystallization with ATP analogues to obtain the structure of nucleotide-bound HmuUV-T. The large shift in the IC50 curves in comparison to the wild-type transporter indicates that rearrangements in the nucleotide-binding site have taken place, and the structure of such a construct might not reflect the native ATP-bound state. No such change in the sensitivity for AMP-PNP was observed for the N162C-N162C' crosslinked BtuCD [1]. The crosslink didn't affect the $K_{0.5}$ for ATP or the cooperativity of the hydrolysis reaction. Surprisingly, the $K_{0.5}$ value decreases and Hill coefficient goes up upon reduction.

Figure 5.8: Functional characterization of HmuUV$_{C131S}$ S176C C229S (S176C$_{Cys16}$). S176C mutation was introduced on the Cys16 background, where 16 indicates the number of cysteine residues remaining in the full transporter. Introduced mutations are detailed in e. Data obtained for the wild-type transporter and/or Cys16 mutant are shown for comparison. a, ATPase activity. Measurements were done at least in triplicate. NS, not significant. b, Inhibition of ATPase activity by AMP-PNP. c, Inhibition of ATPase activity by ADP-AlF$_4$. d, Determination of $K_{0.5}$ for ATP. Data fitted using the Hill equation. Inset represents magnification of the data up to 0.5 mM ATP. e, Table summarizing the parameters obtained from b, c and d. $K_{0.5}$, ATP concentration at half-maximal enzyme velocity; h, Hill coefficient; IC50, half-maximal inhibitory concentration; ND, no data; n, number of experiments.
**A177C - A177C’ crosslink**

As the S176C mutation proved not to be suitable for structural studies, the effects of cysteine mutation and oxidation of the neighboring Ala177 residues were investigated. The A177C-A177C’ link between the HmuV domains caused a ~50-fold decrease in the ATP hydrolysis rate (Figure 5.9a). This is most probably explained by severely restricted conformational flexibility of the D-loop region in the presence of the disulphide bridge. In the apo HmuUV structure, the distance between the Ca atoms of the juxtaposed Ala177 residues is 10.1 Å (Figures 5.1c and 5.6). Limiting the distance to ~6.5 Å might cause a distortion in the adjacent α-helix, thus interfering with the hydrolysis. Upon reduction of the disulphide link, ~50% of the activity was recovered.

![Figure 5.9: Functional characterization of HmuUV C131S A177C C229S (A177CCys16).](image)

A177C mutation was introduced on the Cys16 background, where 16 indicates the number of cysteine residues remaining in the full transporter. Introduced mutations are detailed in e. Data obtained for the wild-type transporter and/or Cys16 mutant are shown for comparison. a, ATPase activity. Measurements were done at least in triplicate. b, Inhibition of ATPase activity by AMP-PNP. c, Inhibition of ATPase activity by ADP-AlF4. d, Determination of K0.5 for ATP. Data fitted using the Hill equation. Inset represents magnification of the data up to 0.5 mM ATP. e, Table summarizing the parameters obtained from b, c and d. K0.5, ATP concentration at half-maximal enzyme velocity; h, Hill coefficient; IC50, half-maximal inhibitory concentration; ND, no data; n, number of experiments.
The sensitivity to AMP-PNP and ADP-AlF₄ was slightly decreased but the shift in the IC50 curves was less pronounced than in the presence of the S176C-S176C' crosslink (Figure 5.9b,c). However, the Hill coefficient indicated a negative cooperativity between the two catalytic sites (Figure 5.9d,e). This implies that, upon binding of ATP at one site, unfavorable, non-native rearrangements at the other site might take place. Thus, also a nucleotide-bound HmuUV-T structure stabilized by the A177C-A177C' crosslink might not correctly reflect the native ATP-bound state.

Ser176 and Ala177 are located in a close proximity of strictly conserved residues crucial for ATP hydrolysis, for example, the catalytic Glu173 (Figure 5.6). Therefore, rearrangements in the catalytic site upon introduction of an adjacent non-native disulphide bridge are highly probable and careful functional analysis of the engineered constructs is necessary to validate them as targets for structural studies.

