Chapter 5 - Processivity Studies.

5.1 Introduction

In the previous chapter length dependency studies determined that Ligase uses facilitated diffusion to locate a nick. What these experiments don’t show is the mechanism of facilitated diffusion adopted by Ligase to locate a nick. To distinguish between two methods of facilitated diffusion: one-dimensional (1D) sliding or three-dimensional (3D) hopping, previous studies on restriction enzymes have carried out processivity studies (Gowers et al 2005).

In this chapter similar processivity studies have been used to determine if Ligase locates a nick by either 1D sliding or by 3D hopping.

5.2 Preparation of DNA substrates.

For these processivity experiments, two different DNA substrates were produced; each contained two nick sites, either the nick sites were in the DNA strand (direct repeat) or a single nick was in each of the DNA strands (inverse repeat). Figure 5.1A is an illustration of the two DNA substrates used in these experiments. On the left (Figure 5.1 Panel A1) is the direct repeat. Red arrows indicate where BbvCI will bind DNA and react to produce a nick. As described before, in these experiments the mutated form of BbvCI (R1+) was used to produce a nick in the bottom strand only, producing head to tail nick sites. On the right (Figure 5.1 Panel A2) is the inverted repeat. The second nick site has been inverted so that a nick will be produced in the opposite strand producing head to head nick sites.

In the first set of experiments direct repeated DNA substrates were used. Figure 5.1B shows the three possible Ligase reaction pathways. Either the reaction is processive and therefore both nicks are sealed at relatively the same time, indicated by the red arrow, or the reaction produces an intermediate product where one nick is sealed first. The second
Figure 5.1 Mechanism of processivity

Panel A. Processivity strategies. Black lines represent the bottom DNA strand. Blue lines represent the top DNA strand. Red arrows represent BbvCI sites. 1. BbvCI sites are repeated on the same strand. 2. BbvCI site is inverted (dashed box) so that sites are on different DNA strands.

Panel B. Cartoon of repeated sites and partial products. A scheme for ligase in which the enzyme can follow a processive (central red arrow) or sequential (side arrows) mechanism. If sequential, the Ligase can either seal the nick between DNA fragments A and B (Blue arrow) or the nick between DNA fragments B and C (green arrow) before sealing the second nick (black arrows).

Panel C. A theoretical graph of a processive reaction. If an enzyme is processive the initial rate (within first 10% of reaction) of the final product ABC (red line) will be higher than the initial rates for intermediate products AB (blue) and BC (green).
pathway is indicated by black arrows and this is a non-processive reaction. The
labelling in Figure 5.1B will be used throughout this chapter. A is the 3’ fragment of the
bottom strand, B is the central fragment, and C is the 5’ DNA fragment. AB and BC are
the intermediate products from Ligase reaction, and ABC is the final product. The top
fragment is denoted abc (not shown in this figure). Figure 5.1C is a graphical
representation of a processive reaction. The initial reaction rates can be used to
determine the processivity factor \((fp)\) of Ligase. If the reaction rate for ABC is faster
than the rate of AB and BC, then the reaction is termed processive. Inverted repeat
DNA substrates can not be used directly to determine processivity. This is because the
intermediate products are the same length (shown later). However, these substrates can
be used to determine which method of facilitated diffusion Ligase adopts. If both nicks
are sealed at the same rate the mechanism will hint at a three-dimensional hopping.
Inverted repeats will also help determine if Ligase has a site preference. If one nick is
sealed faster than the other, then Ligase will have site preference.

For processivity studies, DNA substrates containing two nicks on the bottom strand
were produced. Figure 5.2A shows how the different DNA substrates were named in
this chapter. The table shows the different DNA substrates used; 21dir, 30dir, 36dir,
40dir, 45dir and 75dir, and the length of each DNA fragment as seen when run on a
denaturing polyacrylamide gel. Figure 5.2B shows the DNA substrates used for
facilitated diffusion and site preference experiments; 24inv, 30inv, 34inv, 39inv and
69inv. DNA fragments a and b are the two single stranded fragments produced when
there is a nick in the top strand. DNA fragments C and D are the two fragments
produced when there is a nick in the bottom strand of DNA. The intermediate products
formed during the Ligase reaction are ab and CD. These are both the same length and
will not be able to be separated on a denaturing polyacrylamide gel. The distance
between nick sites is from 24 bases to 69 bases.
A

Directly repeated nicks (dir PCR substrates)

\[
\begin{array}{c}
5' \quad abc \\
5' \\
3' \quad A \\
3' \\
\mid \quad AB \\
\mid \\
3' \quad B \\
3' \\
\mid \quad BC \\
\mid \\
3' \quad C \\
3'
\end{array}
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B

Inverted repeated nicks (inv PCR substrates)

\[
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5' \\
3' \quad C \\
3' \\
\mid \quad ab/CD \\
\mid \\
3' \quad b \\
3' \\
\mid \\
3'
\end{array}
\]

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**Figure 5.2 Naming convention of DNA substrates used in this chapter**

**Panel A.** Direct repeat DNA substrates: 21dir, 30dir, 36dir, 40dir, 45dir, 75dir. Substrates were radiolabelled using $^{33}$P dATP inclusion during PCR. Two nicks were created in the bottom strand of each substrate using BbvCI R1+. The table shows the individual DNA fragments that can be seen when run on denaturing polyacrylamide gels. The important bottom strand is in capitalised letters. (abc is not involved in Ligase reaction but can be seen on denaturing gels).

**Panel B.** Inverted repeat DNA substrates: 24inv, 30inv, 34inv, 39inv, 69inv. Substrates were labelled as above. A single nick in each strand was created using the mutated BbvCI R1+. Table shows the individual DNA fragments that can be seen when run on denaturing polyacrylamide gels.
DNA substrates were made by radiolabelling PCR with $\alpha^{33}$P-dATP. This meant that every possible A had the potential to be radiolabelled, as previously described. To avoid the production of extra (intermediate) bands as seen in Chapter 4, all PCR substrates were purified as per the new protocol. In total 12 plasmids were used to make DNA substrates (PCR product in brackets); pL2 (21dir), pL4 (30dir), pL5 (24inv), pL6 (36dir), pL7 (30inv), pL8 (40dir), pL9 (34inv), pL10 (45dir), pL11 (39inv), pL12 (75dir) and pL13 (69inv). Figure 5.3 shows the DNA purification protocol used for making the DNA in this chapter. The ‘dirty’ PCR products were cleaned using Qiagen columns to remove excess dNTPS and polymerase and eluted into 50 µl T.E. buffer. An additional purification was added at this stage. The ends of DNA were cut using EcoRV to produce blunt ends. This reaction was achieved in 100 µl reactions for 2 hr at 37°C. The cut DNA substrates were then cleaned using Qiagen columns to remove the cut ends and EcoRV, and eluted into 50 µl T.E. Nicks were introduced by digestion with BbvCl R1+, in 200 µl reactions containing reaction buffer B, and 20 µM BbvCl R1+. Reactions were carried out for 2 hr at 37°C. The DNA was cleaned from BbVCl using Qiagen spin columns and eluted into 50 µl T.E. DNAs were then run on 5% polyacrylamide gels for 4 hr at 15 mA. This was to remove any doubly nicked DNA substrates, and to ensure the DNA substrates used in these experiments were as clean as possible (free from intermediate bands). The wet gels were exposed for 20 min and scanned and visualised. The distance that the required DNA had migrated was calculated using ImageGuage. The gel was then measured and the DNA band was excised using a razor. The gel slice was added to 300 µl T.E. DNA was eluted out into the T.E for 8 hr at 37 °C. To confirm that the correct band had been excised the wet gel was re-exposed for 20 min, and rescanned and visualised. Figure 5.3A and B show PCRs 21dir and 30dir before and after the DNA band was excised from the gel. The DNA was then precipitated by ethanol precipitation and resuspended in 50 µl T.E. The
Figure 5.3 Method for purifying DNA
Primers were used to incorporate EcoRV sites at the ends of PCR products. Radiolabelled PCR products were made and cleaned using Qiagen PCR cleanup spin columns. The PCR products were cut with EcoRV to produce blunt ended DNA. DNA was purified from EcoRV using Qiagen spin columns. Two nicks were introduced using BbvCI R1+, 2 hr at 37°C in buffer B. DNA was purified from enzyme using Qiagen columns. Nicked DNA was gel purified on 5% polyacrylamide gel (15 mA, 4 hr). Gels were exposed for 20 min and visualised using ImageGauge. The distance of the required DNA (indicated by black arrows) was calculated and DNA was excised and placed into T.E buffer.

