Integral membrane proteins: fundamental medical biochemistry studies towards novel antibiotic targets

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CHAPTER 7

General Discussion

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It is widely accepted that MPs are central mediators in biological functions. However, the handling of MPs is very difficult, with the consequence that there is still an enormous lack of insight regarding MPs, compared to water-soluble proteins. As an example, out of the ~90,000 protein structures available in the protein data bank (http://www.rcsb.org) to date only ~400 are representing MPs, and of those only a dozen is human MP structures[1]. For many years, the determination of MP structure was seen as a "near-impossible mission". The main difficulty in studying MP structure stemmed from problems associated with the extraction and solubilization of MPs[2,3]. Here, new technologies, new methods, and novel means to maintain the functionality of MPs and to investigate their properties are required.

In this thesis, we used the mild detergent LMNG to characterize a bacterial terminal oxidase. We show that LMNG performed superior to DDM and other detergents regarding the activity of the solubilized enzyme. The reason for this enhanced activity is unclear, but it may be related to improved accessibility of the substrate-binding site. Alternatively, DDM may impair the structural and functional integrity of this terminal oxidase. This interpretation is supported by our finding that LMNG-solubilized cytochrome *bd* maintained higher activity even after detergent removal and reintegration into liposomes. Consistent with this interpretation, LMNG was reported to maintain the structural integrity of the eukaryotic NADH dehydrogenase complex[4]. In that case, purification with DDM led to the loss of a 42 kDa subunit, whereas usage of LMNG prevented the dissociation of this essential subunit from the NADH dehydrogenase complex[4]. The large hydrophobic surface area of the branched-chain LMNG molecule may enable this detergent to hold MP complexes together[5]. It needs to be investigated to which extent the ability to maintain structural integrity of a MP represents a general property of LMNG.

Based on the results with LMNG, we extended our work to develop a methodology for exploring the function of MPs' in a native lipid environment. The functional reintegration of selected MP into liposomes was used to study MPs and characterize vectorial properties. It was observed that cytochrome bo_3, an MP involved in respiration, reintegrated into liposomes without applying a detergent removal step.

Subsequently, we implemented this approach, which we have named **L**MNG-**A**uto-**i**nsertion **R**eintegration (LAiR), on other MPs that are known to be difficult to reconstitute. In comparison to most up-to-date models for the reintegration of MPs into the membrane, LAiR showed higher efficiency, MP enzymatic activity, and long-term stability. In particular, LAiR may form a very interesting platform for target-based drug screening. In these screening efforts, the selection of hits from a library of drug candidates usually is based on a minimum threshold of inhibition of the MP target (often a threshold of 80-85 % is applied). Fragile MPs are prone to partially lose their function during detergent solubilization or during the reintegration procedure; therefore, complete inhibition usually is not achievable. As an example, in chapter 6 (page 142), we used oligomycin to evaluate the integrity of LAiR-reintegrated ATP synthase. Oligomycin inhibited the ATP hydrolysis function by >95 %, whereas ATP synthase reintegrated into liposomes using standard methods typically is inhibited by only 50-85 %[6]. If oligomycin were a drug candidate, it would very likely be disregarded when screening assays based on standard reintegration systems are used. In contrast, a LAiR-based assay would show sufficiently high inhibition to qualify this compound as a hit for potential further characterization or development. As such, target-based screening assays based on LAiR may allow for identifying a broader selection of hits.

LAiR was also suitable for the reintegration of highly fragile and complex mitochondrial MPs, suggesting that it might also be useful for the reintegration of MP super-complexes. Super-complexes consist of two or more MP complexes that are structurally and functionally integrated. Super-complexes are proposed to play a role in the regulation of electron flow in mitochondrial or bacterial respiratory systems[7,8,9]. To my knowledge, no reintegration systems for functional super-complexes have been reported so far. LAiR may also prove suitable for the reintegration of MP complexes from plant chloroplast sources. These MPs are notoriously difficult to re-integrate into liposomes.

Several findings described in this thesis remain unexplained, as the project's time frame did not allow for closer investigation. As mentioned above, the reasons for the superior performance of LMNG in MP purification are not known. Similarly, it is unclear by which mechanism CL activates cytochrome *bd* and other detergent-solubilized MPs. Moreover, the molecular mechanism underlying the transfer of MPs from the detergent shell into liposomes during LAiR is not elucidated yet. For example, it is unknown which parts of the LMNG molecule are essential and which role the lipid composition of the liposomes plays. The phospholipid composition may influence the reintegration efficiency for a given MP and additionally may modulate the MPs' sensitivity for inhibitors. Shining light on these factors may not only contribute new knowledge but can also contribute to further improvement of the LAiR approach.

