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STRUCTURAL AND FUNCTIONAL ANALYSES OF COPPER-SENSING OPERON REGULATOR PROTEIN (CsoRGz) OF Geobacillus zalihae STRAIN T1

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By

ASHWAANI MANGAVELU

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

STRUCTURAL AND FUNCTIONAL ANALYSES OF COPPER-SENSING OPERON REGULATOR PROTEIN ($CsoR_{Gz}$) OF *Geobacillus zalihae* STRAIN T1

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March 2018

Chairman : Normi Binti Mohd Yahaya, PhD
Faculty : Biotechnology and Biomolecular Sciences

Copper sensor regulator protein (CsoR) is widespread in most Gram positive bacteria. It is categorized under metalloregulatory protein that tightly regulates the passage of copper(I) ions to maintain cell viability, metabolism and preventing cell toxicity. Previously, gene annotation of a locally isolated *Geobacillus zalihae* strain revealed the presence of a CsoR-like hypothetical protein ($CsoR_{Gz}$) that shared only 30-38% sequence identity to structurally characterized CsoR proteins of various bacterial strains. This highlights the possibility of structural novelty of $CsoR_{Gz}$. As the actual 3D structure of $CsoR_{Gz}$ is not available, information on possible novelty of its structure as well as its 3D copper(I) binding site and motif is unknown. This study aimed to elucidate the structure of $CsoR_{Gz}$ via X-ray crystallography, identify the functional metal-binding residues and residues involved in dimerization of $CsoR_{Gz}$ protein and compare its structure with other structurally characterized CsoR proteins. For this study, *Escherichia coli* BL21 Star ® (DE3) inserted with pET28b-$CsoR_{Gz}$ vector was used to express the recombinant $CsoR_{Gz}$ protein. $CsoR_{Gz}$ protein was optimally expressed in LB medium at 37°C with IPTG induction at 0.1 mM and purified to homogeneity by affinity chromatography followed by gel filtration. The protein was stable when dialysed at pH 6.5 in the buffer containing 10 mM MES, 100 mM NaCl, 0.2 mM EDTA, 0.2 mM DTT and 5% glycerol. The purified $CsoR_{Gz}$ protein was crystallized via sitting drop vapour diffusion method at 15 °C. Trigonal crystals corresponding to space group $P3_121$ were grown in 5% (v/v) tacsimate TM (pH 7.0), 0.1 M HEPES (pH 7.0), 10% (w/v) polyethylene glycol monomethyl ether 5,000. The crystal was successfully diffracted at 1.83Å using an in-house X-ray beam with completeness of 100% with unit-cell parameters as follows; a=44.73, b=44.73, c=82.37 Å, where α=ß=90° and γ=120°. The Matthew's coefficient analysis revealed that the crystal structure had one molecule per asymmetric unit with the solvent content of 32.82%. Its conserved copper-binding residues, Cys46-His71-Cys75, were

found to be located at α2 helix onwards of the crystal structure similar to other CsoR proteins. The dimeric structure of $CsoR_{Gz}$ was indicated using Protein, Interface and Structure and Assemblies (PISA) whereby the monomers were held together by disulphide bonds, hydrogen bonds and salt bridges and the residues involved in these respective interactions were duly mapped. $CsoR_{Gz}$ showed largely similar global topology to other known Cu(I) bound CsoRs with minimal differences in the characteristics of the Cu(I) binding pocket whereby $CsoR_{Gz}$ had a more hydrophobic environment than other reported CsoRs.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

ANALISA STRUKTUR DAN FUNGSI PROTEIN PENGAWAL ATUR OPERON KUPRUM (CsoR$_{Gz}$) DARIPADA *Geobacillus zalihae* STRAIN T1