Panels A and B: Visualised DNA bands from 21dir (left) and 30dir (right), before (A) and after (B) DNA bands were excised from the gel. DNA was eluted from the gel into 300 µl T.E for 8 hr at 37°C. DNA was purified by Ethanol precipitation and resuspended in T.E

Panel C: DNA purity was determined by UV spectroscopy. UV traces for 21, 30, 36, 40, 45 and 75dir DNA substrates (from top left to bottom right respectively).
purity of each DNA substrate was determined by Nanodrop Spectroscopy. Figure 5.3C shows the spectrophotometry results for PCR 21, 30, 36, 40, 45 and 75dir. The molecular weight of 21dir is smaller than the other PCR products because it is 3 bp shorter at 468 bp, whilst all other PCR products were 471 bp.

5.3 Processivity results on DNA substrates containing two directly repeated nicks

Figures 5.4 to 5.6 show the results for the Ligase reaction on DNAs 21, 30, 36, 40, 45 and 75dir. All reactions were carried out at 37°C with 0.2 nM Ligase, 5 nM DNA, 25 µM NAD⁺ and 1xLigase reaction buffer. All gels represent a 20 min timecourse with time intervals 0, 0.5, 1, 2, 4, 6, 8, 10 and 20 min. All graphs were created directly from the corresponding gel results therefore no error bars are seen. However these experiments were repeated with time intervals from 0 to 60 min at which the Ligase reaction was confirmed to be completed (data not shown). The results from each repeat were collated to give the results later in this chapter. Aliquots at each time interval were added to STOP buffer to terminate the Ligase reaction. The DNA was denatured as previously described and run on pre heated 5% denaturing polyacrylamide gels for 45 min at 70 W. The gels were then dried, exposed, scanned and visualised as described previously.

Figure 5.4 shows the Ligase reaction results for 21dir and 30dir. Panel A shows the polyacrylamide gel; on the left is Ligase reaction with 21dir (lanes 2-10), and on the right is the reaction with 30dir (lanes 12-20). Lanes 1 and 11 are the marker lanes. As the DNA is labelled throughout, the following DNA bands are visible for the 21dir reaction: 468 b (abc and ABC, abc being the intact top strand), 265 b (AB), 244 b (A), 224 b (BC), 203 b (C) and 21 b (B), as indicated by the black arrows. Each substrate, intermediate product and final product are clearly visible on the gel. The following DNA bands are visible for the 30dir reaction 471 b (abc and ABC), 274 b (AB), 244 b
Figure 5.4 Timecourses on 21dir or 30dir DNA.
Reactions at 5 nM DNA, 1x Ligase buffer and 0.2 nM Ligase, 37°C as in Materials and Methods. Samples loaded onto 8% PA urea denaturing gel.

Panel A. Gel showing two timecourses, lane 1 marker (as used in Chapter 4, Figure 4.3C). Lanes 2-10 21dir (0, 0.5, 1, 2, 4, 6, 8, 10, 20). Lanes 11 marker, Lanes 12-20 (timepoints as before). DNA fragments are indicated by black arrows. Panel B: Graph showing results of quantitation of 21dir. Colours as per legend. Panel C: Graph showing results of quantitation of 30dir. Colours as per legend.
(A), 227 b (BC), 197 b (C) and 30 b (B). In both reactions, at time zero a dark DNA band can be seen. This is the intact top DNA strand. The intensity of this band increases as the Ligase reaction proceeds because the amount product ABC is increasing.

The gel was visualised using ImageGauge. Boxes were drawn around each band (all boxes were the same size). The intensity of each band was exported to Excel. As previously explained in Chapter 4, it was assumed that every A was labelled. The number of As was determined for each single-stranded DNA fragment seen on the denaturing polyacrylamide gel. The fraction of ‘hot’ As in each band was determined in relation to the total number of hot As in the intact final product. The intensity of each DNA band on the gel was multiplied by the fraction of hot As, and then multiplied by the known DNA concentration (5 nM). This gave the corrected DNA concentration of every band in the gel. At time zero the 468 b for 21dir and 471 b for 30dir, the DNA concentration starts at 5 nM. This is the intact top strand. As time progresses the intensity of this band increases. This is the production of the nick sealed bottom DNA strand.

The initial rate of each DNA fragment was determined in Excel. A linear regression was fitted to the first 10% of the reaction. The initial rates for 21dir were 0.250 nMmin\(^{-1}\) for 468 b (ABC), 0.531 nMmin\(^{-1}\) for 244 b (A), 0.552 nMmin\(^{-1}\) for 21 b (B), 0.502 nMmin\(^{-1}\) for 203 b (C), 0.372 nMmin\(^{-1}\) for 265 b (AB), 0.467 nMmin\(^{-1}\) for 224 b (BC). The initial rates for 30dir were 0.100 nMmin\(^{-1}\) for 471 b (ABC), 0.416 nMmin\(^{-1}\) for 244 b (A), 0.831 nMmin\(^{-1}\) for 30 b (B), 0.268 nMmin\(^{-1}\) for 197 b (C), 0.391 nMmin\(^{-1}\) for 274 b (AB), 0.125 nMmin\(^{-1}\) for 227 b (BC).

Figure 5.4B and C show the timecourses plotted in prism. A single 1\(^{st}\)-order exponential rise was fitted to the final product formation (468 b for 21 dir and 471 b for 30dir); a single 1\(^{st}\)-order exponential decay was fitted to the nicked fragments A, B and C; the results for AB and BC cannot be fitted as the DNA concentration rises and falls.
during the course of the Ligase reaction. Instead a strikethrough line was added to this data. The Ligase reaction was near completion at 20 min. In figure 5.4C it is clearly seen that the rate of AB is faster than the rate of BC. This hints to a possible site preference by the Ligase.

