ation of repair tracts and newly replicated DNA, respectively. Proteolytic cleavage may expose (or remove) structural determinants that govern interactions with nuclear factors, thereby directing DNA Methylase activity to the repair of replication machinery as required by the cell.


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Kinetic and structural investigations of the replicative fidelity of the Klenow fragment

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Escherichia coli DNA polymerase I (Pol I) is a 109 kDa protein required for repair and replication in vivo (Kornberg, 1980). In addition to a 3'→5' polymerase activity requiring a template to be copied and a primer strand to which nucleotides are added, the enzyme possesses a 3'→5' exonuclease activity capable of removing nucleotides from the primer strand, and a 5'→3' exonuclease activity which removes nucleotides in front of the growing primer strand (Jovin et al., 1969). Limited proteolysis yields a 68 kDa fragment (the Klenow fragment) which retains the polymerase and 3'→5' exonuclease activity (Bruttig et al., 1969; Klenow & Henninger, 1970). This fragment has been studied extensively by a variety of kinetic (McClyre & Jovin, 1975; Bambara et al., 1976; Bryant et al., 1983; Mizrahi et al., 1985, 1986; Kuchta et al., 1987, 1988), stereochemical (Burgers & Eckstein, 1979; Brody & Frey, 1981; Gupta & Benkovic, 1984), genetic (Joyce & Steitz, 1987; Freemont et al., 1986), and structural (Joyce & Steitz, 1987; Ollis et al., 1985) methods and is the subject of this study.

A complete kinetic scheme describing the polymerization of correct and incorrect nucleoside triphosphate (dNTPs) by the Klenow fragment has been developed using short DNA oligomers of defined sequence (Kuchta et al., 1987, 1988). The high fidelity, a selectivity of up to 105 for the correct nucleotide over non-complementary bases, arises from a three-stage mechanism. The first stage of discrimination (101-102) comes primarily from a dramatically reduced rate of phosphodiester bond formation for incorrect nucleotides, but also gains a smaller contribution from selective dNTP binding (1-25-fold). After phosphodiester bond formation, a conformational change slows dissociation of the incorrect DNA products from the Klenow fragment and in conjunction with editing by the 3'→5' exonuclease increases fidelity by further 4-60-fold. Finally, the addition of a correct base on to a mismatched 3' terminus is exceedingly slow providing the exonuclease with a second opportunity to excise the error.

Abbreviations used: dNTP, deoxy nucleoside triphosphate; dNMP, deoxy nucleoside monophosphate; AEDANS-S-dUMP, 5-[(5-[3H]N=2-aminethyl)-5-sulphophenylthalamethylacetamido)xoxyuridine-5-monophosphate; ANS-5-naphthylamine-1-sulphonic acid; NBD, 7-nitrobenz-2-oxa-1,3-diazole.

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X-ray crystallography has provided structural data of the Klenow fragment with substrates and inhibitors at 0.33 nm resolution (Joyce & Steitz, 1987; Ollis et al., 1985). The location of the 3'→5' exonuclease site was determined in crystals containing a nucleoside monophosphate which is a competitive inhibitor of the 3'→5' exonuclease activity. In the absence of metals, single-stranded DNA was observed to bind to this site. Using structural data from X-ray and inference as to the locus of the polymerase activity from enzymes containing single site mutations (Freamont et al., 1986), β-DNA was model built into the enzyme to approximate the location of the polymerase site. One particularly intriguing observation was that the polymerase and exonuclease sites were about 3.0 nm apart, prompting questions of how the two sites work together during DNA synthesis.

The distance separating the polymerase and exonuclease sites was probed by the use of fluorescence energy transfer. To perform a distance measurement by this method, a fluorescent dNMP donor and a fluorescent oligonucleotide acceptor were synthesized. A fluorescent dNMP analogue, 5-[(5-[3H]N=2-amin methyl)-5-sulphonylnaphthalenylacetamido)xoxyuridine-5-monophosphate (AEDANS-S-dUMP), was prepared via a modification of the methods of Ho et al. (1978) and Scheit & Faerber (1978). The fluorescence portion of this analogue, 5-naphthylamine-1-sulphonic acid (ANS), was chosen because of its relatively compact size in an effort to minimize steric problems, and for its compatibility as an energy transfer donor with another compact fluorophore, 7-nitrobenz-2-oxa-1,3-diazole (NBD). The fluorescence emission from AEDANS-S-dUMP excited at 340 nm, is very weak in aqueous solution. However, upon binding to the Klenow fragment, this emission is increased 13-fold and the peak maximum is blue-shifted 28 nm, indicating that the environment surrounding the probe is less polar than the aqueous solvent. A fluorescent oligonucleotide duplex capable of acting as an energy transfer acceptor from AEDANS-S-dUMP was prepared by a modification of the method of Gibson & Benkovic (1987). The duplex system (Scheme 1) consisted of a 20-mer template strand and a primer 11-mer that had been derivatized with NBD. The emission of the NBD-labelled 11/20 duplex was weak in aqueous solution, but was increased 6-fold when bound to the enzyme. Accompanying the increase was a blue-shift in the peak maximum of 19 nm, similar to that observed upon AEDANS-S-dUMP binding.

