MECHANISTIC STUDIES OF *ESCHERICHIA COLI* TRANSKETOLASE

Submitted by

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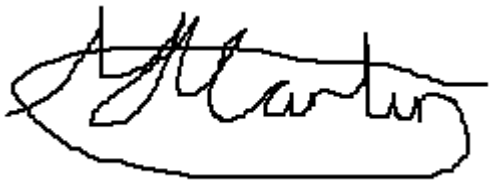
to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Chemistry (December 2008).

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.



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Abstract

The enzyme transketolase is found in nature as part of the Pentose Phosphate Pathway to rearrange large sugar phosphates. It also is an important enzyme for carbon-carbon bond formation for industrial biocatalysis.

The work presented in this thesis describes the purification, crystallisation, characterisation and structural determination of the recombinant *Escherichia coli* transketolase complexed with the substrate hydroxypyruvate and potential inhibitor fluoropyruvate. The native transketolase and the transketolase-hydroxypyruvate structures were solved to a 1.18 and 1.05 Å resolution respectively. The transketolase structures show a chain of ordered water molecules spanning a distance of 20 Å between the two active sites. The water molecules are linked via a network of hydrogen bonds and they are proposed to facilitate proton transfer between the two-thiamine pyrophosphate molecules, thereby providing a method of communication between the two active sites of the enzyme. The transketolase-hydroxypyruvate structure shows the hydroxypyruvate substrate forming a covalent bond to the thiamine pyrophosphate thereby creating a α,β-dihydroxyethyl–thiamine pyrophosphate complex within the enzyme active site. The novel transketolase-fluoropyruvate structure solved to a 1.60 Å resolution, it produced a snapshot image of the ketol donor prior to formation of the active enamine intermediate. The trapped fluoropyruvate molecule is shown to form an angle that varies from the accepted Burgi-Dunitz angle of 109.5° for nucleophilic attack. However, this is inconclusive due to the low occupancy of the fluoropyruvate. In addition, kinetic studies were performed on the recombinant *E. coli* transketolase to investigate the inhibitory role of fluoropyruvate during the enzymatic reaction.

The active site recombinant *E. coli* transketolase mutants H26Y and D469Y have been also been purified and characterised. The mutant H26Y complexed with fluoropyruvate was crystallised and its structure determined to 1.66 Å resolution. This structure has given an insight into why this mutation results in the formation of the opposite D-enantiomer of erythrulose rather than the L-erythrulose produced by the wild-type transketolase enzyme.

The thesis also includes the purification, crystallisation, characterisation and X-ray diffraction studies of the commercially useful oxygenating enzyme, 2,5-diketocamphane 1,2-monooxygenase from *Pseudomonas putida*. The recombinant dimeric oxygenase component of this enzyme has been crystallised and its structure solved to 1.4 Å resolution.

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Abbreviations

2,5-DKMO- 2,5-diketocamphane 1,2-monooxygenase

3,6-DKMO- 3,6-diketocamphane 1,2-monooxygenase

A_{280}- Absorbance at 280 nm

Å- Angstrom

Au- Atomic units

APS- Ammonium persulfate

ATP- Adenosine Triphosphate

BAM- Benzamidine

bp- Base pairs

BSA- Bovine serum albumin

BVMO- Baeyer-Villiger monooxygenase

CCP4- Collaborative Computational Project, number 4

CHMO- cyclohexanone monooxygenase

D469Y- Glutamate mutation to tyrosine at position 469

DMSO- Dimethyl Sulfoxide

EC- Enzyme commission

EDTA- Ethylenediaminetetraacetic acid (disodium salt)

FAD- flavin adenine dinucleotide

FMN- flavin mononucleotide

FFQ- Fast flow Q

FPA- Fluoropyruvic acid

FPLC- Fast protein liquid chromatography

g- Acceleration due to gravity

GA- Glycolaldehyde

GAPDH- Glyceraldehyde 3-phosphate dehydrogenase

GF- Gel filtration

H26Y- Histidine mutation to tyrosine at position 26

HEPES- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

his-tag- Poly histidine tag

HPA- Hydroxypyruvic acid

IPTG- Isopropyl β-D-galactopyranoside

kDa- Kilo Dalton

K_m- Michaelis Constant

LB- Luria-Bertani

MAD- Multiple anomalous diffraction

MES- 2-(*N*-morpholino)ethanesulfonic acid

MW- Molecular weight

MWCO – Molecular weight cut off

NADPH/ NAD^+- Nicotinamide adenine dinucleotide (protonated/deprotonated)

NMR- Nuclear magnetic resonance

OD- Optical density

PAGE- Polyacrylamide gel electrophoresis

PDB- Protein Databank

PEG- Polyethylene glycol

PIPES- 1,4-Piperazinediethanesulfonic acid

PMSF- Phenylmethylsulphonyl fluoride

PPP- Pentose Phosphate Pathway

RMS- Root mean square

rpm- Revolutions per minute

SAD- Single anomalous diffraction

SDS- Sodium dodecyl sulfate

SRS- Synchrotron radiation source

TEMED- N,N,N,N-tetramethylethylene diamide

TFA- Trifluoroacetic acid

TK- Transketolase

TPP- Thiamine pyrophosphate

Tris- Tris(hydroxymethyl)aminomethane

UV- Ultra violet

v/v- Volume to volume

V- Volts

V_{max}- Measure of maximum turnover by an enzyme

w/v- Weight to volume

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