Summary

The objects of the presented PhD thesis were two *Helicobacter pylori* thiol oxidoreductases (HP0231 and HP0377), which belong to the family of Dsb proteins (disulfide bond). The biochemical properties of HP0377 and its functioning in heterologous host *Bacillus subtilis* were analyzed. Additionally, HP0231 impact on virulence processes was documented by analyzing its interaction with AGS cell line.

The bacterial proteins of the Dsb family are responsible for introducing disulfide bonds to extracytoplasmic proteins that are often important virulence factors of pathogenic bacteria. The process of protein oxidative folding determines their spatial structure and consequently their activity. In the model microorganism *Escherichia coli* the functioning of the Dsb system has been well characterized. There are two active metabolic pathways in the periplasmic compartment: the oxidation pathway (catalyzed by EcDsbA and EcDsbB) and the isomerization/reduction pathway (catalyzed by EcDsbC and EcDsbD). Not all proteins require the introduction of disulfide bonds - on the contrary, in strictly oxidative conditions of the periplasm some proteins must remain in their reduced form (the -SH cysteine groups not connected by a disulfide bond). This applies i.a. to apocytochrome c. Maturation of apocytochrome requires heme ligation to the reduced CXXCH motif of apocytochrome. The process of apocytochrome reduction is dependent on monomeric proteins of the Dsb family, referred as CcmG (cytochrome c maturation), which have only one known substrate - apocytochrome. The *Helicobacter pylori* Dsb system is very modest when compared to other bacteria. *H. pylori* does not code the classic DsbA/DsbB oxidation pathway and the DsbC/DsbD reduction isomerization pathway. It possesses two extracytoplasmic proteins (HP0231 and HP0377), which have a thioredoxin domain with a CXXC motif, a characteristic feature of proteins from the Dsb family. The dimeric oxidoreductase HP0231 is a key protein in the disulfide bond formation process. It has oxidative activity despite its structural similarity to EcDsbG - a dimeric protein that protects cysteine residues from oxidation to sulfenic acids. Research conducted in the Department of Bacterial Genetics showed that HP0231 also has chaperone activity. The HP0231 protein is thus an atypical dimeric oxidoreductase that combines the activity of the DsBA and DsbG proteins with no isomerization activity. That is why it has been designated as DsbK protein. The membrane protein HP0595 (DsbI) is partially
responsible for its reoxidation). The HP0377 protein initially described as homologue of EcDsbC resembles CcmG protein in its structure and by its ability to reduce apocytochrome \textit{in vitro}.

In the first part of this work, biochemical, structural and functional characteristics of HP0377 were conducted. At the beginning, the $pK_a$ value was measured for both cysteines of CXXC motif. The $pK_a$ value of cysteine allows to determine in what pH the cysteine is in equilibrium in the protoned form and in the form of a thiol anion. Oxidoreductases are active in disulfide bond introduction reactions when the first cysteine of the CXXC motif is in the form of a thiol anion. HP0377 was shown to be the first described CcmG protein to have extremely low $pK_a$ (3.46 ± 0.24), close to that of \textit{Escherichia coli} main oxidase DsbA (3.5) and the isomerase EcDsbC (4.1). Subsequent tests of isomerization and oxidation activities \textit{in vitro} showed that the investigated oxidoreductase, unlike other CcmG proteins, which are involved exclusively in the cytochrome biogenesis process, is a multifunctional protein. It has high isomerization activity and is devoid of oxidative activity. All known Dsb proteins which are isomerases exist in dimeric forms. The chemical crosslinking reaction carried out using glutaraldehyde and the molecular filtration showed that HP0377 also forms dimers and occurs in a thermodynamic equilibrium between the monomeric and dimeric forms. This fact inspired the analysis of the structure of the HP0377 complex with its substrate (apocytochrome c). The mutated version of HP0377 was used in this test. The introduced mutation (replacement of the C-terminal cysteine of the CXXC motif with alanine) prevents breaking of the protein-substrate complex. SDS-PAGE analysis and molecular filtration showed that dimeric form is combined with one molecule of apocytochrome. The next stage of the research was to identify the redox partner (the protein responsible for maintaining HP0377 in its active, reduced form). \textit{H. pylori} proteome contains HP0265 protein, which is described as a CcdA protein (truncated form of DsbD). A comparison of the HP0377 redox state in wild type and \textit{hp0265} mutant showed that HP0377 in the mutated strain is in the oxidized state and in the wild type in the reduced form.

