The Properties of the Fatty Aldehyde Decarbonylase from *Synechocystis* PCC6803

Submitted by

Robert James Kalibala

to the University of Exeter as a thesis for the degree of Masters by Research in Biological Science, (March 2012)

This thesis is available for Library use on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgment.

I certify that all materials in this thesis which is not my work has been identified and that no materials has been submitted and approved for the award of a degree by this or any other University.

Signature……………………………………

Robert James Kalibala

ABSTRACT

Alkanes dominate the constituents of gasoline, diesel, and jet fuel and are naturally produced by diverse species; saturated and unsaturated fatty acids are converted to alkanes and alkenes respectively by the enzyme aldehyde decarbonylase (AD). Here we describe the over-expression, purification, data collected and X-ray crystal structure solved for the AD protein from *Synechocystis* PCC6803.

This report describes the optimisation of over-expression, protein purification and characterization and crystallisation of the *Synechocystis* cyanobacterial AD enzyme (SynADC) has been carried out. The optimisation of protein expression has been carried out using the pET160, pET22b and pCold$^{TM\ II}$. Expression of soluble protein was obtained with all vectors. The initial Lumio^{TM} tag on pET160 prevented the protein from crystallising; the pCold$^{TM\ II}$ vector with a small His-tag was used for high soluble protein over-expression. The purification of the SynADC was optimized and the enzyme was characterised biochemically, SynADC was found to be a dimer of 29 kDa molecular weight. Metal contents were investigated using ICP-MS, SynADC protein was found to contain; Zn, Fe, Ni and Mn metals in a ratio (2.37, 1.16, 0.137, and 0.032) mg/l respectively.

The enzyme has been assayed using a series of ferredoxin assays of (C_8, C_{10}, C_{12}, C_{13}, C_{16} and C_{18}) and activity has been determined using C_{13} aldehyde and C_{18} aldehyde.

The enzyme has been successfully crystallised with four different ligands (valeric acid, Hexanoic acid, C_4 and C_8) using the microbatch method and metal soaking, this has allowed the X-ray structure to be determined. Based on this structure predication of electron transfer mechanism, a mutagenesis experiment has been carried out with the change of Asp143 to Asn, Leu and Ala. The enzyme has been assayed using PMS. Experiments to determine potential proteins, which could interact with SynADC, have been carried out. Positive results have been obtained using SDS-PAGE however, more protein is required for mass spectrometric determination.

This project was part of a larger study to clone and solve the structure of the *Synechocystis* Cyanobacterial AD in order to understand its substrate specificity and mechanism. Work carried out in collaboration with others is clearly mentioned in this thesis.

Acknowledgements

Firstly, I would like to thank Prof. Nicholas Smirnoff and Prof. Jenny Littlechild for giving me the opportunity to undertake this project and for the continuous help and guidance throughout. Thank you for giving me the freedom to develop the project in directions that interested challenged me to the limit.

Secondly, I would like to thank Dr. Misha Isupov for carrying out the data processing and structure refinement, Dr Christoph Edner for his supervision in the lab and for getting me back on track with my practical work when I was at my wits end and for reminding me what it felt like for practical work to run as it should, Mr. Kevin for help me with ICP – MS experiments and Dr Hannah Florence for help me with Mass spectrometry experiments.

Thirdly, one of the biggest thank you has to go to the "Mezz" and my beloved colleagues of "Biocats". You know who you are and your help, patience, friendship and morale support. I only hope and wish I could manage to do for you all what you did for me.

And finally to my friends and family: George for support and distracting me when my brain was ready to implode, as Dr. Tomas, Dr. Paul and Prof. Jenny Littlechild for believing in me always, for your unconditional support and encouragement you're truly made a huge difference in my life thank you ever so much.

