Biochemical and structural characterisation of a thermophilic Aldo-Keto Reductase from *Thermotoga maritima*

Submitted by Simon Carl Willies, to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Biosciences, March 2009.

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

..... (signature)

Abstract

The Aldo-Keto Reductases (AKR) are a group of oxidoreductase enzymes structurally and mechanistically distinct from the Alcohol Dehydrogenases (ADH). The AKRs are of importance for their ability to produce industrially useful compounds induding chiral secondary alcohols. The ADH family have traditionally been exploited for chiral alcohol production; the AKR family have currently been underexploited for chiral alcohol production and present the opportunity to search for novel oxidoreductases with properties and substrate specificities distinct from the ADH enzymes.

The AKR studied here, from the hyperthermophilic bacteria *Thermotoga maritima* has been characterised with respect to its biochemical and structural properties, and its potential as a biocatalyst evaluated. This enzyme is the second example of a thermophilic AKR to have its three dimensional structure solved, the other also being from *Thermot. maritima*. The AKR studied exhibits high stability with respect to temperature and moderate amounts of organic solvents. A large preference for the reduction reaction compared to the oxidation reaction was found, which has previously been observed in other AKRs. The X-ray crystal structure was solved to 2.6Å resolution in the apo form. The final structure has three loop sections which were not located due to disorder within the crystal, which are expected to become ordered upon cofactor and substrate binding. A section of one of these missing loops was found to bind at the active site of the enzyme, with a glutamate occupying the site of substrate carbonyl binding. The formation of a dimer, increased helix-dipole stabilisation and long distance ion pair interactions all act to increase thermostability of the AKR with respect to its mesophilic homologues.

The X-ray crystal structure of *Escherichia coli* bacterioferritin has also been solved to 1.9Å resolution, which was co-purified along with the recombinant AKR enzyme. This structure shows the symmetrical binding of a heme molecule on the local two-fold axis between subunits and the binding of two metal atoms to each subunit at the ferroxidase centre. These metal atoms have been identified as zinc by the anaylsis of the structure and X-ray data and confirmed by microPIXE experiments. For the first time the heme has been shown to be linked to the internal and external environments via a cluster of waters positioned above the heme molecule. This information has provided a greater insight into the function and mechanism of bacterioferritin.

Acknowledgements

I would like to thank all those who have helped and supported me through the course of my PhD. Specific mention should be given to some. Misha Isupov, who has provided his help, knowledge and experience throughout all the stages of X-ray crystallography work, and without whom some of the problems encountered along the way might not have been solved. I would like to thank my supervisors Jenny Littlechild and Matt Bycroft who have provided helpful conversations and suggestions throughout the project. Thank you also to Jen, who has whilst I've been writing this thesis made me feel like I should be working a bit harder.

List of Contents

Abstract.....2
Acknowledgements.....2
List of Contents.....4
List of Figures.....5
List of Tables.....7
List of Equations.....8
Abbreviations.....9

List of Figures

Fig.	Title	Page
1.1	Application of biotransformation processes	15
1.2	Naming conventions for chiral molecules	17
1.3	Types of enzymes used in industrial biotransformation reactions	21
1.4	Enzymatic regeneration of NAD(P)H cofactor	22
1.5	Structural diversity of the alcohol dehydrogenase family	24
1.6	The common structural fold adopted by the AKR family	26
1.7	Spatial arrangement of the catalytic residues and the cofactor within the AKR active site	27
1.8	The acid-base catalytic mechanism of aldehyde reduction by the AKR family enzymes	29
1.9	Pompeii Worm and Water bear	33
1.10	Cell membrane phospholipids	34
1.11	An ultrathin layer electron microscope image of a *Thermotoga maritima* cell	35
1.12	The covalent modification mechanisms that occur in protein denaturation	38
1.13	Alteration of ΔG_{stab} vs temperature to give increased thermostability	40
1.14	The intersubunit ionic bonds in *A. pernix* ADH	42
1.15	The helix dipole stabilisation in *Thermotoga maritima* phosphoglycerate kinase	46
1.16	The growth of the Protein Data Bank since 1980	49
2.1	SDS-PAGE analysis of induction studies of TmAKR	59
2.2	SDS-PAGE analysis of heat lysis trials of TmAKR	60
2.3	Hydrophobic interaction chromatography of the TmAKR protein	61
2.4	SDS-PAGE analysis of hydrophobic interaction chromatography	62
2.5	Ion exchange chromatography of the TmAKR protein	62
2.6	SDS-PAGE analysis of ion exchange chromatography of TmAKR	63
2.7	Size exclusion chromatography of the TmAKR protein	64
2.8	SDS-PAGE analysis of size exclusion chromatography of the TmAKR protein	64
2.9	TmAKR and Bacterioferritin separated by gel filtration chromatography on Superdex 200	65
2.10	SDS-PAGE of size exclusion chromatography from Fig 2.9	66
3.1	Confirmation of activity of TmAKR towards potential substrates from GC analysis	73
3.2	Substrates to which TmAKR has been shown to be active	74
3.3	pH optimum of the forward reaction	75
3.4	pH optimum of the reverse reaction	75
3.5	Temperature optimum for the alcohol to carbonyl reaction of TmAKR	76
3.6	Temperature stability of TmAKR	77
3.7	Stability of TmAKR with respect to a variety of organic solvents	78
3.8	Michaelis-Menten kinetics of TmAKR catalysis of THBA	81
4.1	Vapour phase diffusion method of protein crystallisation	82
4.2	Microbatch crystallisation	83
4.3	Phase diagrams for protein crystallisation	84
4.4	A single crystal of *E. coli* Bacterioferritin	87
4.5	Crystals of TmAKR obtained from microbatch screening	88
4.6	Optimised crystals of TmAKR	89
4.7	Further optimised crystal of TmAKR	90