5.4.4 Cytoplasmic gate II in HmuUV

A previously unrecognized cytoplasmic gate formed by the loops connecting the TM2 and TM3 was observed in the AMP-PNP bound BtuCD-F structure and named cytoplasmic gate II [1] (Figure 5.2a). It was tested by disulphide crosslinking, whether the closed conformation of the presumed cytoplasmic gate II can also be detected in HmuUV. Two conserved residues in the loops connecting TM2 and TM3 were exchanged to cysteines and subjected to oxidation by Cu²⁺. The L84C mutation was introduced on Cys16 and Cys10 backgrounds, the N82C mutation on Cys16 background only.

Independent of the background, a crosslinking efficiency of up to ~90% was achieved for the L84C mutation, albeit only in the presence of nucleotide and excess binding protein HmuT (Figure 5.10). Mg²⁺ didn't affect the crosslinking efficiency. No spontaneous crosslinking in...
the absence of the oxidizing agent was observed and the maximum crosslinking efficiency without nucleotide addition was ~40% only. This indicates that both HmuT- and nucleotide-induced conformational changes are needed to bring the introduced cysteine residues close enough for the crosslinking reaction to take place. The induced rearrangements are considerable, as they allow for the crosslinking of the residues located 19.1 Å apart in the apo HmuUV structure (Figure 5.2b). The results are consistent with the observations made with BtuCD-F and strongly suggest that the loop connecting TM2 and TM3 also operates as a cytoplasmic gate in HmuUV.

Only ~30% crosslinking efficiency was achieved, when the Asn82 was replaced with cysteine even in the presence of excess HmuT and nucleotide. Similarly, the corresponding N83C mutation in BtuC yielded only minor amounts of crosslinked BtuC dimer [1]. Thus, the introduced cysteine residues are not in an optimal orientation for crosslinking even after the closure of the cytoplasmic gate II. Therefore, the L84C mutant was chosen to study the effects of trapping the cytoplasmic gate II in a closed conformation.

Surprisingly, the L84C-L84C' link between the HmuU subunits caused an increase in ATPase activity, while a 6.5-fold reduction was observed for the corresponding modification in BtuCD [1]. The effect was particularly pronounced, when Cys10 background was used (Figure 5.11a). This suggests that the six additional native cysteines in the Cys16 transporter might have undergone considerable oxidative modifications in the presence of the 4 mM CuCl2 required to achieve a near-complete crosslinking of the introduced cysteines. This interferes with the subsequently assayed ATPase activity of the crosslinked mutant.

The crosslink in the cytoplasmic gate II might restrict the outward movement of the coupling helices (Figure 5.2), thus keeping the NBD domains in a close proximity even after ATP has been hydrolyzed and the products released. The increased ATPase activity in the presence of the engineered disulphide bond indicates that the closure of the NBD domains, i.e. formation of the two composite catalytic sites, might be the rate limiting step in the HmuV-catalyzed ATP hydrolysis reaction. In BtuD, a distinct step might be limiting. For example, the release of the hydrolysis products and re-opening of the NBD dimer, which would be further slowed down by the restricted movement of the coupling helices. The incomplete return of the L84C\textsubscript{Cys10} ATPase rate to the background level upon reduction with DTT might be attributed to occasional re-formation of the disulfide bond in solution in the presence of ATP and Mg\textsuperscript{2+} used in the assay.
Figure 5.11: Figure 5.7: Functional characterization of L84C_Cys16 and L84C_Cys10. L84C mutation was introduced on the Cys16 or Cys10 background, where 16 and 10 indicate the number of cysteine residues remaining in the full transporter. The introduced mutations are detailed in f. Measurements were performed with detergent-solubilized, preparatively gel-filtrated HmuUV-T complex. Data obtained for the wild-type transporter, Cys16 and/or Cys10 mutants are shown for comparison. a, ATPase activity. Measurements were done at least in triplicate. **P ≤ 0.01. b, Determination of K_{0.5} for ATP. Data fitted using the Hill equation. Inset represents magnification of the data up to 0.5 mM ATP. c and d, Inhibition of ATPase activity by AMP-PNP measured for the L84C_Cys10 and L84C_Cys16, respectively. e, Inhibition of ATPase activity by ADP-AlF_4. f, Table summarizing the parameters obtained from b, c, d and e. K_{0.5}, ATP concentration at half-maximal enzyme velocity; h, Hill coefficient; IC50, half-maximal inhibitory concentration; ND, no data; n, number of experiments.
In contrast to the NBD-linking disulphide bridges, increased sensitivity to the ATP analogues was observed. This was independent of the background the L84C mutation was introduced on and true for both AMP-PNP and ADP-AlF$_4$ (Figure 5.11c-f). No considerable changes in the K$_{0.5}$ for ATP or Hill coefficient were observed (Figure 5.11b and f). These characteristics make the L84C-L84C' construct a suitable candidate for the co-crystallization with ATP analogues to determine the HmuUV-T structure in the nucleotide bound state. On the other hand, the minor fraction of the non-crosslinked species and the increased ATPase activity might be problematic. ADP-AlF$_4$ and other transition state analogues might be a better choice for crystallization than AMP-PNP, as AMP-PNP is slowly hydrolyzed even by the catalytic glutamate mutant E173Q. The crystallization trials, including trials with L84C$_{Cys10}$, are summarized in the annex.