ABBREVIATIONS

°C	Degree centigrade
A	Amps
Å	Angstrom (10^{-10}m)
A_{280}	Absorbance at 280nm
A_{600}	Absorbance at 600 nm
APS	Ammonium persulphate
BLAST	Basic local alignment search tool
DMSO	dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic Acid
g	Acceleration due to gravity
g	grams
GF	Gel filtration
hr	Hour
IPTG	Isopropyl ß-D-galactopyranoside
K	Kelvin
kDa	Kilo Dalton
mg	milligrams
min	Minute
ml	milliliter
MW	Molecular weight
nm	nanometer
NMR	Nuclear Magnetic resonance
No.	Number
OD	Optical density
PAGE	Poly acrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDB	Protein date bank
PMSF	phenylmethylsulfonyl fluoride
PI	Isoelectric point
ppt	Precipitate
rtm	Room Temperature
s	Second

SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TEMED	N, N, N, N- tetramethylethylene diamide
Tris	Tris [hydroxymethyl] aminomethane
UV	Ultra violet
V	Volts
v/v	volume per volume
V_0	Initial velocity
V_{max}	Maximum velocity
Vol.	Volume
w/v	Weight to volume

Organism abbreviations

E. coli	*Escherichia coli*

CONTENTS

Title page *i*
Abstract *ii*
Acknowledgements *iii*
Abbreviations *iv*
List of contents *vii*
List of Figures *xi*
List of Tables *xiii*
List of Appendices *xiii*

CHAPTER 1: INTRODUCTION.

1.1 Hydrocarbons 1
1.1.2 Historical background of microbial hydrocarbons 1
1.1.3 The role of hydrocarbons in organisms 2
1.2 Intracellular hydrocarbons of microorganisms 3
1.3 Extracellular hydrocarbon of microorganisms 4
1.3.1 long chain hydrocarbon 4
1.4 Hydrocarbon synthesis pathways in organisms 5
1.5 Cyanobacteria aldehyde decarbonylase (AD) 6
1.6 P450 monooxygenases 11
1.7 History of enzymology 13
1.7.1 Enzymes and biotechnology 13
1.7.2 Enzyme classification 14
1.8 Aim and objectives 14

CHAPTER 2: PROTEIN SEQUENCE ANALYSIS

2.1 Introduction 16
2.2 Materials and Methods 17

2.2.1 Primary sequence analysis 17
2.2.2 conserved domains of sll0208 17
2.3 Results 17
2.3.1 Aldehyde decarbonylase protein present in database 17
2.3.2 Conserved domains *Synechocystis* AD (sll0208) 18
2.3.3 Evolution background of decarbonylase enzyme in nature 22
2.3.4 Comparisons between (sll0208) and some of other fatty aldehyde decarbonylase 23

CHAPTER 3: PROTEIN EXPRESSION AND PURIFICATION

3.0 Introduction 25

Section 1 Over-expression of *Synechocystis* fatty aldehyde decarbonylase

3.1 Materials and Methods 25
3.1.1 Reagents grade chemicals 25
3.1.2 Growth media 26
3.1.3 Cell culture 26
3.1.4 Expression SynADC 27
3.1.5 Handling and storage of protein solutions 27
3.1.6 SDS- polyacrylamide gel electrophoresis (SDS-PAGE) 27
3.1.7 Pre -made SDS gels 28
3.1.8 SDS-PAGE gel running procedure 28
3.1.9 SDS-PAGE staining and de-staining procedure 28
3.1.10 Purification of SynADC 28
3.1.10.1 Introduction to purification 28
3.1.10.2 Sample preparation 30
3.1.10.3 Purification of recombinant SynADC 30
3.1.10.3 Affinity Chromatography 31
3.1.10.4 Gel Filtration 31

3.1.10.5 Protein concentration determination 33
3.1.10.6 Determination of metal content of SynADC 33
3.2 Results And Discussion 34
3.2.1 Over-expression of SynADC 34
3.2.2 Gel filtration chromatography 34
3.2.3 GF elution profiles under different buffer conditions 36
3.2.4 Protein purification 38
3.3 **Discussion** **38**

