Insights into the function and mechanism of a novel type sulfide:quinone oxidoreductase

Ph.D. Thesis

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INTRODUCTION

Sulfur is essential for cells. Organic or inorganic compounds of sulfur are prevalent as gaseous, liquid and solid forms. Sulfide is the most reductive form of sulfur, which was important constituent of primordial soup. Sulfide is toxic above a certain concentration for living organisms thereby it can inhibit the respiratory chain. Despite its well-known toxicity sulfide plays essential physiological role in eukaryotes and prokaryotes. In case of eukaryotes sulfide has important function in the central nervous system, cardiovascular system, angiogenesis and regulation of muscle tone. In case of prokaryotes sulfide serves as electron donor for several phototrophic microorganisms. Detoxification, homeostasis and utilization of sulfide as electron donor are catalyzed by sulfide oxidizing enzymes. Flavocytochrome c sulfide dehidrogenase (FCSD) and sulfide:quinone oxidoreductase (SQR) are widespread sulfide oxidase flavoproteins belong to ancient disulfide reductase enzyme family. SQR proteins present on every kingdom of life except plants. Despite of diversity of SQRs the structure of these proteins are conserved. According to phylogenetic and structure-based classifications there are six types of sulfide:quinone oxidoreductase proteins. SQRs catalyze electron transfer from sulfide to the membrane quinone pool. For catalytic process SQRs require FAD cofactor and redox-active disulfide bridge formed between conserved essential cysteines. The number and position of cysteines are significantly different between SQR groups. The structure, function and catalytic mechanism of SQR type enzymes are poorly known. The most characterized SQR group is type I. Members of type VI family is hardly studied and type IV is uncharacterized group yet. In several cases a microorganism has more than one SQR proteins belonging different SQR groups. During my research my aim was to expand the knowledge about sulfide:quinone oxidoreductase enzymes by biochemical and functional analyses of a type VI SQR enzyme.

APPLIED METHODS

DNA modifications, site directed mutagenesis and the constructions of vectors were carried out according to the manufacturer's instructions. Plasmids were transformed into *E. coli* and conjugated into *T. roseopersicina* strains.

The sulfide:quinone oxidoreductase genes (*sqrD, sqrF*) were identified by *in silico* analysis and their deduced protein products were classified by multiple alignment and phylogenetic analysis.

The role of the *T. roseopersicina* SqrD and SqrF proteins in the sulfur metabolism of the microorganism was determined by analysis of *in vivo* sulfide utilization of SQR protein containing and mutant strains using gas chromatograph and was proven by measurement of sulfide dependent quinone reducing activity of membrane fractions of the wild type and SQR mutant cells using spectrophotometer.

SqrD and SqrF proteins fused to StrepII affinity tag were expressed in *E. coli* and *T. roseopersicina*. The recombinant SqrF was purified from membrane fraction of *T. roseopersicina* via affinity chromatography.

The analytical studies on the purified SqrF protein samples were performed by denaturing and native gradient polyacrylamide gel electrophoresis. The recombinant SQR proteins were detected specifically by Western-blot hybridization via StrepII antibody.

The covalent bond between the protein and the FAD cofactor was proven by fluorescent signal detection of the denatured SqrF protein band in SDS-polyacrylamide gel. Furthermore, the covalent connection of FAD was supported by study of cofactor release from denatured SqrF protein.

The redox-active FAD cofactor content of the purified SqrF was determined using absorption and fluorescent spectrophotometer. Sulfide dependent quinone reducing activity of the enzyme was measured using UV-Vis spectrophotometer.

Kinetic constants of *T. roseopersicina* SqrF enzyme were determined using curve-fitting to the experimentally determined enzyme activity data with nonlinear regression analysis by MatLab software.

The role of the cysteine residues in SqrF was studied by biochemical analysis of single cysteine mutant enzyme variants constructed by site directed mutagenesis. Furthermore, inhibition of the wild type and cysteine mutant enzymes were studied using sulfhydryl group blocking reagent.