The significance of biochemical characterization of MP properties is illustrated by the fact that there is an emerging trend in developing novel therapeutic strategies targeting MPs in diseases, such as cancer,

neurodegeneration, and infectious diseases[10,11,12]. As an example of an infectious disease, *Mycobacterium tuberculosis* poses a severe global health threat. Within the last decade alone it has been attributed to over 1.3 million deaths per year[13]. In particular, combating drug-resistant strains of *M. tb* is highly challenging or may even be impossible. Inhibitors of energy metabolism show great promise as next-generation antibacterials for the treatment of drug-resistant tuberculosis. BDQ, a specific inhibitor of mycobacterial ATP synthase, has revolutionized tuberculosis drug development and triggered the discovery of other small molecules that are proven or suspected inhibitors of respiration or ATP synthesis, e.g., Q203 or pretomanid.

The characterization of these new (candidate) drugs has mainly been done by evaluating the impact on growth or viability of *M. tb* or the model strain *M. smegmatis*. To find the targets, typically mutagenesis studies and the characterization of 'resistance mutants' have been carried out. Inverted membrane vesicles (IMVs), often isolated from *M. smegmatis,* have been used intensively for drug characterization and quantifying the inhibitory effect on the target pathway, e.g. respiratory chain function or ATP synthesis. Results from IMV assays have been highly useful in this regard and have strongly contributed to better insight into the mechanism of action of next-generation tuberculosis drugs.

However, IMV assays have two disadvantages. First, this approach assesses respiratory chain function disconnected from other metabolic pathways in the bacterial cell and it does not include information on whether a drug can penetrate the bacterial cell envelope. To address these issues, in this thesis, we used a phenotypic assay that measures respiration by whole *M. tb* bacteria. Our assay is based on the previous results by Lamprecht et al[14], who

found that Q203, an inhibitor of the mycobacterial cytochrome *bcc* complex, actually caused activation of oxygen consumption[14]. This increased respiration was mediated by enhanced function of the parallel cytochrome *bd* respiratory branch. It was postulated that decreased ATP levels upon inhibition of cytochrome *bcc* via feedback loops in bacterial metabolism, in particular activation of glycolytic functions, caused the observed enhanced oxygen consumption[14,15]. In this thesis, we confirmed respiratory activation by Q203 (chapter 3 - page 65). In line with the previous interpretation that intracellular feedback loops cause the enhanced electron flow, we did not find activation but inhibition for Q203 in IMV assays (chapter 2 - page 41). Interestingly, this phenotypic activation is also observable upon the usage of KCN, an inhibitor of cytochrome aa_3, which triggers the activation of oxygen consumption.

As such, phenotypic assays like the one we used here may be applied to characterize unknown inhibitors and to distinguish between inhibitors of cytochrome *bcc* or cytochrome aa_3 on the one hand (causing respiratory activation) and inhibitors of NADH dehydrogenase (causing inhibition). The assays described here can also be used to identify cytochrome *bd* inhibitors, as small molecules targeting this MP have only little effect when used alone, but are expected to strongly inhibit respiration in combination with a cytochrome *bcc* or cytochrome aa_3 inhibitor.

The second disadvantage of IMV assays is that the results give information about the target pathway, but do not determine the drug target, as any MP in the investigated pathway can be the target.

To pinpoint the target of new inhibitors of energy metabolism, it is required to characterize the drug's interaction with its (proposed) target in a

purified state. The availability of purified components of energy metabolism from *M. tb* and/or *M. smegmatis* will also facilitate the biochemical characterization of these MPs. To date, expression and purification of MPs from *M. tb* for structural and functional studies remains a difficult task due to poor or undetectable levels of expression and other difficulties in detergent solubilization and purification. Recent progress includes the purification of the cytochrome *bcc-aa*$_3$ complex[16] and the structure determination of this complex by cryo-electron microscopy[8,9]. These studies utilized a hybrid complex consisting of the cytochrome *bcc* from *Mycobacterium tuberculosis* and the cytochrome *aa*$_3$ from *Mycobacterium smegmatis*[16]. Very recently, the ATP synthase from *Mycobacterium smegmatis* has been purified[17]. Here, purification protocols need to be established for other components of the mycobacterial ETC. Moreover, the purified MPs need to be reintegrated from the detergent-solubilized state into liposomes to investigate their vectorial properties in a lipid environment that mimics their native physiological state.

LMNG may turn out as a prime candidate for solubilization and purification of MPs from *M. tb*. To optimize purification protocols for these MPs, the role of CL on MP function and the usability of CL or other (phospho)lipids to stabilize the solubilized MPs should be assessed. Reintegration of purified, detergent-solubilized *M. tb* MPs using LAiR would allow for target-based drug screening in a native-like lipid environment.

A deeper understanding of MPs' functional properties in (myco-)bacterial energy metabolism and how drugs can influence these MPs may open the gate to developing new combination regimens based on drug synergy. The combination therapy described in this thesis, targeting both the cytochrome *bcc-aa*$_3$ complex and cytochrome *bd*, may be expanded to triple-drug

combinations, in which an inhibitor of cytochrome *bd* likely plays an important role. Such a drug combination may qualify as a next-generation chemotherapy regime for the treatment of multi-drug resistant tuberculosis.

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