Bisubstrate Kinetics and Processivity Measurements on *Escherichia Coli* DNA Ligase A

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Abstract

DNA ligases are essential repair enzymes required for maintaining genomic integrity in cells. The first ligase to be discovered was *Escherichia* coli DNA ligase; a 670 amino acid, 74 kDa, NAD^+ dependent ligase. This work reports a series of studies into the behaviour of His-tagged *E.coli* ligase.

Order-of-addition studies on singly-nicked oligoduplexes under steady state conditions revealed that ligase undergoes an obligatory off-step from the DNA after sealing a break in a phosphodiester strand before readenylation in solution. These results corroborate the findings of Lehman that a sequential model is the normal mode of Ligase operation. Ligase affinity for its substrates NAD^+ and DNA were 3.5 μM and 3.5 nM respectively.

Length dependency studies on singly-nicked PCR substrates revealed that when two different DNA lengths were in the same solution, the initial association rate was always faster for the longer DNA substrate. For example, 40 bp versus 902 bp gave initial rate values 0.06 nM/min (40 bp) and 0.28 nM/min (902 bp); increasing the length 22 fold increased the initial rate 4 fold. This hints that Ligase uses DNA flanking a nick to locate its specific site.

Processivity studies were achieved to determine the one- or three-dimensional pathway of Ligase using doubly-nicked DNA. Nicks were either directly repeated (on the same DNA strand) or inverted (opposite strands). Results revealed Ligase is weakly processive; 32% processive. However, when beta-clamp and gamma-loader were added to the reaction processivity significantly increased.

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Abbreviations and notations

Units, prefixes, parameters

1D, 3D	one or three-dimensional motion
Å	Angstrom (1×10^{-10} m)
A_{260}, A_{280}	UV absorbance at 260 or 280 nm
bp	base pair(s)
Da	Daltons (kDa)
fp	fractional processivity
g	gram (mg, µg, ng)
hr, min, sec	hour(s), minutes(s), second(s)
k, k_{cat}	rate constant (s^{-1}, min^{-1})
K_d, K_m	dissociation/Michaelis constant (µM, nM)
l	litres(s) (ml, µl)
M	molar concentration (mM, µM, nM)
psi	pounds per square inch (15 psi ~ 1 kg/cm^2)
rpm	revolutions per minute
RT	room temperature
T_m	melting temperature (50% annealing)
V_{max}	maximal enzyme velocity

Reagents

AMPS	ammonium persulphate
BSA	bovine serum albumin
DTT	dithiothreitol
DW	distilled water (>16 MOhm)
E. coli	*Escherichia Coli* strain HB101
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
EtOH	ethanol
IPTG	isopropyl-β-D-thiogalactopyranoside

SDS	sodium dodecyl sulphate
TAE	Tris-acetate EDTA buffer
TBE	Tris-borate EDTA buffer
TE	Tris-EDTA buffer
TEMED N,N,N',N'	tetramethylethylenediamine
Tris	2-amino-2-hydroxymethylaminomethane
Techniques	
EMSA	electrophoretic mobility-shift assay
HPLC	high-pressure liquid chromatography
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
Nucleic acids	
^{33}P	^{33}P-labelled phosphorous atom
A, T, C, G	adenine, thymine, cytosine, guanine
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dsDNA	double-stranded DNA
MCS	multiple-cloning site
NAD^+	Nicotinamide adenine dinucleotide (oxid)
RNA	ribonucleic acid
Enzymes	
β-clamp	*E.coli* beta-sliding clamp
BRCT	BRCA1-C-terminal domain
HhH	Helix-turn-helix
His-tag	N-terminal polyhistidine tag
LigA	*E.coli* DNA ligase A
OB	oligomer binding
PNK	polynucleotide kinase
Zn	zinc finger motif

Plasmids

Details of all plasmids used in this work, including sequences, are given in the Appendices (page 175 onwards). Brief details are given below:

pL1	Plasmid with one BbvCI site cloned into the mutilple clong site of pUC19. This was used to make PCR fragments containing a single nick (Chapters 3 and 4).
pL2, 4, 6, 8, 10, 12	Plasmids with two BbvCI sites (21, 30, 36, 40, 45, 75 b apart respectively), derived from plasmid pL1. Following PCR, each BbvCI site was cut in one strand only to give a defined DNA nick. These Ligase substrates had two directly-repeated nicks, so were termed dir substrates. These plasmids were used in processivity experiments (Chapter 5).
pL5, 7, 9, 11, 13	Plasmids with two BbvCI sites (24, 30, 34, 39, 69 b apart respectively), derived from plasmid pL1. Following PCR, each BbvCI site was cut in one strand only to give a defined DNA nick. These Ligase substrates had two inverted repeated nicks, so were termed inv substrates.These plasmids were used in processivity experiments (Chapter 5).
pRB20	*Eco*LigA over-expression plasmid to produce His-tagged DNA ligase. This plasmid was a kind gift from Dr Richard Bowater (UEA).

Declaration

Whilst registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.