Figure 5.5 shows the Ligase reaction results for 36dir and 40dir. Panel A shows the polyacrylamide gel; on the left is Ligase reaction with 36dir (lanes 2-10), and on the right is the reaction with 40dir (lanes 12-20). Lanes 1 and 11 are the marker lanes. As the DNA is labelled throughout, the following DNA bands are visible for the 36dir reaction: 471 b (abc and ABC, abc being the intact top strand), 280 b (AB), 244 b (A), 227 b (BC), 191 b (C) and 36 b (B), as indicated by the black arrows. Each substrate, intermediate product and final product are clearly visible on the gel. The following DNA bands are visible for the 40dir reaction 471 b (abc and ABC), 284 b (AB), 244 b (A), 227 b (BC), 187 b (C) and 40 b (B). In both reactions, at time zero a dark DNA band can be seen. This is the intact top DNA strand. The intensity of this band increases as the Ligase reaction proceeds because the amount product ABC is increasing. The intensity of B in both 36dir and 40dir is lighter than the other DNA bands. This is because the number of hot As is smaller; 9 As for 36dir and 9 As for 40dir.

The gel was visualised using ImageGuage. Boxes were drawn around each band (all boxes were the same size). The intensity of each band was exported to Excel. As previously explained above, it was assumed that every A was labelled. The number of As was determined for each single-stranded DNA fragment seen on the denaturing polyacrylamide gel. The DNA concentration was determined for every band on the gel. The initial rate of each DNA fragment was determined in Excel. A linear regression was fitted to the first 10% of the reaction. The initial rates for 36dir were 0.814 nMmin⁻¹ for 471 b (ABC), 1.960 nMmin⁻¹ for 244 b (A), 1.201 nMmin⁻¹ for 36 b (B), 1.043 nMmin⁻¹ for 191 b (C), 1.489 nMmin⁻¹ for 280 b (AB), 0.242 nMmin⁻¹ for 224 b (BC). The initial
Figure 5.5 Timecourses on 36dir or 40dir DNA.
Reactions at 5 nM DNA, 1x Ligase buffer and 0.2 nM Ligase, as in Materials and Methods.
Panel A. Gel showing two timecourses, lane 1 marker. Lanes 2-10 36dir (0, 0.5, 1, 2, 4, 6, 8, 10, 20). Lanes 11 marker. Lanes 12-20 40dir (timepoints as before). DNA fragments indicated by black arrows.
Panel B: Graph showing results of quantitation of 36dir using Prism. Colours as per key. Panel C. Graph showing results of quantitation of 40dir. Colours as per key.
rates for 40dir were 0.601 nMmin$^{-1}$ for 471 b (ABC), 1.906 nMmin$^{-1}$ for 244 b (A), 1.091 nMmin$^{-1}$ for 40 b (B), 0.793 nMmin$^{-1}$ for 187 b (C), 0.995 nMmin$^{-1}$ for 284 b (AB), 0.320 nMmin$^{-1}$ for 227 b (BC).

Figure 5.5B and C show the timecourses plotted in prism. A single 1$^{st}$-order exponential rise was fitted to the final product formation 471 b for both 36dir and 40dir; a single 1$^{st}$-order exponential decay was fitted to the nicked fragments A, B and C; the results for AB and BC cannot be fitted as the DNA concentration rises and falls during the course of the Ligase reaction. Instead a strikethrough line was added to this data. The Ligase reaction was near completion at 20 min. In figure 5.5C it is seen clearly that the rate of AB is faster than the rate of BC. This was also observed for 30dir, further suggesting that the Ligase has a preference for the nick between A and B over the nick between B and C. The initial reaction rate for nick sealed intermediate DNA product AB is greater than three times that of the BC product for both 36dir and 40dir.

Figure 5.6 shows the results of the Ligase reaction on 45dir and 75dir. Panel A shows the polyacrylamide gel; on the left is Ligase reaction with 45dir (lanes 2-10), and on the right is the reaction with 75dir (lanes 12-20). Lanes 1 and 11 are the marker lanes. As the DNA is labelled throughout, the following DNA bands are visible for the 45dir reaction: 471 b (abc and ABC), 289 b (AB), 244 b (A), 227 b (BC), 182 b (C) and 45 b (B), as indicated by the black arrows. Each substrate, intermediate product and final product are clearly visible on the gel. The following DNA bands are visible for the 75dir reaction 471 b (abc and ABC), 319 b (AB), 244 b (A), 227 b (BC), 152 b (C) and 75 b (B). In both reactions, at time zero a dark DNA band can be seen. As described before, this is the intact top DNA strand. The intensity of the intermediate product bands AB and BC first increases as the first nick is sealed reaching its peak at 10 min for both DNA substrates (lanes 9 and 19), and then the intensity decreases as the second nick is sealed to produce 471 b (ABC) product.
Figure 5.6 Timecourses on 45dir or 75dir DNA.
Reactions at 5 nM DNA, 1x Ligase buffer and 0.2 nM Ligase, 37°C as in Materials and Methods.
Samples loaded onto 8% PA urea denaturing gel.
Panel A. Gel showing two timecourses, lane 1 marker. Lanes 2-10 45dir (0, 0.5, 1, 2, 4, 6, 8, 10, 20).
Lanes 11 marker. Lanes 12-20 74dir (timepoints as before). DNA fragments indicated by black arrows.
Panel B: Graph showing results of quantitation of 21dir. Colours as per key. Panel C. Graph showing
results of quantitation of 30dir. Colours as per key.
The gel was visualised using ImageGauge. Boxes were drawn around each band (all boxes were the same size). The intensity of each band was exported to Excel. As previously explained above, it was assumed that every A was labelled. The number of As was determined for each single-stranded DNA fragment seen on the denaturing polyacrylamide gel. The DNA concentration was determined for every band on the gel. The initial rate of each DNA fragment was determined in Excel. A linear regression was fitted to the first 10% of the reaction. The initial rates for 45dir were 0.647 nMmin$^{-1}$ for 471 b (ABC), 0.810 nMmin$^{-1}$ for 244 b (A), 0.903 nMmin$^{-1}$ for 45 b (B), 1.591 nMmin$^{-1}$ for 182 b (C), 0.743 nMmin$^{-1}$ for 289 b (AB), 0.231 nMmin$^{-1}$ for 227 b (BC). The initial rates for 75dir were 0.681 nMmin$^{-1}$ for 471 b (ABC), 1.841 nMmin$^{-1}$ for 244 b (A), 1.597 nMmin$^{-1}$ for 75 b (B), 1.155 nMmin$^{-1}$ for 157 b (C), 1.130 nMmin$^{-1}$ for 319 b (AB), 0.360 nMmin$^{-1}$ for 227 b (BC).

Figure 5.6B and C show the timecourses plotted in Prism. A single 1st-order exponential rise was fitted to the final product formation 471 b for both 45dir and 75dir; a single 1st-order exponential decay was fitted to the nicked fragments A, B and C; a strikethrough line was plotted to the results for AB and BC. The Ligase reaction was near completion at 20 min. In figure 5.6C it is seen clearly that the rate of AB is faster than the rate of BC. This was also observed for 30, 36 and 40dir, it seems highly possible that the Ligase has a preference for the nick between A and B over the nick between B and C. As previously seen, the initial reaction rate for nick sealed intermediate DNA product AB is greater than three times that of the BC product for both 45dir and 75dir.