CHAPTER4: SCREENING OF DIFFERENT VECTOR CONSTRUCTS

4.0 Introduction 40
4.1 SynADC – Tag 40
4.2 Materials And Methods 40
4.2.1 Expression of SynADC- Tag 40
4.2.2 SDS-PAGE samples 41
4.2.3 Ammonium sulfate fractionation and protein purification 41
4.2 Cold-Shock expression Vector pCold^{TM} II DNA 41
4.2.1 Cloning SynADC into pCold vector and expression in *E.coli* 42
4.2.2 Screening of IPTG concentration for optimum induction 42
4.2.3 Purification of SynADC protein 43
4.2.4 Quantification of SynADC concentration and activity 43
4.3 Results and Discussion 44
4.3.1 Discussion 47

CHAPTER 5: SPECTROSCOPY STUDIES

5.0 Introduction 49
5.1 Materials and Methods 50
5.1.1 Spectrophotometer scanning 50

5.1.2 Pull down assays to determine other proteins,
Which interact with *SynADC* 51
5.1.3 Preparation of protein extract of the *Synechocystis* wild type 52
5.1.4 Binding SynADC to PROBOND resin 53
5.1.5 Challenge resins with *Synechocystis* extract 54

5.2 Results And Discussion 56
5.2.1 Spectrophotometer scanning 56
5.2.2 Protein interactors with SynADC 57
5.2.3 Discussion 58

CHAPTER 6: PROTEIN CRYSTALLISATION

6.1 Introduction 60
6.1.1 X-ray crystallography 62
6.2 Materials And Methods 65
6.2.1 Expression of SynADC using a pCold vector 65
6.3 Protein purification of SynADC (pCold SynADC) 65
6.3.1 Cell lysis 65
6.3.2 Nickel affinity and gel filtration chromatography 66
6.4 SynADC cleavage of N-terminal His-tag using AcTEV protease 66
6.4.1 Sample preparation for His-tag cleavage 66
6.4.2 Protein concentration determination 67
6.5 Crystallisation of SynADC 67
6.5.1 Initial crystal trials (using microbatch method) 67
6.5.2 Using vapour diffusion method 68
6. 6 Preparation of apo-SynADC (stripping off metals) 68
6.7 Crystallisation Optimization 68
6.8 Microseed Matrix Screening 69
6.9 Soaking of protein crystals in metals ions (Fe^{2+} and Zn^{2+}) 70
6.10 Soaking of protein crystals with ligands 70

6.11 Co-crystallization with ligands 70

6.12 Preparing crystals for data collection 71

6.13 X-Ray data collection 71

6.14 Results and Discussion 71

6.14.1 Expression of the SynADC protein 71
6.14.2 Protein concentration determination 71
6.14.3 Co-crystallization with ligands 72
6.14.4 Protein Purification 72
6.14.4.1 Nickel affinity and gel filtration chromatography 72
6.14.4.2 SynADC cleavage of N-terminal His-tag using AcTEV protease 73
6.3.2 Crystallization Results 73
6.3.3 Soaking of protein crystals with ligands 77
6.3.4 Co-crystallization with ligands 78
6.3.5 Data collection 80
6.3.6 Structural analysis 81
6.15 Discussion 88

CHAPTER 7: SITE DIRECTED MUTAGENESIS OF SynADC BASED ON CRYSTAL STRUCTURE

7.0 Introduction 89
7.1 Materials and Methods 89
7.1.1 Site directed mutagenesis of SynADC 89
7.1.2 Amino acids residues for mutagenesis 91
7.1.3 Expression of mutant proteins 91
7.1.4 Activity assay for SynADC mutant proteins 92
7.2 Results 92
7.2.1 Site-directed mutagenesis of Asp143 93
7.2.2 Expression of D143N SynADC 93

7.2.3 Expression of D143L SynADC 94
7.2.4 Expression of D143A SynADC 95
7.2.5 Activity assays for SynADC mutant proteins 95
7.3 Overview 97