4.8	Variety of crystals produced from optimisation of needle like TmAKR crystal conditions	91
5.1	Schematic representation of a rotating anode X-ray generator	93
5.2	Reflection of incident X-ray beams from a pair of parallel lattice planes	95
5.3	Friedel's law and its disruption by anomalous scattering effects	98
5.4	Theoretical X-ray absorption curve for the Fe K edge	99
5.5	Single representative X-ray diffraction image for dataset Si18	109
5.6	Single Representative X-ray diffraction image for dataset SCW10	110
5.7	Ramachandran plot for TmAKR dimer	114
5.8	EXAFS scan of the Fe K edge	116
5.9	Representative X-ray diffraction image for *E. coli* bacterioferritin	117
5.10	Figure showing the μPIXE point spectra for *E. coli* bacterioferritin	119
5.11	Ramachandran plot for *E. coli* bacterioferritin	121
6.1	Overall fold of TmAKR	123
6.2	Topology diagram of TmAKR	123
6.3	Secondary structure summary for TmAKR	124
6.4	Cα-backbone trace of TmAKR	125
6.5	Asymmetric unit showing the crystallographic dimer	126
6.6	Biological unit of TmAKR	128
6.7	Ion-pair network present at the subunit interface in TmAKR	129
6.8	The arrangement of residues forming the catalytic tetrad	131
6.9	A stereoview of the density found within the active site	132
6.10	A schematic representation of the predicted hydrogen bonds and other significant contacts between $NADP^+$ and TmAKR	134
6.11	TM1009 dimer	139
6.12	Ion-pair network at the dimer interface in TmAKR	140
7.1	Topology diagram of the *E. coli* bacterioferritin monomer	147
7.2	Structure sequence summary of *E. coli* bacterioferritin	148
7.3	Dimer of *E. coli* bacterioferritin	149
7.4	A view along a 3-fold axis of the *E. coli* bacterioferritin	151
7.5	A view down a 4-fold axis of the *E. coli* bacterioferritin	152
7.6	A stereo view of the heme in *E. coli* bacterioferritin	155
7.7	$2F_C$-F_0 map of *E. coli* bacterioferritin	156
7.8	Location of the heme water channel in the *E. coli* bacterioferritin	158
7.9	The hydrogen bonding network within the heme water cavity of *E. coli* bacterioferritin	158
7.10	The ferroxidase centre in *E. coli* bacterioferritin	159
7.11	The ferroxidase cavity and channel of *E. coli* bacterioferritin	161
7.12	View of a 3-fold axis channel of *E. coli* bacterioferritin from the internal cavity	162
7.13	An external view of the B-channel in *E. coli* bacterioferritin	164
8.1	Alternate NADPH binding	166
8.2	Superposition of NADPH with sulphate positions	167
8.3	NADPH binding	168
8.4	Variation in loops 3 and 4 of the AKRs	170
8.5	Steroid substrate binding	171
8.6	Sites identified for iterative saturation mutagenesis	173