Each of the above experiments was repeated three times (individual data not shown). The results were correlated and put in a table in figure 5.7A. With the exception of 21dir, every reaction showed a site preference to the nick between DNA fragments A and B. The initial reaction was at least three times faster than the initial rate of the nick.
A

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(average in nMmin⁻¹)

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Figure 5.7
Panel A: Table of initial rates for every DNA substrate (three experiment repeats were collated into one result) The results were averaged and added to the table. Columns show initial rates in nMmin⁻¹. Final two columns show the total rate for substrate loss and for intermediate gain.
Panel B: Table of initial rates plotted into processivity equations 5.2 and 5.3 (described on page 134). The results were multiplied by 100 to give processivity (fp) as a percentage.
sealed product BC. The initial rate for DNA fragment A was the fastest rate compared to other nicked fragments (again with the exception of 21dir). The processivity factor was determined by putting this data into equation 5.3 shown below:

\[ fp = \frac{\text{Amount of final product}}{\text{Start and intermediate products}} \]  

Equation 5.1

\[ fp = \frac{(A + C - AB - BC)}{(A + B + AB + BC)} \]  

Equation 5.2

\[ fp = \frac{(ABC)}{(ABC + AB + BC)} \]  

Equation 5.3

Equation 5.1 is the worded processivity equation. Equation 5.2 is the equation used in other processivity experiments (Terry et al. 1985, Stanford et al. 2000) and equation 3 has been adapted from the previous equation to show the processivity factor for nicked substrates. To compare these equations, the data was applied to both equations 5.2 and 5.3. Figure 5.7B shows the initial rates applied to each equation. The results show that as the intersite distance increases, the Ligase reaction is more processive. Processivity was calculated to be 30-35%.

**5.4 Changing salt concentrations**

To determine how ionic strength affected processivity experiments were conducted in which the salt concentration was varied. PCR substrates 45dir and 75dir were produced as described earlier. Ligase (50 µl) reactions were carried out at 37°C, 1x Ligase buffer 25 µM NAD\(^+\) and with either 1 mM or 4 mM MgCl\(_2\), with either 0, 20 or 40 mM NaCl. Ligase reactions were achieved for up to 120 min. At set time intervals the Ligase reaction was terminated by adding aliquots of reaction to STOP buffer. The DNA was denatured, and run on 5% denaturing polyacrylamide gels, as previously
described. The gels were dried, exposed, scanned and visualised. Figures 5.8 and 5.9 show the results of the Ligase reaction at different salt concentrations. Reactions were carried out using 625 bp DNA fragments, produced using primer pairs 45dir and 75 dir. These reactions were conducted consecutively with the processivity experiments in Section 5.3. The 625 bp substrates were used because previous experiments (601 bp in Chapter 3.7) showed that these PCRs produced the cleanest DNA. The 625 bp contain two off-centre nicks (closer to 5’ on the bottom strand). These substrates were not gel purified and some extra bands are seen on the gels. These bands were not significant enough to affect the results of each Ligase reaction.

Figure 5.8 shows the results for Ligase reaction at 1 mM MgCl₂ with varying NaCl concentrations. Panel A shows the denaturing polyacrylamide gel for 45 and 75dir Ligase reaction with 1 mM MgCl₂ and 0 mM NaCl. Lanes 1-6 is the Ligase reaction for 75dir and lanes 7-12 is the timecourse for 45dir; time intervals 0.5, 2, 5, 15, 30 and 60 min. As the DNA is labelled throughout, the following DNA bands are visible for the 45dir reaction: 625 b (abc and ABC), 499 b (AB), 454 b (A), 171 b (BC), 126 b (C) and 45 b (B), as indicated by the green arrows. Each substrate, intermediate product and final product is clearly visible on the gel. The following DNA bands are visible for the 75dir reaction 625 b (abc and ABC), 529 b (AB), 454 b (A), 171 b (BC), 96 b (C) and 75 b (B), as indicated by blue arrows. In both reactions, at time zero a dark DNA band can be seen. As described before, this is the intact top DNA strand. This gel was slightly over exposed, as indicated by the very dark DNA bands at 625 b. Using ImageGuage the intensity of the gel can be reduced so that all bands can been seen clearly for analysis.

Figure 5.8B shows the denaturing polyacrylamide gel for 45dir and 75dir Ligase reactions with 1 mM MgCl₂ and 20 mM NaCl in the buffer. Lanes 1-6 show the Ligase
Figure 5.8 Timecourses on 45dir or 75dir. Reactions: 1 mM MgCl$_2$ and either 0, 20 or 40 mM NaCl.
Reactions at 5 nM DNA, 1x Ligase buffer and 0.2 nM Ligase, 37°C. Samples loaded onto 8% PA urea denaturing gel.

Panels A, B and C. Gels showing 6 timecourses, lane 1-6 75dir (A) and 45dir (B and C) Lanes 7-12 45dir (A) and 75dir (B and C); Timepoints in A(0.5, 2, 5, 15, 30, 60 min), B(0.5, 5, 15, 30, 60, 90 min) and C(0.5, 10, 30, 60, 90, 120 min). DNA fragments indicated by green/blue arrows. Panel D and E. Graphs showing results of quantitation of above gels. Colours as per key.
reaction for DNA substrate 45dir, lanes 7-12 show the Ligase reaction for 75dir; time intervals 0.5, 5, 15, 30, 60 and 90 min. DNA bands are clearly distinguishable and are as described above; blue arrows indicate DNA fragments for 75dir, and green arrows indicate DNA fragments for 45dir. Panel C shows the denaturing polyacrylamide gel for 45dir and 75dir Ligase reactions with 1 mM MgCl$_2$ and 40 mM NaCl in the buffer. Lanes 1-6 show the Ligase reaction for DNA substrate 45dir, lanes 7-12 show the Ligase reaction for 75dir; time intervals 0.5, 10, 30, 60, 90 and 120 min. DNA bands are clearly distinguishable and are as described previously; blue arrows indicate DNA fragments for 75dir, and green arrows indicate DNA fragments for 45dir. As the ligase reaction progresses the intensity of DNA bands A, B and C decreases, since the nick is being sealed producing intermediate products AB and BC. Equally the intensity of DNA band ABC (625 b) increases as time progresses. The intensity of each DNA band on each gel was quantified on ImageGuage. Boxes were drawn around every band and the quantatative result was transferred to Excel. The data were plotted as a timecourse. A linear regression were fitted to the first 10% of the reaction to calculate the initial rates. These initial rates have been collated and entered into the table in Figure 5.10 Panel A. Figure 5.8D shows the timecourses for 45dir with 1 mM MgCl$_2$ with either 0, 20 or 40 mM NaCl plotted into graphs using Prism. The Ligase reaction did not reach completion in any experiment. Figure 5.8 E shows the 75dir timecourses for 1 mM MgCl$_2$ and 0, 20, or 40 nM NaCl. Figure 5.9 shows the results for 45dir and 75dir with 4 nM MgCl$_2$ and 0, 20 or 40 mM NaCl. Figure 5.9A Lanes 1-6 is the Ligase reaction for 45dir and lanes 7-12 is the timecourse for 75dir; time intervals 0.5, 2, 5, 15, 30 and 45 min. As the DNA is labelled throughout, the following DNA bands are visible for the 45dir reaction: 625 b (abc and ABC), 499 b (AB), 454 b (A), 171 b (BC), 126 b (C) and 45 b (B), as indicated by the green arrows. Each substrate, intermediate product and final product is clearly
Figure 5.9. Timecourses on 45dir or 75dir. Reactions: 4 mM MgCl₂ and either 0, 20, or 40 nM NaCl. Reactions at 5 nM DNA, 1x Ligase buffer and 0.2 nM Ligase, 37°C. Samples loaded onto 8% PA urea denaturing gel. Panels A, B and C. Gels showing 6 timecourses, lane 1-6 45dir and lanes 7-12 75dir; Timepoints in A(0.5, 2, 5, 15, 30, 45 min), B(0.5, 10, 30, 60, 90, 120 min) and C(0.5, 10, 30, 60, 105, 120 min). DNA fragments indicated by green/blue arrows. Panel D and E. Graphs showing results of quantitation of above gels. Colours as per key.
visible on the gel. The following DNA bands are visible for the 75dir reaction 625 b (abc and ABC), 529 b (AB), 454 b (A), 171 b (BC), 96 b (C) and 75 b (B), as indicated by blue arrows. In both reactions, at time zero a dark DNA band can be seen, this is the intact top DNA strand. Figure 5.9B shows the denaturing polyacrylamide gel for 45dir and 75dir Ligase reactions with 4 mM MgCl$_2$ and 20 mM NaCl in the buffer. Lanes 1-6 show the Ligase reaction for DNA substrate 45dir, lanes 7-12 show the Ligase reaction for 75dir; time intervals 0.5, 10, 30, 60, 90 and 120 min. DNA bands are clearly distinguishable and are as described above; blue arrows indicate DNA fragments for 75dir, and green arrows indicate DNA fragments for 45dir. Panel C shows the denaturing polyacrylamide gel for 45dir and 75dir Ligase reactions with 4 mM MgCl$_2$ and 40 mM NaCl in the buffer. Lanes 1-6 show the Ligase reaction for DNA substrate 45dir, lanes 7-12 show the Ligase reaction for 75dir; time intervals 0.5, 10, 30, 60, 105 and 120 min. As the ligase reaction progresses the intensity of DNA bands A, B and C decreases, since the nick is being sealed producing intermediate products AB and BC and final product ABC. The intensity of each DNA band on each gel was quantified on ImageGuage. Boxes were drawn around every band and the quantitative result was transferred to Excel. The data was plotted as a timecourse. A linear regression was fitted to the first 10% of the reaction to calculate the initial rates. These initial rates have been collated and entered into the table in Figure 5.10 Panel A. Figure 5.9D shows the timecourses for 45dir with 4 mM MgCl$_2$ with either 0, 20 or 40 mM NaCl plotted into graphs using Prism. The Ligase reaction did not reach completion in any experiment. Figure 5.9E shows the timecourses for 75dir with 4 mM MgCl$_2$ with either 0, 20 or 40 mM NaCl plotted into graphs using Prism. All results show that the rate of reaction was slower with increasing salt concentration (ionic strength).

