

The Properties of the Fatty Aldehyde Decarboxylase 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 pColdTM II. Expression of soluble protein was obtained with all vectors. The initial LumioTM tag on pET160 prevented the protein from crystallising; the pColdTM 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 (C₈, C₁₀, C₁₂, C₁₃, C₁₆ and C₁₈) and activity has been determined using C₁₃ aldehyde and C₁₈ aldehyde.

The enzyme has been successfully crystallised with four different ligands (valeric acid, Hexanoic acid, C₄ and C₈) 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 Smirnov 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 β-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

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

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 decarboxylase protein present in database	17
2.3.2 Conserved domains <i>Synechocystis</i> AD (sll0208)	18
2.3.3 Evolution background of decarboxylase enzyme in nature	22
2.3.4 Comparisons between (sll0208) and some of other fatty aldehyde decarboxylase	23

CHAPTER 3: PROTEIN EXPRESSION AND PURIFICATION

3.0 Introduction	25
-------------------------	-----------

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

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™ II DNA	41
4.2.1	Cloning SynADC into pCold vector and expression in <i>E.coli</i>	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 <i>SynADC</i>	51
5.1.3	Preparation of protein extract of the <i>Synechocystis</i> wild type	52
5.1.4	Binding <i>SynADC</i> to PROBOND resin	53
5.1.5	Challenge resins with <i>Synechocystis</i> extract	54
5.2	Results And Discussion	56
5.2.1	Spectrophotometer scanning	56
5.2.2	Protein interactors with <i>SynADC</i>	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 <i>SynADC</i> using a pCold vector	65
6.3	Protein purification of <i>SynADC</i> (pCold <i>SynADC</i>)	65
6.3.1	Cell lysis	65
6.3.2	Nickel affinity and gel filtration chromatography	66
6.4	<i>SynADC</i> 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 <i>SynADC</i>	67
6.5.1	Initial crystal trials (using microbatch method)	67
6.5.2	Using vapour diffusion method	68
6.6	Preparation of apo- <i>SynADC</i> (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 <i>P. marinus</i>	9
1.3	Comparison of the similar three-dimensional structure of a cyanobacterial AD and ribonucleotide reductase R2 from <i>E. coli</i> .	10
1.4	Proposed Microbial Biosynthesis of Alkanes	12
1.5	Sequence alignment of fatty aldehyde decarbonylase	18
1.6	conserved domains of sll0208	18
1.7	The alignment between <i>Synechocystis</i> AD (16331419) with fatty aldehyde decarbonylase from <i>P. marinus</i>	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 <i>P. marinus</i>	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 <i>Synechocystis</i>	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