The distance separating two fluorophores, R, is measured as a function of R, the distance at which there is 50% energy transfer:

\[ R_0 = \left[ \frac{8.79 \times 10^{-5} \text{K} \cdot n \cdot \Phi_{dNMP}}{\Phi_{NBD}} \right]^{1/6} \]
The quantum yield of the donor, AEDANS-S-dUMP, bound to the polymerase, \( \Phi_p \), was determined by the method of Parker & Rhees (1960) and found to be 0.15. Given the measured spectral overlap integral, \( I_{\text{ov}} \), of 3.23 \( \times 10^{-14} \) cm\(^2\)/mol nm\(^2\) and the assumed values for the refractive index of the intervening medium, \( n \), of 1.35 and orientation factor, \( K_2 \), of 2/3, an \( R_0 \) value of 0.31 nm is obtained for this pair of fluorophores.

The actual distance separating the two fluorophores on the ternary complex of bound primer-template and dUMP was determined by measuring the quenching of the donor's emission in the presence of the acceptor. Using the measured efficiency of energy transfer, \( E \), of 0.79 and the calculated \( R_0 \) value of 0.31 nm, the actual distance was found to be 0.25 nm:

\[ R = \left( \frac{1}{E} - 1 \right)^{1/6} R_0 \]

The apparent separation of the polymerase and exonuclease sites by 2.5 nm within the ternary complex of enzyme-DNA-dNMP supports the hypothesis that for a dNMP to be excised from the primer by the exonuclease activity of the enzyme, the DNA duplex must translocate to that site as opposed to the two sites coming closer together.

Joyce & Steitz have suggested that the translocation of the DNA has two components—a sliding of the DNA over a distance of approximately 8 bp and a melting of the 3-4 bp of the primer terminus strand along the lines of the fraying requirement for exonuclease activity originally proposed by Kornberg (Joyce & Steitz, 1987; Joyce et al., 1988). DNA duplexes in which the two strands are covalently cross-linked have been prepared (Webb & Matteucci, 1986a, 1986b). Such structures would seem to provide an ideal test of the structural requirements for the polymerase and exonuclease activity of the Klenow fragment. A modification of the method of Webb & Matteucci (1986b) was employed to convert 5'-dimethoxymethylthymidine-3'-O-acyclohexyl-N-diisopropylphosphoramidite to a 4-triazole derivative which was then displaced to form the 4-azidine. The latter was incorporated into the desired oligonucleotide that was then annealed with a complimentary strand and allowed to cross-link. The structure of the cross-linked duplex is shown in Fig. 1.

The ability of the Klenow fragment to remove dTMP and dAMP from the cross-linked duplex was examined in the presence of dATP and dTTP, respectively, to limit the hydrolytic degradation to a single 3'-end. The duplex was 5'-end labelled so that the rate of exonuclease action could be monitored by gel electrophoresis of aliquots removed at various times (Kuchta et al., 1988). A control reaction was conducted to demonstrate that the Klenow fragment can completely degrade the uncross-linked duplex formed by mixing an equimolar solution of the template/primer constituting I. Identical experiments were conducted with the T4 polymerase.

The cross-linked duplex I was found to be a competent substrate for the exonuclease and polymerase activities of the Klenow fragment. The Klenow fragment removes one base (dTMP) from the primer strand of the duplex, but is incapable of removing bases four or less from the cross-linked base pair. Gel-electrophoretic analysis of the products formed by the action of the Klenow fragment on the duplex revealed that the reaction was pseudo-first order and that the rate of the exonuclease activity was similar to that observed with other uncross-linked duplexes \( (10^{-3}-7 \times 10^{-4} \text{s}^{-1}) \) (Kuchta et al., 1988). The enzyme was able to remove one additional base from the primer strand, but this action is exceedingly slow, less than \( 5 \times 10^{-5} \text{s}^{-1} \). The Klenow fragment also was able to remove six nucleotides of dAMP from the blunt end of the cross-linked duplex. Finally, in the control reaction with uncross-linked duplex the exonucleolytic activity completely degraded the primer strand within the anticipated time span.

T4 DNA polymerase (Kornberg, 1980) also utilized the cross-linked duplex I as a substrate for exonuclease activity. This enzyme has an exceedingly active 3'→5'-exonuclease activity, about 400 times faster than the Klenow fragment (T. Capson, personal communication), and accordingly displays quantitatively as well as qualitatively different activity with the cross-linked duplex. A lower concentration of T4 polymerase removes bases from the primer strand faster than the Klenow fragment, and the enzyme is capable of removing bases up to one base from the cross-link. When the cross-
linked duplex was subjected to the exonuclease activity of T4 DNA polymerase for short times, a 'ladder' of products was formed, representing duplexes shorter than 7 by one or more bases. After destruction of the T4 enzyme and addition of Klenow, a mechanistic combination of sliding/fraying to achieve editing by the Klenow fragment (Fig. 2).

The collective results provide support for the hypothesis that the polymerase and exonuclease sites of the Klenow fragment are separate and have different structural requirements for DNA to act as a substrate. From the fluorescent energy transfer experiments, the distance between sites is about 3 nm, in good agreement with that estimated from the X-ray model. In terms of nucleotides, the distance between the exonuclease and polymerase sites is approximately nine bases. Of these, approximately four bases must be melted out from the duplex to access the exonuclease site. Thus there is a mechanistic combination of sliding/fraying to achieve editing by the Klenow fragment (Fig. 3a and b).

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The three-dimensional structure of interleukin-1β

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The three-dimensional structure of human recombinant interleukin-1β has been determined at 0.24 nm resolution by X-ray crystallographic techniques. The partially refined model has a crystallographic R-factor of just under 19%. The structure is composed of 12 β-strands forming a complex network of hydrogen bonds. The core of the structure can best be described as a tetrahedron whose edges are each formed by two antiparallel β-strands. The interior of this structure is filled with hydrophobic side-chains. There is a