References

6. Conclusions and outlook

Successful iron acquisition is a determinant of bacterial virulence [1]. The translocation of iron compounds across the bacterial plasma membrane is, however, poorly understood at a molecular level. In this study, the molecular mechanism of heme transport by *Yersinia pestis* heme importer HmuUV-T was investigated. The *Yersinia pestis* heme transporter HmuUV-T has been functionally characterized *in vivo* [2], and is therefore a well-suited representative of type II ABC importers catalyzing uptake of iron compounds into the cytoplasm of bacterial pathogens.

To understand the molecular details of transporter-catalyzed substrate translocation, quantitative measurements of the transport activity are indispensable. Reconstitution of purified transporter proteins into lipid vesicles provides means of studying the transport reaction in a controlled environment while the protein resides in a native-like lipid bilayer. The transport measurements are, however, often compromised by the hydrophobicity of the translocated substrates, such as the numerous structurally diverse hydrophobic drugs extruded by the ABC multidrug exporters. Likewise, heme is a lipophilic molecule. Furthermore, the determination of the *in vitro* transport rates is complicated by such factors as unknown vectorial reconstitution ratio of the proteins and the possible presence of multilamellar proteoliposomes and nonfunctional transporters.

In the course of this thesis project, an *in vitro* heme transport assay, used to quantitatively measure HmuUV-catalyzed heme uptake into proteoliposomes was developed. The hydrophobic heme moiety was confined to the binding pockets of the substrate-delivery protein HmuT, demonstrated to bind to two heme molecules with nanomolar affinities. To enable the calculation of the *in vitro* transport rates, a simple, direct method for the quantitation of the accessible, transport-competent fraction of HmuUV was developed. The method relies on tight association of fluorescently labeled binding protein with the 'right-side-out' reconstituted transporter and is very likely applicable for other type II ABC importers, characterized by formation of high-affinity transporter-binding protein complexes.

Typically, radiolabeled substrates are used to monitor transport reactions due to the high sensitivity of the radioactive detection method and its compatibility with diverse reaction environments. Radiolabeled heme is, however, currently not commercially available. Two
alternative approaches were taken to enable determination of nanomolar concentrations of heme in the presence of the high-affinity heme-binding protein HmuT. First, a previously reported chemiluminescence-based method for heme determination by reconstitution with horseradish peroxidase (HRP) apo-enzyme was adapted for our purpose [3, 4]. Second, a simple, convenient method for enzymatic insertion of a radioactive iron isotope into PPIX ring to generate $^{55}\text{Fe}$-labeled heme was developed. The required ferrochelatase enzyme was expressed and purified in-house using standard procedures and the amount of equipment required for the labeling reaction was kept to a minimum. Thus, the established procedure can be generally applied to produce radiolabeled heme for functional assays avoiding extensive radioactive contamination of the lab instrumentation as in the commonly used biosynthetic preparation.