List of Tables

Table	Title	Page
3.1	Ketone and aldehyde substrates used for screening assays with TmAKR	69
3.2	Polarity index of solvents tested	78
3.3	Summary of kinetic parameters of the TmAKR enzyme	81
5.1	Composition of mother liquors used for soaking crystals of TmAKR prior to flash freezing	103
5.2	Data collection parameters for 'Holo' crystals of TmAKR	104
5.3	Data collection statistics for three TmAKR data sets	108
5.4	Refinement statistics for final model of TmAKR	112
5.5	Processing statistics for peak and remote data sets of *E. coli* bacterioferritin	115
5.6	Refinement statistics for *E. coli* bacterioferritin	118
6.1	Amino acid composition	136
6.2	α-helix stabilisation	138
7.1	Summary of the atomic contacts made by the two alternate conformations of heme with subunits A and B of *E. coli* bacterioferritin	154
8.1	Summary of sites identified for iterative saturation mutagenesis	174

List of Equations

Equation	Title	Page
1.1	Equilibrium model of enzyme inactivation	39
2.1	Estimation of native molecular weight	57
3.1	Kinetic constants of one substrate one product reaction	79
3.2	Michaelis-Menten equation	79
3.3	The Michaelis constant	79
3.4	The catalytic constant	80
5.1	Bragg's law	94
5.2	Structure factor	95
5.3	Atomic scattering contribution	96
5.4	Scattering density	96
5.5	Anomalous scattering	98
5.6	Structure factor equation with anomalous scattering	99
5.7	Reliability factor	101
7.1	The Fenton reaction	144
7.2	Bacterioferritin reduction of O_2	144
7.3	Bacterioferritin reduction of H_2O_2	144
7.4	Net reaction of bacterioferritin	144

Abbreviations

%	Percentage
°C	Degrees centigrade
µ	Micro
Å	Angstrom
ADH	Alcohol Dehydrogenase
AKR	Aldo-Keto Reductase
APS	Ammonium persulfate
atm	Atmospheres
C	Centi
Da	Daltons
DMSO	Dimethyl sulfoxide
E_{act}	Active enzyme
E_{inact}	Inactive enzyme
eV	Electron volts
EXAFS	Extended X-ray Absorption Fine Structure
FFQ	Fast Flow Q-sepharose
g	Gram
IPTG	Isopropyl β-D-1-thiogalactopyranoside
K	Kelvin
k	Kilo
K_{cat}	Catalytic constant
k_d	Dissociation constant
K_{eq}	Equilibrium constant
K_{inact}	Inactivation constant
K_m	Michaelis constant
L	Litre
LB	Liquid broth
m	Metre
m	Milli
M	Molar
MAD	Multiwavelength anomolous dispersion
MDR	Medium chain dehydrogenase/reductase
MIR	Multiple isomorphous replacement
mol	Mole
MR	Molecular replacement
n	Nano
NAD^+	Nicotinamide adenine dinudeotide
NADH	Nicotinamide adenine dinudeotide (reduced form)
$NADP^+$	Nicotinamide adenine dinudeotide phosphate

NADPH	Nicotinamide adenine dinudeotide phosphate (reduced form)
OD_x	Optical density at wavelength x
Pcp	Pyrrolidone carboxyl peptidase
PIXE	Proton induced X-ray emission
rpm	Revolutions per minute
S	Seconds
SDR	Short chain dehydrogenases /reductases
SDS-PAGE	Sodium-dodecyl sulfate polyacrylamide gel electrophoresis
TEMED	Tetramethylethylenediamine
T_{eq}	Equilibrium temperature
THBA	1,2,3,6-tetrahydrobenzaldehyde
Tm	Melting temperature
TmAKR	*Thermotoga martima* aldo keto reductase
U	Units of activity
v/v	Volume per volume
Vmax	Maximum reaction velcoity
W	Watts
w/v	Weight per volume
ΔG^*_{inact}	Activation energy of inactivation
ΔG_{stab}	Energy of stabilisation
$\Delta\Delta G_{desolvation}$	Change in free energy of desolvation

Organism Abbreviations

A. pernix	*Aeropyrum pernix*
B. amyloliquefaciens	*Bacillus amyloliquefaciens*
B. halodurans	*Bacillus halodurans*
B. subtilis	*Bacillus subtilis*
C. tenuis	*Candida tenuis*
D. lebanonensis	*Drosophila lebanonensis*
E. coli	*Escherichia coli*
Pyroc. furiosus	*Pyrococcus furiosus*
S. acidalcaldarius	*Sulfolobus acidalcaldarius*
S. cerevisiae	*Saccharomyces cerevisiae*
S. solfataricus	*Sulfolobus solfataricus*
Thermoc. litoralis	*Thermococcus litoralis*
Thermot. martima	*Thermotoga martima*
Thermu. thermophilus	*Thermus thermophilus*