Figure 5.10 shows all the results for Ligase reaction at varying salt concentrations. Figures 5.10A and B are tables of the initial rates of each DNA fragment, that is the first
### Panel A

1 mM MgCl₂

<table>
<thead>
<tr>
<th>45dir</th>
<th>ABC</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>AB</th>
<th>BC</th>
<th>fp</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM NaCl</td>
<td>0.007</td>
<td>0.097</td>
<td>0.146</td>
<td>1.425</td>
<td>0.109</td>
<td>0.070</td>
<td>3.76</td>
</tr>
<tr>
<td>20 mM NaCl</td>
<td>0.040</td>
<td>0.0172</td>
<td>0.179</td>
<td>0.168</td>
<td>0.156</td>
<td>0.043</td>
<td>16.74</td>
</tr>
<tr>
<td>40 mM NaCl</td>
<td>0.010</td>
<td>0.102</td>
<td>0.091</td>
<td>0.045</td>
<td>0.091</td>
<td>0.028</td>
<td>7.75</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>4 mM MgCl₂</th>
<th>45dir</th>
<th>ABC</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>AB</th>
<th>BC</th>
<th>fp</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM NaCl</td>
<td>0.068</td>
<td>0.171</td>
<td>0.328</td>
<td>0.188</td>
<td>0.138</td>
<td>0.051</td>
<td>26.46</td>
<td></td>
</tr>
<tr>
<td>20 mM NaCl</td>
<td>0.038</td>
<td>0.075</td>
<td>0.052</td>
<td>0.031</td>
<td>0.029</td>
<td>0.011</td>
<td>32.20</td>
<td></td>
</tr>
<tr>
<td>40 mM NaCl</td>
<td>0.027</td>
<td>0.138</td>
<td>0.071</td>
<td>0.020</td>
<td>0.088</td>
<td>0.036</td>
<td>17.88</td>
<td></td>
</tr>
</tbody>
</table>

### Panel B

1 mM MgCl₂

<table>
<thead>
<tr>
<th>75dir</th>
<th>ABC</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>AB</th>
<th>BC</th>
<th>fp</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM NaCl</td>
<td>0.061</td>
<td>0.176</td>
<td>0.267</td>
<td>0.188</td>
<td>0.133</td>
<td>0.068</td>
<td>23.28</td>
</tr>
<tr>
<td>20 mM NaCl</td>
<td>0.063</td>
<td>0.169</td>
<td>0.107</td>
<td>0.067</td>
<td>0.089</td>
<td>0.024</td>
<td>35.80</td>
</tr>
<tr>
<td>40 mM NaCl</td>
<td>0.001</td>
<td>0.127</td>
<td>0.030</td>
<td>0.072</td>
<td>0.113</td>
<td>0.035</td>
<td>0.67</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>4 mM MgCl₂</th>
<th>75dir</th>
<th>ABC</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>AB</th>
<th>BC</th>
<th>fp</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM NaCl</td>
<td>0.015</td>
<td>0.036</td>
<td>0.146</td>
<td>0.087</td>
<td>0.073</td>
<td>0.046</td>
<td>11.19</td>
<td></td>
</tr>
<tr>
<td>20 mM NaCl</td>
<td>0.030</td>
<td>0.069</td>
<td>0.080</td>
<td>0.048</td>
<td>0.041</td>
<td>0.014</td>
<td>35.29</td>
<td></td>
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<tr>
<td>40 mM NaCl</td>
<td>0.016</td>
<td>0.076</td>
<td>0.064</td>
<td>0.013</td>
<td>0.084</td>
<td>0.034</td>
<td>11.9</td>
<td></td>
</tr>
</tbody>
</table>

### Panel C

Figure 5.10. All results of varying salt concentrations.

**Panel A:** Table of initial results for 45dir at various NaCl and MgCl₂ concentrations. The initial rate (first 10% of reaction) was calculated for each DNA fragment. Results were applied to the processivity equation 5.3 (page 134) and the processivity value was calculated and shown in column fp.

**Panel B:** Table of initial results for 75dir at various NaCl and MgCl₂ concentrations. Results were applied to the processivity equation, as above, and results shown in column fp.

**Panel C:** Graphical results of processivity values for 45 and 75dir substrates. 1 mM MgCl₂ plotted as red squares, and 4 mM MgCl₂ plotted as blue triangles. The graphs represent singular experiments and therefore no error bars are observed.
10% of reaction calculated in Excel. Processivity was calculated using the equation 5.3. The 45dir substrates results (Panel A) show Ligase was very weakly processive when MgCl₂ was 1 mM; 3.76% and 7.75% when NaCl concentration was 0 and 40 mM respectively. However substrate 75dir was weakly processive when MgCl₂ was 4 mM. For both DNA substrates ligase was most processive at 20 mM NaCl, about 30-35%, results that mirror those from Section 5.3. Figure 5.10C shows graphical results for processivity at vary salt concentrations. Both graphs show that processivity peaked at 20 mM NaCl. Ligase reaction was very inefficient at 40 mM NaCl.