Using the newly established assay, an in vitro heme transport rate of $1.0 \pm 0.1$ nmol heme min$^{-1}$ mg$^{-1}$ transporter was determined for the reconstituted wild-type HmuUV at 37ºC. Assuming the same transport rate in vivo, ~8000 HmuUV transporters would have to be expressed in the plasma membrane of Yersinia pestis to satisfy the iron requirement of the pathogen which is ~$10^5$-$10^6$ iron ions per bacterial cell per generation [5, 6]. Therefore, the in vivo transport rate is most probably higher than measured in our in vitro setup. On the other hand, due to the absolute requirement for iron, several acquisition systems operate in most pathogenic bacteria, including Yersinia pestis. Hence, the hmu system does not have to satisfy the iron requirement of the cell alone.

Furthermore, an apparent stoichiometry of ~300 ATP molecules per transported heme was calculated. Such an uncoupling of energy consumption from substrate translocation is normally considered to be deleterious for the cell. The 300:1 stoichiometry could be attributed to the high basal ATPase rate in vitro. While ABC type II importers are characterized by a high constitutive hydrolysis activity in the absence of substrate, type I importers hydrolyze ATP rapidly only upon docking of substrate-loaded binding protein. Generally, type I importers catalyze uptake of readily available nutrients required by the cells in large amounts, such as carbohydrates and amino acids. In contrast, the substrates of the type II iron siderophore and heme importers are scarcely available. Therefore, it might be possible that even in vivo ATP is constantly hydrolyzed at a low rate to keep the protein in a transport competent state. It has been demonstrated for the type II vitamin B$_{12}$ importer, that
docking of a substrate-loaded binding protein to the transporter in the absence of nucleotide results in the loss of the untranslocated substrate to the environment.

Due to the various parameters that distinguish the in vitro setup from the in vivo situation, such as the lipid composition, membrane potential and chemical gradients, the physiological relevance of the in vitro transport rates remains an open question. The true value of the in vitro measurements in a well-controlled environment lies in the relative quantitation of the effects caused by changes like point mutations of selected residues or addition of selected compounds to the reaction mixture.

The availability of the HmuUV crystal structure, determined in our lab in parallel to this thesis work by B.A. Götz, J.S. Woo and K.P. Locher [7], allowed for the identification of the residues lining the heme translocation pathway of HmuUV. Two of those, Gly164 and Arg176, were found to be uniquely conserved in ABC type II heme importers, raising the question whether the transmembrane subunits might contribute to the substrate specificity additionally to the high-affinity substrate binding pockets of the periplasmic binding proteins. A site-directed mutagenesis study was carried out to investigate the function of the conserved residues. A combination of transport activity measurements, ATPase activity assays and studies of the HmuUV-T complex formation was applied for this purpose. The Arg176, located at the periplasmic entrance of the translocation pathway, was found to participate in the generation of the specific recognition interface with HmuT. However, the inspection of HmuT structure did not reveal an obvious interaction partner for Arg176. Therefore, direct structure determination of the HmuUV-T complex is required to clarify this point. Gly164, marking the narrowing point in the entrance funnel of the translocation pathway, was found to have a role in screening the substrate for size. Thus, a tight fit for the transported substrate, but not a high-affinity binding pocket seems to be optimal for an efficient translocation.