Because the inactivation of the \textit{H. pylori} \textit{hp0377} gene is lethal for the cells, the characterization of HP0377 was carried out in heterologous host \textit{Bacillus subtilis}. Performing experiments in \textit{E. coli} cells, a model organism for the study of Dsb pathways, was impossible due to significant differences in cytochrome maturation pathways between \textit{H. pylori} and
The B. subtilis cytochrome c maturation pathway is identical to that of H. pylori. In addition, bioinformatic and structural analyzes have shown a high similarity of HP0377 to ResA - Bacillus subtilis homologue of CcmG. Analysis of the presence of active cytochromes showed that HP0377 replaces the lack of ResA. This result was the first evidence of HP0377 ability to reduce apocytochrome in vivo (earlier conclusions were based on in vitro experiments).

The final stage of this part of the work was to analyze the activity of HP0377 selected mutants in in vivo and in vitro experiments. Studies have shown that the HP0377 protein with changed single cysteines to serine in the CSYC catalytic motif does not lose its in vitro and in vivo activity, whereas when single cysteines are replaced with alanine, the protein becomes inactive. Replacing both cysteines with serine/alanine also resulted in the loss of activity. Replacement of the threonine in the cis-proline loop (TcP) did not affect the activity in the in vitro tests, while the conversion of TcP to TcT negatively affected the activity of the protein in vivo. So, the effect of mutation on interaction with the redox partner was checked. It turned out that the HP0377TcT is in oxidized form in B. subtilis cells as opposed to the wild version of the protein, which proves that cis-proline participates in interaction with the redox partner.

In the second part of this work an analysis of the impact of the Dsb system on the process of oxidative folding of HopQ protein (Helicobacter outer protein) was performed. In silico analyzes revealed the presence of outer membrane proteins with even numbers of cysteine residues in their amino acid sequences, indicating that they might be the target of Dsb protein system. Preliminary experiments concerning the effect of Dsb proteins on the process of H. pylori interaction with AGS cells (epithelial gastric cancer cells) have documented that of the three analyzed mutants (Δhp0231, Δhp0377, Δhp0595) only H. pylori Δhp0231 strain was not able to induce changes in eukaryotic cell physiology. In addition, it has previously been shown that in the periplasmic subproteome of the hp0231 mutated strain there is a significantly elevated amount of two outer membrane proteins: HopQ and HopD. For further experiments aimed at understanding the details of oxidative folding of the outer membrane proteins, a HopQ protein was selected. HopQ has six cysteine residues that form three disulfide bonds. It is known that HopQ plays an important role in pathogenesis processes, it influences the process of transporting the H. pylori CagA protein (the main pathogen oncprotein) to the host cells through the IV secretion system by interacting with the CEACAM1 receptors. The identification of HP0231 substrates carried out in Department
of Bacterial Genetics using "reverse purification" method showed that HopQ protein (and 6 other Hop proteins) is among the obtained proteins. The research presented in the publication included in this doctoral dissertation showed that HopQ in the hp0231 mutated strain is in the reduced form (in the wild strain in the oxidized form) and localized mainly in the periplasm (in the wild strain in the outer membrane). Both results documented that HP0231 not only determines the redox state but also the correct location of HopQ. The HopQ lacking disulfide bonds (site-directed mutagenesis) is unable to interact with the CEACAM1 receptors presented on the surface of AGS cells and the H. pylori strains producing mutated versions of HopQ do not affect the signaling pathways of eukaryotic cells of AGS. Only two out of three HopQ disulfide bonds are necessary for its proper functioning.

In summary, in the presented PhD thesis it was documented that HP0377 is an atypical Dsb oxidoreductase that combines the activity of CcmG and DsbC proteins. It occurs in equilibrium between the dimeric and monomeric forms and reacts as a dimer with its substrate - apocytochrome. HP0265 protein of the CcdA family, which is a shortened form of the DsbD protein is responsible for keeping HP0377 in its active, reduced form. The proline in the cis-proline loop is responsible for the proper interaction of the HP0377 protein with its redox partner. Identification of HP0231 substrates (Department of Bacterial Genetics studies) has proven that outer membrane proteins belonging to the Hop family are substrates of Dsb proteins. For HopQ protein, it was shown that disulfide bonds occurring in outer membrane proteins might be responsible for their correct location and function.