5.5. Results on DNA substrates containing two inverted nicks.

Results in Section 5.3 have shown that the Ligase reaction is 30-35% processive on DNA substrates containing two nicks on the same DNA strand, that is the bottom strand. In this section PCR products were made containing two inverted nicks, one nick in each strand of a DNA molecule. Figures 5.11 to 5.13 show the results for the ligase reaction on DNA substrates 24, 30, 34, 39 and 69inv. All reactions were achieved at 37°C, 0.2 nM Ligase, 5 nM DNA, 25 μM NAD⁺, 1xLigase reaction buffer. At set time intervals 0, 0.5, 1, 1.5, 2, 5, 10, 15 and 90 min, Ligase reaction was terminated by adding aliquots to STOP buffer. DNA was denatured as previously described, and run on 5% denaturing polyacrylamide gels. Gels were dried, exposed, scanned and visualised. The intensity of each band was determined in ImageGauge, and data transferred to Excel and the DNA concentration in each band was calculated. Timecourses were plotted in Excel and a linear regression was fitted to the first 10% to determine the initial rate of reaction on each DNA fragment. Timecourses were also plotted into Prism, and these graphs are shown in Figures 5.11 to 5.13. Each graph is directly comparable to the corresponding gel, therefore no error bars are seen. However, each experiment was repeated and all results were collated in the final analysis.
Figure 5.11 shows the Ligase reaction for 24inv and 30inv. Panel A shows the 5% denaturing polyacrylamide gel. Each DNA fragment is clearly visible as named and indicated by the black arrows. The 471 b band represents the two intact intermediate products ab and CD. These two fragments cannot be separated as they are the same length. This means that the initial rates for each intermediate product cannot be calculated individually. It is because of this that inverted nick repeat DNA substrates cannot be directly used to determine processivity. However, the rate at which nicked fragments decreases can be used to estimate the rate at which the intermediate product increases. For example, DNA fragments 268 (a) and 203 (b) represent a nick in the top strand of DNA. When this nick is sealed 471 b product is made. In theory, these two rates should be the same.

Figures 5.11B and C show the timecourses for 24inv (B) and 30inv (C) plotted in Prism. Both reactions were completed within 50 min. Graphs were colour coordinated as in the legend. Timecourse data was plotted in Excel and the initial rates were calculated (described above). Only the initial rates for the 471 b DNA band will be described here, other results were correlated and are shown later in this section. The initial rate of reaction for 471 b in the 24inv DNA substrate was 0.167 nMmin$^{-1}$, and in 30inv the initial rate was 0.186 nMmin$^{-1}$.

Figure 5.12 shows the Ligase reaction for 34inv and 39inv. Reactions were achieved as described previously. Panels A and B show the 5% denaturing polyacrylamide gels for each reaction. Each DNA fragment is clearly visible as named and indicated by the black arrows. The DNA concentration of each band was calculated and the timecourse was plotted in Prism. Figures 5.12C and D show the timecourses for the corresponding gels. Both Ligase reactions were completed within 50 min. Each timecourse was also plotted in Excel to determine the initial rate of Ligase reaction. The rate at which 471 b
Figure 5.11. Timecourses on 24inv or 30inv DNA.
Reactions at 5 nM DNA with 1xLigase buffer and 0.2 nM Ligase, 37°C as in Materials and Methods. Samples loaded onto a 5% polyacrylamide urea denaturing gel.
Panel A. Gel showing two timecourses, lane 1 marker (as in Figure 4.3). Lanes 2-10 24inv (timepoints 0, 0.5, 1, 1.5, 2, 5, 10, 15, 90 min). Lane 11 is the marker. Lanes 12-20 30inv (timepoints as before). DNA fragments indicated by black arrows. Panel B: Graph showing results for quantitation of 24inv. Colours as per key. Panel C: Graph showing results of quantitation for 30inv. Colours as per key.
Figure 5.12. Timecourses on 34inv or 39inv DNA.

Reactions at 5 nM DNA with 1xLigase buffer and 0.2 nM Ligase, 37°C as in Materials and Methods. Samples loaded onto 5% PA urea denaturing gel.

Panel A. Gel showing one timecourse, lane 1 marker (from Figure 4.3C). Lanes 2-10 34inv (time points 0, 0.5, 1, 1.5, 2, 5, 10, 15, 90 min). DNA fragments are indicated by black arrows.

Panel B: Lane 1 marker. Lanes 2-10 39inv (timepoints as before).

Panels C and D: Graphs showing results of quantitation for 30inv and 39inv respectively. Colours as per legend.
increased in DNA substrate 34inv was 0.2618 nMmin$^{-1}$. For DNA substrate 39inv the initial rate was 0.3682 nMmin$^{-1}$.

Figure 5.13 shows the Ligase reaction for 69inv. The reactions were conducted as described previously. Panel A show the 5% denaturing polyacrylamide gel for the reaction. Each DNA fragment is clearly visible as named and indicated by the black arrows. The DNA concentration of each band was calculated and the timecourse was plotted in Prism. Figures 5.13B shows the timecourse for the corresponding gel. The Ligase reaction was completed within 50 min, a trend noticed in every reaction on inverted nick DNA substrates. The timecourse was also plotted in Excel to determine the initial rate of Ligase reaction. The rate at which 471 b increased in DNA substrate 69inv was 0.315 nMmin$^{-1}$.

Figure 5.14 shows all the results for inverted nick repeat DNA substrates 24, 30, 34, 39 and 69inv. Panel A is a summary of the initial rates of each DNA fragment. There is a clear trend; as intersite distance increases the initial rate increases. This is true for every DNA fragment, for example DNA fragments a and b for each DNA substrate, increased from 0.103 (a) and 0.160 (b) nM/min for 24inv to 0.22 and 0.31 nM/min$^{-1}$ for 39inv. Interestingly the same trend was found when nicks were on the same DNA strand although overall the rate of reaction was slower when the second nick site was inverted. The Ligase reaction in both direct and inverted experiments has shown a clear site preference. Nick AB in direct repeats is the same nick as CD in inverted repeats (as shown in Figure 5.2). In both series this nick was sealed at a faster rate than the other nick.

5.6 Processivity results for DNA substrates containing inverted nicks.

The inverted repeat DNA substrates in the previous section could not be analysed for processivity. This is because the intermediate products can not be analysed separately.
Figure 5.13. **Timecourses on 69inv DNA.**
Reactions at 5 nM DNA with 1xLigase buffer and 0.2 nM Ligase, 37°C. Samples were loaded onto a 5% polyacrylamide urea denaturing gel.

**Panel A.** Gel showing the timecourse for 69inv, lane 1 marker (as in Figure 4.3C). Lanes 2-10 69inv (time points 0, 0.5, 1, 1.5, 2, 5, 10, 15, 90 min). DNA fragments indicated by black arrows.