Recently, convincing structural and functional evidence has led to the conclusion that type II ABC importers operate by a mechanism distinct from the simple two-state alternating access model thought to be shared by all ABC transporters. A peristaltic transport mechanism was proposed for the vitamin B₁₂ importer BtuCD-F [8]. Whether it is representative for the whole ABC type II importer family is, however, unclear. To elucidate the conformational changes of HmuUV during the transport cycle and to visualize the HmuUV-HmuT recognition interface, I aimed to solve a high-resolution structure of the HmuUV-T complex. Initial HmuUV-T
crystals were obtained both in the nucleotide-bound and apo states. The diffraction quality was, however, insufficient for structure determination. The observations that both HmuUV and BtuCD adapt an outward-facing conformations in the nucleotide-free state [7, 9] and that the cytoplasmic gate II, recently identified in BtuCD-F, could also be detected in HmuUV by disulphide crosslinking studies, suggest that the general mechanism might be shared. The mechanistic details might, however, vary as indicated by the observed differences in the kinetics of the ATP hydrolysis reaction and by the demonstrated adaptation of the translocation pathway to the size of the transported substrate. Indeed, a transport mechanism diverging from that of the BtuCD has been proposed for the MolBC transporter suggested to transport the ~10-fold smaller substrate molybdate [10]. Thus, further structural and biochemical studies are needed to elucidate the transport mechanism of the ABC type II importers and to appreciate the amount of mechanistic diversity within this transporter family.

References

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PUBLICATIONS


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Appendix: Crystallization of the HmuUV-T complex

Crystallization experiments were carried out with following HmuUV-T constructs:

1. **Wilde-type HmuUV** where the TM subunit HmuU contains an N-terminal decahistidine tag combined with either untagged binding protein HmuT or HmuT with a C-terminal His6-tag.

2. **HmuU<sub>C67A C205I V68V C131S C229S T</sub>** with a reduced number of cysteines - five of the native cysteines per half-transporter are exchanged to other amino acids. Five more cysteines per half-transporter (three in HmuU and two in HmuV) remain. Histidine tags form HmuU and HmuT were removed by 3C protease cleavage.

3. **HmuU<sub>C67A L84C C205I V68V C131S C229S T</sub>** - on the background described above a cysteine residue was introduced in the loop linking the TM2 and TM3 (cytoplasmic gate II). The introduced cysteines were oxidized to form a disulphide bridge between the juxtaposed TM2-TM3 loops of the two TM subunits (described in chapter 5). Histidine tags form HmuU and HmuT were removed by 3C protease cleavage.

The plasmid design and the procedures for the expression, purification, 3C protease cleavage and disulphide crosslinking of the proteins are described in detail in chapters 3 and 5.

Results of the crystallization experiments are briefly described and summarized in the tables 1-3 below.
Wild-type HmuUV-T complex

The different sample preparation strategies are outlined in the legend for the table 1. Crystal grown in K-citrate 100 mM pH 5.4, Mg-acetate 50 mM, 15 % PEG 3350, 2 mM DTT was directly frozen in liquid nitrogen and diffraction data were collected at the protein crystallography beam line X06SA at the Swiss Light Source (SLS). Diffraction to about 8 Å was observed (Figure 1). Efforts to optimize the conditions for crystal growth were unsuccessful; no crystals showing diffraction beyond the 8 Å of the initial hit were obtained.

Table 1: Growth conditions yielding hits in the in-house crystal screen (LLX screen, six 96-well-plates) at 20°C, 150 + 150 nl or 200 + 100 nl (protein + reservoir) sitting drops.

<table>
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<th>Untagged HmuT</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-citrate 100 mM pH 5.4</td>
<td>Mg-acetate 100 mM</td>
<td>10 - 15 mg/ml</td>
<td>+ 1 mM DTT</td>
<td>+ 2 mM DTT</td>
</tr>
<tr>
<td>Mg-acetate 50 mM</td>
<td>25-35% PEG 400 (1 &amp; 3)</td>
<td>+</td>
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<td>+</td>
</tr>
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<td>KCl 200 mM</td>
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<td>(NH$_4$)$_2$SO$_4$ 300 mM</td>
<td>15% PEG 3350</td>
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<tr>
<td>MES 100 mM pH 6.2</td>
<td>KCl 200 mM</td>
<td>15% PEG 3350</td>
<td>-</td>
<td>+</td>
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</table>

10 - 15 mg/ml HmuUV-T complex in 10 mMTris-HCl pH 8.0, 150 mM NaCl, 0.5 mM EDTA-NaOH pH 8.0 and:

1 - 0.01% DDM. Excess HmuT-His$_5$ added to HmuUV after solubilization → Ni$^{2+}$ affinity purification → desalting into buffer containing 1 mM DTT → preparative SEC → concentration → crystallization;