RESULTS OF THE THESIS

During my research on sulfide:quinone oxidoreductases, I identified two SQR protein coding genes (*sqrD*, *sqrF*) in *Thiocapsa roseopersicina* genome. The deduced proteins are classified into type IV (SqrD) and type VI (SqrF) SQR groups, which are the mostly undercharacterized SQR subfamilies.

I. I proved that the promoter of *sqrD* and *sqrF* is sulfide inducible by gene expression analysis of *T. roseopersicina* cells treated with different concentration sulfides The maximum gene expression level of *sqrD* was measured in case of 1mM sulfide, while the maximum gene expression of *sqrF* was detected in cells incubated on 2,5 mM Na_2S containing medium.

II. I created SqrD or SqrF mutant *T. roseopersicina* strains to analyse the physiological role of these proteins in the sulfur metabolism of the cells.

III. I demonstrated that both of *T. roseopersicina* SQR proteins participate in the complex sulfur metabolism, since lack of SqrD or SqrF enzyme caused slower utilization of sulfide as electron donor.

IV. Both of C- or N-terminal StrepII affinity tag fused *T. roseopersicina* SQR proteins were expressed in *E. coli*, but the produced recombinant proteins were aggregated in inclusion bodies in this cell. However, the expression of the recombinant proteins in *T. roseopersicina* were successful. The SQR proteins were detected in the membrane fraction of these recombinant cells.

V. *T. roseopersicina* SQR proteins are membrane associated, thus I developed a purification method for membrane-bound SqrD and SqrF. Only the recombinant SqrF protein could be solubilized successfully from membrane fraction and purified via affinity chromatography.

VI. I determined the mono- di- and trimer form of purified SqrF by Western-blot on native gradient PAGE.

VII. Based on the absorption and fluorescent spectra of purified SqrF I proved the FAD cofactor content of the protein, which was redox-active and covalently bound.

VIII. I demonstrated that the SqrF catalyze sulfide dependent quinone reduction. The enzyme is able to utilize only quinone molecules as electron acceptor. Based on the investigation of quinone preference of SqrF I determined that the enzyme prefers ubiquinone type quinones (decilubiquinone, duroquinone).

IX. Using duroquinone and decylubiquinone I studied the SqrF catalytic properties: I determined the pH- and temperature dependence of SqrF enzyme activity. Based on enzyme activity data of SqrF measured on physiological temperature range I calculated the activation energy of SqrF enzyme. Based on the sulfide and co-substrates dependence of SqrF activity I determined the kinetic parameters of the studied enzyme.

X. I measured the temperature stability of SqrF enzyme. According to the results *T. roseopersicina* SqrF is moderately termostable enzyme.

XI. I identified three conserved (C121, C272, C332) and one unique (C49) cysteines in *T. roseopersicina* SqrF. I created the single cysteine mutant protein variants expressing *T. roseopersicina* strains and purified the cysteine mutant enzymes to analyse the function of cysteines in the catalytic mechanism of this type VI SQR enzyme.

XII. Based on the absorption spectra of the SqrF variants I found that the C121 residue has essential role in FAD cofactor binding of the protein.

XIII. I measured the sulfide dependent quinone reducing activity of the SqrF enzyme variants and I determined the kinetic parameters of the active mutant enzymes. Based on the kinetic parameters I concluded that C272 could play role in the reductive part of the enzyme reaction. Additionally I confirmed that C332 has important but not essential functional role in the enzyme activity. Presumably this cysteine takes part of the coordination of the oxidized sulfur compound produced during the reaction cycles of the enzyme.

XIV. I studied the inhibition of the wild type and the cysteine mutant enzymes with sulfhydryl group blocking reagents (iodoacetamide, iodoacetic acid) proving the role of cysteines in enzyme catalysis.

XV. I determined that the C121 is essential for both FAD cofactor binding and mechanism of sulfide oxidation proving the crucial role of the FAD binding covalent bond in the catalysis in case of type VI SQR enzymes.