**Panel B:** Graph showing the results of quantitation for 69 inv. Colours as per legend.
### A

<table>
<thead>
<tr>
<th>PCR</th>
<th>ab/CD</th>
<th>a</th>
<th>b</th>
<th>C</th>
<th>D</th>
<th>Total Rate (a+b+C+D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24inv</td>
<td>0.160</td>
<td>0.103</td>
<td>0.161</td>
<td>0.198</td>
<td>0.217</td>
<td>0.679</td>
</tr>
<tr>
<td>30inv</td>
<td>0.194</td>
<td>0.114</td>
<td>0.176</td>
<td>0.237</td>
<td>0.271</td>
<td>0.798</td>
</tr>
<tr>
<td>34inv</td>
<td>0.361</td>
<td>0.216</td>
<td>0.340</td>
<td>0.474</td>
<td>0.443</td>
<td>1.473</td>
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<tr>
<td>39inv</td>
<td>0.349</td>
<td>0.220</td>
<td>0.311</td>
<td>0.459</td>
<td>0.231</td>
<td>1.221</td>
</tr>
<tr>
<td>69inv</td>
<td>0.247</td>
<td>0.094</td>
<td>0.226</td>
<td>0.415</td>
<td>0.317</td>
<td>1.052</td>
</tr>
</tbody>
</table>

Average (nMmin⁻¹): 1.045

### B

<table>
<thead>
<tr>
<th>PCR</th>
<th>(a+b)/2</th>
<th>(C+D)/2</th>
<th>Average overall rate (nMmin⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24inv</td>
<td>0.132</td>
<td>0.208</td>
<td>0.17</td>
</tr>
<tr>
<td>30inv</td>
<td>0.160</td>
<td>0.254</td>
<td>0.207</td>
</tr>
<tr>
<td>34inv</td>
<td>0.278</td>
<td>0.458</td>
<td>0.368</td>
</tr>
<tr>
<td>39inv</td>
<td>0.266</td>
<td>0.345</td>
<td>0.305</td>
</tr>
<tr>
<td>69inv</td>
<td>0.160</td>
<td>0.366</td>
<td>0.263</td>
</tr>
</tbody>
</table>

**Figure 5.14**  
Panel A: Table of initial rates for DNA substrates 24 to 69inv. Results for every DNA fragment are shown. Three experiment repeats were collated into a single result, therefore the table shows the average results for repeated experiments. The average for the total rate was calculated.  
Panel B: Table of initial rates for nicks ab and CD for DNA substrates 24 to 69inv. The average of initial rates for DNA fragments a and b represent the initial rate for nick ab, and C and D represent nick CD.
and therefore can not be placed in the processivity factor equation. To overcome this hurdle, experiments were carried out by labelling either the top or bottom DNA strand. Results in this section show the Ligase reaction on 24inv and 34inv DNA substrates.

DNA substrates were made by radiolabelling the 5’ end of either the top or bottom strand. In any single reaction, when visualised on denaturing polyacrylamide gels, only one of the DNA strands will be visible. Labelling the top strand would allow us to see DNA fragments a, b and ab. Labelling the bottom strand would show only the DNA fragments C, D and CD. By performing two Ligase reactions consecutively, and by combining the results, the initial rates of the intermediate products can be calculated and therefore can be applied to the $fp$ equation. The Ligase reaction were achieved as described previously, and DNA was run on 8% denaturing polyacrylamide gels.

Figure 5.15 shows the preliminary results for processivity reactions on 24inv and 34inv DNA substrates. Figure 5.15A shows the Ligase timecourse on 24inv DNA substrate, time intervals; 0, 0.5, 1, 1.5, 2, 5, 10, 15 and 30 min. On the left of this gel only the bottom strand is labelled so only DNA fragments D and CD are visualised. The right side of the gel was top strand radiolabelled, only DNA fragments a and ab are seen, indicated by blue arrows. The intensity of each band was determined and the DNA concentration was calculated. The timecourse was plotted into a graph in Prism (Figure 5.15C). The reaction was fast, completed within 8 min. To see the initial part of Ligase reaction results clearly time interval 30 min was eliminated from the graph. The timecourse was also plotted in Excel to calculate the initial reaction rate. These results are seen in Panel E.

Figure 5.15B shows the Ligase timecourse on 34inv DNA substrate, time intervals; 0, 0.5, 1, 5, 10, 15, 20 and 30 min. On the left of this gel only the bottom strand is labelled so only DNA fragments D and CD are visualised. The right side of the gel was top strand radiolabelled, only DNA fragments a and ab are seen, indicated by green
arrows. The intensity of each band was determined and the DNA concentration was calculated. The timecourse was plotted into a graph in Prism (Figure 5.15D). Ligase reaction was completed within 20 min. The timecourse was also plotted in Excel to calculate the initial react rate. These results are seen in Panel E.

The initial rates of each DNA fragment is seen in figure 5.15E. The 24inv substrate follows the trend previously seen, that is the nick CD was sealed faster than the nick ab. This again shows that ligase has a site preference.

5.7. Preliminary beta-clamp and gamma-loader studies.

In this chapter it has been determined that Ligase is a weakly processive enzyme, $fp$ value of 30-35% on direct substrates (dir). In 2001 the O’Donnell group reported that the polymerase sliding clamp (beta-clamp) associated with EcoLigA (López et al. 2001). ATP ligases have been reported to associate with PCNA (sliding clamp in eukayotes) during replication (Levin et al., 2000). In this section preliminary experiments were conducted to determine if Ligase forms a protein:protein interaction on nicked DNA with the beta-clamp to improve processivity of the enzyme.

Initial reactions were conducted with DNA Ligase present in the reaction, no Beta-clamp was added at this point. A new buffer was made, Beta buffer, which contained every component required for both Ligase and beta-clamp to work efficiently (see Table 2.14). To ensure that the Ligase reaction rate didn’t change within the new Beta buffer, a Ligase reaction timecourse was conducted either in Ligase buffer or Beta buffer. There was no significant difference in the Ligase reaction when using either buffer (data not shown). Adding beta-clamp alone to a Ligase reaction does not affect the reaction rate as the clamp requires gamma clamp-loader to load beta-clamp onto a DNA molecule. Figure 5.16 shows the results of the Ligase reaction in the presence of beta-clamp with varying concentrations of gamma clamp-loader.
Figure 5.15 Timecourses with either labelled top or bottom DNA strands.

Reactions at 5 nM DNA, 1xLigase buffer and 0.2 nM Ligase, 37°C. Samples loaded onto 8% polyacrylamide urea denaturing gels.

**Panel A.** Gel showing two timecourses, lane 1 marker. Lanes 2-10 ‘bot’ 24inv (time points 0, 0.5, 1, 5, 10, 15, 20, 30 min). Lane 11 marker. Lanes 12-20 ‘top’ 24inv time points as before DNA fragments indicated by green arrows.

**Panel B.** Gel showing two timecourses, lane 1 marker. Lanes 2-10 ‘bot’ 34inv time points as above. Lane 11 marker. Lanes 12-20 ‘top’ 34inv DNA fragments indicated by blue arrows.

**Panels C and D:** Graphs showing results of quantitation of both 24inv substrates and both 34inv substrates. Colours as per legend.