2 - 0.016% DDM. Excess untagged HmuT added to purified HmuUV → sample re-run over an NiNTA column, the excess HmuT flows through → eluate containing the HmuUV-T complex is desalted and concentrated → sample split into two parts, 2 mM DTT added to one of the aliquots → crystallization;

3 - 0.01% DDM. Excess untagged HmuT added to HmuUV after solubilization → Ni$^{2+}$ affinity purification in the presence of 5 mM β-mercaptoethanol in all buffers → desalting into buffer containing 1 mM DTT → preparative SEC → concentration → sample split into two parts, 2 mM ADP added to one of the aliquots → crystallization.

+ Crystal growth observed; - no crystal growth.

The different sample preparation strategies are outlined in the legend for the table 1. Crystal grown in K-citrate 100 mM pH 5.4, Mg-acetate 50 mM, 15 % PEG 3350, 2 mM DTT was directly frozen in liquid nitrogen and diffraction data were collected at the protein crystallography beam line X06SA at the Swiss Light Source (SLS). Diffraction to about 8 Å was observed (Figure 1). Efforts to optimize the conditions for crystal growth were unsuccessful; no crystals showing diffraction beyond the 8 Å of the initial hit were obtained.

Figure 1: Diffraction patterns and image of a single wild-type HmuUV-T crystal. Diffraction to ~8 Å was observed.
HmuUC_{67}A C_{205}I V_{C68}V C_{131}S C_{229}S T complex

<table>
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<th>BUFFER</th>
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<tbody>
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<td>10% PEG 3350</td>
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<td>Mg-acetate 100 mM</td>
<td>25% PEG 400</td>
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<td>Na-citrate 100 mM pH 5.4</td>
<td>KCl 200 mM</td>
<td>15% PEG 3350</td>
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<td>Glycine 100 mM pH 9.4</td>
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<td>15% PEG 3350</td>
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<td>MES 100 mM pH 6.2</td>
<td>(NH_{4})<em>{2}SO</em>{4} 300 mM</td>
<td>35% PEG 400</td>
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<td>HEPES 100 mM pH 7.5</td>
<td>K-iodide 100 mM</td>
<td>15% PEG 3350</td>
</tr>
<tr>
<td>TRIS 100 mM pH 8.2</td>
<td>Mg(NO_{3})_{2} 200 mM</td>
<td>15% PEG 3350</td>
</tr>
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</table>

Table 2: Growth conditions yielding hits in the in-house crystal screen (LLX screen, six 96-well-plates) at 20°C, 200 + 200 nl (protein + reservoir) sitting drops. Cysteine-reduced HmuUV-T complex in 10 mMTris-HCl pH 8.0, 150 mM NaCl, 0.5 mM EDTA-NaOH pH 8.0 and 0.01% DDM. Untagged HmuT was added to purified HmuUV at a 1.15:1 molar ratio prior to the concentration step. After concentration to ~15 mg/ml protein, sample was supplemented with a small additional amount of HmuT (0.1:1 HmuT:HmuUV molar ratio) to account for possible losses during the concentration. No reducing agents were used in any of the purification steps.

Although crystal growth in more than 10 conditions was observed (Table 2), the crystals were too small for diffraction measurements and/or irregularly grown. A subsequent broader in-house screen (LLX v10.2, twelve 96-well plates) gave a hit in one condition only (HEPES 100 mM pH 7.5, K-iodide 100 mM, 15% PEG8000). Thus, further screening and optimization of growth conditions is needed to obtain larger diffraction quality crystals.

Notably, HmuU_{C67}A C_{205}I V_{C68}V C_{131}S C_{229}S T transporter in the absence of the binding protein HmuT readily crystallized in growth conditions found to be optimal for the wild-type transporter by B.A. Götz (specifically, 0.05 M Tris-HCl pH 8.6, PEG 400 26%, 3 + 2 µl (protein + reservoir) sitting drop, 20°C) [1, 2]. The diffraction properties of the crystals were alike those observed for the wild-type HmuUV transporter. Diffraction pattern was anisotropic, diffraction to ~3.3 Å was observed in the best direction and to ~6.5 Å after 90° rotation.