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Protein pengawal atur kuprum (CsoR) didapati dengan meluas dalam kebanyakan bakteria Gram positif. Ia dikategorikan di bawah protein pengawal atur logam yang mengawal atur keluar dan masuk kuprum(I) untuk mengekalkan daya tahan dan metabolisme sel dan mencegah ketoksikan sel. Anotasi genom strain *Geobacillus zalihae* yang dipencilkan tempatan mendedahkan kehadiran protein hipotetikal seakan CsoR (CsoR$_{Gz}$) yang berkongsi hanya 30-38% identiti jujukan dengan protein CsoR yang telah dicirikan strukturnya daripada pelbagai strain bakteria. Ini menggariskan kemungkinan struktur CsoR$_{Gz}$ yang novel. Memandangkan struktur 3D asal CsoR$_{Gz}$ masih tiada, maklumat berkaitan kemungkinan struktur novel dan juga tapak dan motif pengikat kurpum(I) 3Dnya masih tidak diketahui. Jadi, kajian ini bertujuan untuk merungkaikan struktur CsoR$_{Gz}$ melalui kristalografi sinar-X, memetakan residu fungsian pengikat logam dan yang terlibat di dalam pendimeran protein CsoR$_{Gz}$ dan membandingkan strukturnya dengan protein CsoR lain yang telah dicirikan strukturnya. Untuk tujuan ini, protein rekombinan CsoR$_{Gz}$ telah dihasilkan di dalam *Escherichia coli* BL21 Star ® (DE3) yang telah dimasukkan dengan pET28b-CsoR$_{Gz}$. Pengekspresan protein CsoR$_{Gz}$ telah dihasilkan secara optimum dalam media LB pada 37°C dengan induksi IPTG pada 0.1 mM dan dipencilkan sehingga homogeneti dengan menggunakan kromatografi afiniti diikuti dengan penapisan gel. Protein tersebut adalah stabil apabila didialisis pada pH 6.5 di dalam penimbal yang mengandungi 10 mM MES, 100 mM NaCl, 0.2 mM EDTA, 0.2 mM DTT dan 5% gliserol. Protein CsoR$_{Gz}$ yang dipencilkan telah dihablurkan melalui kaedah resapan wap *sitting drop* pada 15 °C. Hablur trigonal sepadan dengan kumpulan ruang $P3_121$ telah ditumbuhkan dengan 5% (v/v) tacsimate TM pH 7.0, 0.1 M HEPES pH 7.0, 10% (w/v) polietilena glikol monometill eter 5,000. Hablur tersebut berjaya dibelaukan kepada resolusi 1.83 Å menggunakan sunber sinar X *in house* dengan kelengkapan 100% dengan parameter sel unit seperti berikut; a = 44.73, b = 44.73, c = 82.37 Å, di

mana α=ß=90° dan γ=120°. Analisis nilai pekali Matthew menunjukkan bahawa hablur ini mempunyai satu molekul per unit asimetri dengan kandungan pelarut sebanyak 32.82%. Residu terpelihara yang mengikat kuprum, iaitu Cys46-His71-Cys75, telah dijumpai terletak pada heliks α2 dan seterusnya serupa dengan struktur protein CsoR yang lain. Struktur dimer $CsoR_{Gz}$ telah dianalisis dengan menggunakan program Protein, Antara Muka dan Struktur dan Perhimpunan (PISA) di mana dua monomer dipegang bersama oleh ikatan disulfida, ikatan hidrogen dan penyambung garam. Struktur $CsoR_{Gz}$ memperlihatkan topologi global yang sama seperti protein CsoR yang lain dengan perbezaan yang sedikit dalam ciri-ciri poket mengikat Cu(I)nya di mana $CsoR_{Gz}$ mempunyai persekitaran hidrofobik yang lebih tinggi berbanding dengan CsoR lain yang telah dilaporkan.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

α	Alpha
Å	Angstrom
ATP	Adenosine Tri-phosphate
APS	Ammonium persulfate
Au	Aurum
bp	Base pair
β	Beta
Cd	Cadmium
$CaCl_2$	Calcium chloride
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
Crp	cAMP receptor Protein
csc	Chromosomally encoded sucrose catabolism genes
Co	Cobalt
Cu(I)	Copper (I) ion
CsoR	Copper Sensitive Operon Repressor
°C	Degree Celcius
DNA	Deoxyribonucleic acid
DUF	Domain of Unknown Function
EDTA	Ethylene-diamine-tetraacetic acid
Fe	Ferrum
g	Gram
HTH	Helix Turn Helix
His	Histidine