**Panel E:** Table showing the initial rates of each DNA fragment.

<table>
<thead>
<tr>
<th>PCR</th>
<th>ab</th>
<th>CD</th>
<th>a</th>
<th>D</th>
<th>Total rate (ab+CD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24inv</td>
<td>1.296</td>
<td>1.699</td>
<td>1.379</td>
<td>1.365</td>
<td>2.995</td>
</tr>
<tr>
<td>34inv</td>
<td>1.379</td>
<td>1.365</td>
<td>1.379</td>
<td>1.365</td>
<td>2.744</td>
</tr>
</tbody>
</table>

Average (nMmin⁻¹) 2.869
A

**75** dir

0.2 nM | 5 nM

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |

B

**75** dir

20 nM | 200 nM

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |

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**Fragment lengths (from top of both gels downwards)**

471 (ABC+abc), 319 (AB), 244 (A), 227 (BC), 152 (C), 75 (B)

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C

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D

<table>
<thead>
<tr>
<th>Gamma (nM)</th>
<th>ABC</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>AB</th>
<th>BC</th>
<th>fp</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>0.069</td>
<td>0.088</td>
<td>0.225</td>
<td>0.138</td>
<td>0.063</td>
<td>0.031</td>
<td>0.1</td>
</tr>
<tr>
<td>5</td>
<td>0.048</td>
<td>0.081</td>
<td>0.098</td>
<td>0.022</td>
<td>0.127</td>
<td>0.047</td>
<td>21.6</td>
</tr>
<tr>
<td>20</td>
<td>0.066</td>
<td>0.335</td>
<td>0.257</td>
<td>0.199</td>
<td>0.263</td>
<td>0.077</td>
<td>16.3</td>
</tr>
<tr>
<td>200</td>
<td>0.225</td>
<td>0.562</td>
<td>0.413</td>
<td>0.314</td>
<td>0.376</td>
<td>0.109</td>
<td>34.7</td>
</tr>
</tbody>
</table>

---

**Figure 5.16 Ligase reaction with beta-clamp and gamma-loader**

Reactions at 5 nM DNA, 1x beta buffer and 0.2 nM Ligase, 37°C. Samples loaded onto 8% PA urea denaturing gel. **Panels A and B.** Gel showing two timecourses, lane 1 marker. Lanes 2-10 0.2 nM loader (A) and 20 nM (B). Lane 11 marker. Lanes 12-20 *5 nM loader (A) and 200 nM (0, 1, 5, 10, 15, 30, 45, 60, and 90 min). **Panel C:** Graphs showing results of quantitation of 471 b (ABC) results at each [loader]. Colours as per legend. **Panel D:** Table showing the initial rates of each DNA fragment and processivity factor calculated using equation 5.3.
Reactions were 0.2 nM Ligase, 5 nM beta-clamp, 1x Beta buffer, 37°C and 5 nM 75dir. At set time intervals (0, 1, 5, 10, 15, 30, 45, 60 and 90 min) aliquots of reaction mix were added to STOP buffer to terminate the reaction. DNA was run on 5% denaturing polyacrylamide gels, 70 W, and then dried, exposed, scanned and visualised, as previously described. Figure 5.16A and B shows the resulting denaturing polyacrylamide gels. All DNA fragments are clearly visible on each gel, the migration and size indicated by black arrows. The intensity of each band was quantified and data was plotted in Prism. Panel C shows only the rise of the final Ligase reaction product ABC (471 b). When ligase and beta-clamp are saturated with gamma clamp-loader the reaction was faster. This is clearly seen as indicated by the red line/red squares plotted data for 200 nM loader. In fact the reaction rate is four times faster. The data was also plotted into Excel to calculate the initial rates. Figure 5.16D shows the rates of the first 10% of reaction for every DNA fragment.

5.8 Discussion.

The purpose of undertaking processivity measurements in this chapter started with the aim of determining the 1D or 3D pathway of Ligase on DNA using dir and inv substrates and finished with an unexpected finding that Ligase can interact with the beta sliding clamp.

The results for processivity measurements on the directly (dir) repeated substrates (Figures 5.4 to 5.6) are summarised in Figure 5.7 and show fp values of 22, 16, 32, 31, 40, and 31% over the six intersite distances measured. This gave an average of 28.8% processive reactions. This is not a significantly high proportion of processive Ligase molecules; restriction enzymes have fp values of almost double this (Standford et al. 2000, Gowers et al 2005). Still, nearly 30% is an fp value that is above a token minimal threshold (perhaps 10%) to observe a 1D or 3D mechanism. The average total initial
rate on the dir substrates (A+B+C, from Panel A Figure 5.7) was 3.148 nMmin$^{-1}$. The average intermediate total rate (AB+BC, same figure) was 1.144 nM/min. The processivity values were calculated by two different methods shown in Figure 5.7.

The difficulty with measuring inverted (inv) processivity measurements was that the intermediate products that are crucial for $fp$ calculations (see equations 5.2 and 5.3) are not separated on a denaturing gel from the finial intact products (Figures 5.11 to 5.13). However two strategies were used to overcome this.

The first, shown in Figure 5.11, was to compare the total initial rates of a+b and C+D formation against the rate of intermediate formation (AB and BC) on the dir substrates. If the inv rates are significantly faster than the intermediate dir rates then reactions on the inv substrates do not produce significant amounts of intermediates, and ligase would be processive over 3D routes. The overall reaction rate (a+b+C+D) for all inv substrates (24-69inv) was 1.045 nMmin$^{-1}$ as derived from the table in Figure 5.14B. This overall rate is about a third of the total rate of that for the dir substrates (3.148 nMmin$^{-1}$). Thus ligase operated more slowly under the same reaction conditions on the inv substrates. The reason for this is unknown since all reactions went to completion and it is difficult therefore to draw a clear conclusion of whether the total rate measured on the inv substrates was more like the total rate of the dir substrates or the total intermediates rate on the dir substrates.

A second preliminary attempt to compare dir with inv substrates was to label each strand (a+b or C+D) separately and again compare the rate at which the nicked form of that strand was sealed to the intact form of that strand. The results derived from the table in Panel E of Figure 5.15 indicate that the rate of intact strand formation (now measured separately and then summed for each strand at two inter-nicked distances of 24 and 34 bases) was now 2.869 nM/min (a+b+C+D). This is much closer to the total rate observed for the dir substrates (3.148 nMmin$^{-1}$). The initial rate for a+b formation
(1.296 nMmin\(^{-1}\)) was close to that of AB+BC formation for the dir substrates (1.144 nMmin\(^{-1}\)) leading to the conclusion that the enzyme was carrying out separate reactions on the inv substrates. The rate for C+D formation (1.699 nMmin\(^{-1}\)) was also close to that of AB+BC formation for the dir substrates (1.144 nMmin\(^{-1}\)), leading to the same conclusion, that the enzyme was carrying out separate reactions on the inv substrates.

This is an interesting and novel conclusion, although an indirect measurement. It appears that DNA ligase may prefer to operate by a 1D sliding mechanism rather than possessing an ability to move between strands on the same DNA molecule. The crystal structure of Ligase (Nandakumar et al 2007), like Human DNA ligase I, shows a monomeric protein encircling DNA (Figure 1.4). This may support the proposed mechanism of the protein acting on one DNA strand in preference to a structure which may more easily associate and disassociate with DNA.

The kind provision of two replication proteins (beta-clamp and gamma-loader) from Prof. Mike O’Donnell, allowed us to test whether Ligase processivity on the dir substrates was increased with these factors. As shown in Figure 5.16 the presence of the gamma-loader at four different concentrations gave a tantalising hint that there is a specific interaction between Ligase, beta-clamp and gamma-loader. If the beta-clamp acted as a processivity factor we would expect the \(fp\) values to significantly increase. The overall reaction rate appeared to be less than that observed on the absence of clamp and loader, with processivity values 0.1, 21.6, 16.3 and 34.7% with 0.2, 5, 20 and 200 nM clamp-loader concentrations. The reason for this in unknown but the complex is clearly operating much faster than in the absence of the additional two proteins. These require further experiments for kinetic characterisation.