ABSTRACT

Living organisms can be distinguished from inanimate matter by their ability to energy conversion. In cells, the role of a universal energy carrier belongs to the ATP, which is synthesized by ATP synthase. For the enzymatic activity, this enzyme utilizes the protonmotive force, which is generated by the transfer of electrons coupled with the translocation of protons across the membrane. This is accomplished by the involvement of specialized membrane protein complexes capable of catalysing oxidation-reduction (redox) reactions. These proteins are organized in the so-called electron transport chain, where cytochromes from the *bc* family play a crucial role - transferring electrons from quinol to the high-potential acceptor. Interestingly, various groups of bacteria do not have genes encoding cytochromes from the *bc* family. Instead, they use the Alternative Complex III (ACIII), which performs the same function as *bc* enzymes but significantly differs in subunit composition. Recently, the structure of ACIII was determined with the use of cryo-EM, showing no structural similarity to cytochrome bc_1/b_6f. Based on the obtained structural models, and available biochemical data, the possible electron transfer path within ACIII was proposed. However, the suggested mechanism has not been confirmed so far. Explaining the proposed model of ACIII mechanism was difficult and limited due to the lack of a system for the genetic modification of this enzyme.

As a main part of this dissertation, the task of identifying electron transfer pathways within ACIII heme cofactors was undertaken. The mutagenesis of genes encoding individual ACIII subunits was employed. *F. johnsoniae* was chosen as the research model due to its susceptibility to genetic manipulations, which was proven in previous works. Moreover, the *bd* quinol oxidase provides an alternative route for quinol oxidation and helps to maintain bacterial metabolism in the case of ACIII gene deletion.

The first research task was to improve the available genetic methods for the manipulation of *F. johnsoniae* genes and to construct a **plasmid-based system for deletion and complementation of alternative complex III genes**. The study revealed that overexpression of the previously deleted proteins is lethal for bacteria. Therefore, there was a need to adjust the expression level of these genes. For this purpose, several expression vectors with different *omp*A promoter activity were constructed. This allowed ACIII proteins to be expressed at the desired non-lethal levels. The developed genetic system allows for the removal of selected genes in *F. johnsoniae* and enables the **regulation of protein expression level**.

Herein, the consequences of deletion of genes encoding heme subunits of alternative complex III (ActA and ActE) were described and analysed. EPR oximetry measurements showed that the removal of the ActA subunit (ΔA) results in the lack of activity of aa_3 oxidase (the redox partner of ACIII in *F. johnsoniae*). In contrast, the deletion of *actE* (ΔE) did not alter cytochrome aa_3 activity, indicating that the **ActE subunit is not required for electron transfer between ACIII and the aa_3 oxidase**. Nevertheless, the lack of ActE negatively affected the bacterial growth rate, suggesting the existence of another, yet unidentified, function of this subunit. This work discusses possible explanations for these observations, including a hypothesis of the **branching of electron transfer paths at the interface of ActA and ActE subunits**. Complementation of the deleted genes (expressed from plasmid) restores the oxidase activity and bacterial growth rate to the WT level. Interestingly, analysis of the oxygen consumption rate of the ΔA mutant revealed the existence of an additional, oxygen-independent, and cyanide-sensitive electron transfer pathway. The obtained results suggest that *F. johnsoniae* utilizes at least three independent pathways to oxidize the menaquinone pool.
The idea of branching of electron transfer paths at the ActA interface assumes the involvement of the N-terminal mobile domain of ActA - mdA - which would enable directing electrons to different pathways. To verify the proposed role of the mdA domain, a mutant lacking this domain was constructed. The mutated strain does not show aa_3 activity, which indicates that the electron transfer from ACIII to cytochrome aa_3 does not occur. This confirms that **mdA heme is a direct electron donor for the aa_3 oxidase**.

In the second part of this thesis, the developed genetic system was used to attach the Strep-tag to the ActE subunit, which allowed for efficient purification of ACIII from *F. johnsoniae* mutants. Additionally, *E. coli* was employed for the expression of ActE and mdA in the form of water-soluble cytochromes. Purified proteins were subjected to potentiometric titrations, which allowed for the **determination of equilibrium redox potentials** for ACIII hemes.

The third part of the thesis involved **an attempt to measure ACIII** activity in selected mutant strains. The obtained preliminary results indicate that such measurements have to be carried out in strictly anaerobic conditions.

The presented system for genetic manipulation of ACIII from *F. johnsoniae* is a new tool for studying the molecular mechanism of action of this enzyme. Moreover, after further modification, it may help to study other proteins of this bacterium and related strains. The obtained results provided the first insight into the molecular mechanism of ACIII

and contributed to the elucidation of electron transfer paths within the ACIII-*aa*$_3$ supercomplex.