XVI. I created a proposed model of catalytic mechanism of sulfide oxidation in *T. roseopersicina* SqrF enzyme.

PUBLICATIONS

Article on which the thesis was based:

1) **Ágnes Duzs**, András Tóth, Brigitta Németh, Timea Balogh, Péter B. Kós, Gábor Rákhely: A novel enzyme of Type VI sulfide:quinone oxidoreductases in purple sulfur photosynthetic bacteria, Applied Microbiology and Biotechnology, (2018) *accepted, under publishing* **IF: 3,42**

Article which is closely related to the thesis, but wasn't used in the thesis:

2) Csaba I. Nagy, Imre Vass, Gábor Rákhely, István Zoltán Vass, András Tóth, **Ágnes Duzs,** Loredana Peca, Jerzy Kruk, Péter B. Kós: Coregulated Gene Link Sulfide:Quinone Oxidoreductase and Arsenic Metabolism in *Synechocystis* sp. Strain, Journal of Bacteriology, (2014) 196: 3430-3440 **IF: 2,808**

Other publications:

3) **Ágnes Duzs**, András Tóth, Paula Dobrotka, András Dobos, Kornél L. Kovács, Gábor Rákhely. Sulfide oxidation in a puprle sulfur photosynthetic bacterium, Thiocapsa roseopersicina BBS. Acta Microbiologica et Immunologica Hungarica 60:(supp1) p. 17. (2013)

4) **Duzs Ágnes**, Témavezetők: Tóth András, Rákhely Gábor. Kénanyagcserében részt vevő szulfid-oxidáló enzimek jellemzése egy fotoszintetikus bíbor kénbaktériumban. XXX. Országos Tudományos Diákköri Konferencia Budapest 2011.

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8) András Tóth, **Ágnes Duzs**, Enikő Kiss, Brigitta Németh, Kornél. L. Kovács, Gábor Rákhely.Sulfide oxidase enzymes in photosynthetic purple sulfur bacteria. 4th Central European Forum for Microbiology, Keszthely 2013.

9) András Tóth, **Ágnes Duzs**, Enikő Kiss, Brigitta Németh, Gábor Rákhely. Biochemical and functional analysis of sulfide oxidase enzymes in purple sulfur bacteria. 1. Biomedica Minikonferencia, Szeged 2013.

10) Enikő Kiss, András Tóth, **Ágnes Duzs**, Brigitta Németh, Gábor Rákhely. Biochemical and functional analysis of the Flavocytochrome c in a photosynthetic purple sulfur bacterium. I. Innováció a Természettudományban, Szeged 2014.

11) **Ágnes Duzs**, András Tóth, Brigitta Németh, Vivien Tejsi, Timea Balogh, Gábor Rákhely. Catalytic properties of a type VI sulfide quinone oxidoreductase Microbial sulfur metabolism, Helsingor, Denmark 2015.

12) **Ágnes Duzs**, András Tóth, Kornél L. Kovács, Gábor Rákhely. Identification and characterization of the flavocytochrome c sulfide oxidase in a purple sulfur photosynthetic bacterium. ISIRR 2010 11th International Symposium Interdisciplinary Regional Research Szeged 2010.

13) Timea Balogh, **Ágnes Duzs**, Enikő Kiss, András Tóth, Gábor Rákhely. Expression analysis of sulfide oxidizing enzymes and characterization of flavocytochrome-c sulfide

dehydrogenase in a purple sulfur photosyntethic bacterium. Microbial sulfur metabolism, Helsingor, Denmark 2015.

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17) Roland Tengölics, Edit Győri, Lívia Mészáros, Zsolt Doffkay, András Tóth, **Ágnes Duzs**, Kornél, L. Kovács, Gábor Rákhely. Connection between sulfur metabolism and Hyn hydrogenase of Thiocapsa roseopersicina. EMBO Workshop on Microbial Sulfur Metabolism, Leeuvenhorst Hollandia 2012.