IPTG	Isopropyl-Beta-D-Thiogalactoside
kb	Kilobase
kDA	KiloDalton
L	Litre
LB	Luria-Bertani
Mn	Manganese
M	Molar
µL	Microliter
µm	Micrometer
mM	Millimolar
Ni	Nickel
A_{600nm}	Optical density at wavelength 600 nanometer
%	Percentage
$NaPO_4$	Sodium phosphate
NaCl	Sodium chloride
SDS - PAGE	Sodium deodecyl sulphate Polyacrylamide gel electrophoresis
3D	Three Dimensional
TCS	Two Components Regulatory System
Zn	Zinc

CHAPTER 1

INTRODUCTION

Copper is required as a micronutrient for all living cells, as it is known as an essential component for large numbers of metalloenzymes. However, elevated level of copper ions in micro and macro organisms can be detrimental (Liu *et al.*, 2006). Therefore, tightly controlling the levels of cellular metal ion via homeostasis is required to avoid cell toxicity as well as for cell viability and metabolism.

To cope with unfavourable copper concentration, bacteria utilize copper-induced defence mechanisms. An example of such copper homeostasis mechanism involves CsoR protein. It is a metalloregulatory protein that that functions to regulate the copper ion concentration in bacterial cells. It is responsible in controlling gene expression which allows organisms to adapt to critical level of essential metal ions, particularly copper, thus enabling cells tolerate heavy metal pollutants present in their environment (Giedroc & Arunkumar, 2007). It functions as a copper-sensing repressor, regulates expression of copper-homeostasis genes. It was first discovered in *Mycobacterium tuberculosis* (Liu *et al.*, 2007). Besides *M. tuberculosis*, CsoR-like proteins are also widespread in other prokaryotes such as in *Bacillus subtilis* (Smaldone & Helmann, 2007), *Staphylococcus aureus* (Baker *et al*., 2011), *Streptomyces lividans* (Chaplin *et al*., 2015) and *Listeria monocytogenes* (Corbett *et al*., 2011).

In general, *csoR* gene is located upstream of *copZA* operon that encodes a copper-ATPase and a copper chaperone. Copper chaperone function to transport the excess copper to copper ATPase, while copper ATPase function to remove excess copper out of the cell (Hirooka *et al.,* 2012). When copper is scarce, CsoR represses the transcription by binding to the operator of the *copZA* operon which overlaps *copZ* promoter. When copper concentration exceeds, CsoR binds to the excessive copper ions and detaches itself from the operator, thus unblocking the promoter region for transcription of *copZA* to take place (Rademacher & Masepohl, 2012). Previously, a scan on the genome sequence of *G. zalihae* revealed the presence of a CsoR-like hypothetical protein gene located at the upstream of the *copZA* operon. Bioinformatics analysis done by Musa (2016) on the protein sequence of CsoR-like protein of *G. zalihae* showed that it contained a CsoR-DUF domain similar to other well-characterized CsoR proteins. However, $CsoR_{Gz}$ only shared up to 30-38% sequence identity to structurally characterized CsoR proteins of various bacterial origins (Musa, 2016). This suggests the possibility of its structure being novel compared to other structurally characterized CsoR proteins.

It is important to note that the 3D structure of $CsoR_{Gz}$ have not been determined via X-ray crystallography as well as metal-binding ligands interaction using docking method. Whilst several tools such as isothermal titration calorimetry is able to shed light on the ability of the protein to interact with metals, it is not able to identify which

amino acids the metals interact with. Combination of X-ray crystallography and docking is able to give forth such information upon the interaction of proteins with metals. Hence, this present study aims to:

1. elucidate the structure of $CsoR_{Gz}$ via X-ray crystallography
2. identify the functional metal-binding residue and dimer interface of $CsoR_{Gz}$.
3. compare the similarities and differences of $CsoR_{Gz}$ with other structurally characterized CsoR proteins.

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