Furthermore, crystal screens with the HmuU_{C67}A C_{205}I V_{C68}V C_{131}S C_{229}S T construct in meso (Lipidic Cubic Phase) were set-up. Protein sample, prepared as described in the legend for table 2, was mixed with monoolein to a final volume ratio of 40% protein and 60% monoolein. Ten 96-well plate commercial screens for membrane protein crystallization were
set up by Beat Blattmann (NCCR Crystallization Facility) using 100 nl of the protein-
monoolein mixture and 400 nl mother liquor. Screens were incubated at 20°C and ~20 μm
long needles were observed after 1 week in 0.05 M Tris 8.4, 0.5 M KCl, 35% PEG400
(MacKinnon Membrane Protein Screen). Thus, lipidic cubic phase crystallization might be a
valuable alternative to explore in the attempts to determine a high-resolution HmuUV-T
structure.

**HmuU<sub>C67A L84C C205I V<sub>68V C131S C229S T</sub></sub> complex featuring engineered
disulphide bridge at the cytoplasmic gate II**

After the disulphide crosslinking reaction in the presence of excess HmuT, 2 mM ATP and 4
mM CuCl₂ (see chapter 5 for details), the desalted sample was concentrated and subjected to
preparative SEC. HmuUV-T fraction was collected and either AMP-PNP or ADP-AlF₄ was
added to the final concentration of 2 mM. Subsequently, a second concentration step was
carried out to bring the protein concentration up to ~10-15 mg/ml. In-house crystal screens
were set up. Fast growth (within a few days) of irregular crystals was observed in many
conditions. After analyzing aliquots of the collected SEC fractions on the SDS-PAGE it
became apparent that the HmuT to HmuUV ratio in the samples is too low. Evidently, the
HmuUV affinity for HmuT is decreased due to the presence of bound ATP from the
crosslinking reaction and/or the crosslink itself. Therefore, in a subsequent crystallization
experiment, the sample was supplemented with additional HmuT after the final concentration
step yielding a final molar ratio of ~1.3:1 HmuT:HmuUV. A combination of 2 mM ADP-
AlF₄ and 3 mM MgCl₂ was used for co-crystallization in this experiment. Again, fast growth
of irregular crystals was observed in even more conditions than before. The results are
summarized in the table 3. On many occasions, the growth of crystals in the same conditions
irrespective of the HmuT:HmuUV ratio was observed. This observation and the irregular
shape of the crystals indicate that a mixture of species might be incorporated into crystals
rather than an ordered array of one identical unit. Alternatively, one of the components might
crystallize individually. Indeed, when control wells containing HmuT alone at 3 mg/ml,
corresponding to the final HmuT concentration in the HmuUV-T sample, were set up, under
certain conditions crystal growth was observed. Thus, care has to be taken when preparing the
HmuUV-T samples for crystallization, especially in the presence of nucleotide. A covalent
disulphide crosslink might be necessary to stabilize the HmuUV-T complex in order to avoid
the stoichiometry issues and the dissociation of the complex under the crystallization conditions.

Table 3: Growth conditions yielding hits in the in-house crystal screen (LLX screen, six 96-well-plates) at 20°C, 150 + 150 nl (protein + reservoir) sitting drops. Cysteine-reduced HmuUV-T complex with an introduced disulphide bridge in the cytoplasmic gate II in 10 mMTris-HCl pH 8.0, 150 mM NaCl and 0.01% DDM. Sample preparation is described in the text above. Precipitant is PEG3350 unless indicated otherwise in the table. + Crystal growth observed; - no crystal growth.

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<th>2 mM ADP-AlF&lt;sub&gt;4&lt;/sub&gt; 1 mM Mg&lt;sup&gt;2+&lt;/sup&gt;</th>
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<td>+</td>
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<tr>
<td></td>
<td>K-tartrate 100 mM</td>
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Precipitant: PEG 3350 10-20%
References