CHAPTER 8: CONCLUDING COMMENTS AND FUTURE WORK

8.1 Summary and Concluding Comments 98
8.2 Future Work 100

LIST OF FIGURES

1.1 Pathway for the hydrocarbon biosynthesis by sulfate-reducing bacteria 6
1.2 Structure of cAD from *P. marinus* 9
1.3 Comparison of the similar three-dimensional structure of a cyanobacterial AD and ribonucleotide reductase R2 from *E. coli*. 10
1.4 Proposed Microbial Biosynthesis of Alkanes 12
1.5 Sequence alignment of fatty aldehyde decarbonylase 18
1.6 conserved domains of sII0208 18
1.7 The alignment between *Synechocystis* AD (16331419) with fatty aldehyde decarbonylase from *P. marinus* 19
1.8 The alignment between insects alkanal (fatty aldehyde) decarbonylase and Cyanobacterial alkanal (fatty aldehyde) decarbonylase. 20
1.9 Sequence alignment for the 20 fatty aldehyde decarbonylase protein found on NCBI database 21
1.10 A phylogeny tree 22
1.11 Comparisons between (sll0208) and some of other fatty aldehyde decarbonylase proteins present in NCBI database 23
1.12 List of GF and Sample buffers used during protein purification 32
1.13 SDS-PAGE analysis of the over-expression of SynADC 35
1.14 SDS-PAGE analysis of SynADC after Ni- affinity column 35
1.15 GF elution profiles under different buffer conditions 36

1.16	SDS-PAGE analysis (after GF chromatography)	38
1.17	Activity assays (SynADC)	45
1.18	The Fe-S centres of iron-sulfur proteins	48
1.20	Visible absorption spectra of SynADC protein	54
1.21	SDS-PAGE, analysis of SynADC protein interactors	55
1.22	Silver stained SDS-PAGE, analysis of SynADC protein interactors	56
1.23	The phase diagram, the solubility of the protein as the precipitant concentration changes	59
1.24	Conditions that satisfy Bragg's law	62
1.25	Ewald's Sphere	63
1.26	SynADC cleavage of N-terminal His-tag using AcTEV protease	71
1.27	Needle like crystals of SynADC	72
1.28	Protein crystals obtained by micro batch method from JCSG	73- 75
1.29	Protein crystals soaked with ligands	75
1.30	Co-crystallisation with ligands	76 - 77
1.31	X-ray diffraction pattern for SynADC	78
1.32	Ribbon representation of SynADC dimer	79
1.33	Superimposition of SynADC with AD from *P. marinus*	80
1.34	Ribbon representation of SynADC monomer	81
1.35	Experimental electron density and metal coordination around the enzyme active site	82
1.36	The coordination of metal ions in the active site	83
1.37	Analysis of the electrostatic potential	84
1.38	Amino acid residues mutated	89
1.39	Activity assay for SynADC mutant proteins	92
1.40	Vector Map of pET160/ GW/D-TOPO	98
1.41	Cloning site of PET160/GW/D-TOPO	98
1.42	Superdex 200 gel filtration column calibration	99
1.43	pCold II DNA (Vector Map of pCold II DNA	100
1.44	Cloning site of pCold II DNA	101
1.45	Cloning site of PET160/GW/D-TOPO	99
1.46	Superdex 200 gel filtration column calibration	100
1.47	pCold II DNA (Vector Map of pCold II DNA	100

1.48 pCOLD_SynADC.ape Translation 23 amino acids 101
1.49 Confirmation of mutagenesis 102

LIST OF TABLES

1.1 List of intracellular hydrocarbon of microorganisms 6
1.2 Shows buffers used during affinity chromatography 30
1.3 Screening of IPTG concentrations for optimum induction 45
1.4 Summary of preparation extracts of *Synechocystis* 53
1.5 GF buffers used 65
1.6 Statistics from X-ray diffraction 78
1.7 Summary of crystallographic data collected for SynADC protein 85
1.8 QuikChange Lightning Site-Directed Mutagenesis reaction solutions 88