Resonance Energy Transfer Measurements between Substrate Binding Sites within the Large (Klenow) Fragment of *Escherichia coli* DNA Polymerase I†

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Received March 1, 1989; Revised Manuscript Received June 14, 1989

ABSTRACT: Resonance energy transfer was used to determine separation distances between fluorescent derivatives of substrates for Klenow fragment and a unique sulfhydryl, cysteine 907, on the enzyme. Fluorescent derivatives of duplex DNA, deoxynucleotide triphosphates (dNTP), and deoxynucleotide monophosphates (dNMP), modified with aminonaphthalenesulfonates (ANS), served as energy-transfer donors to the fluorophore used to modify cysteine 907, 4-[*N*-[(iodoacetoxy)ethyl]-*N*-methylamino]-7-nitrobenz-2-oxa-1,3-diazole (IANBD). The labeling of cysteine 907 with NBD caused no decrease in the enzyme's polymerase activity, suggesting that the probe did not significantly alter the conformation of the enzyme. The efficiency of singlet–singlet resonance energy transfer was determined from the quantum yield of the donor in the presence and absence of acceptor. By Förster's theory, the measured distances between cysteine 907 and binding sites for duplex DNA, dNTP, and dNMP were 25–39, 19–28, and 17–26 Å, respectively. As the fluorophores, attached to the substrates via a tether arm, are separated from the substrates by approximately 12 Å, the distances measured between binding sites are subject to this uncertainty. To measure the separation between binding sites for duplex DNA and dNMP, and to reduce the uncertainty introduced by the tether arm, two experiments were carried out. In the first, duplex DNA was labeled with the acceptor fluorophore NBD and used with the donor ANS-modified dNMP to yield a measured distance separating these two sites of 19–28 Å. In the second, polymerization of the NBD-labeled duplex by six base pairs increased the separation distance between these two sites to 26–41 Å, supporting the placement of the DNA duplex in the cleft of the large domain of the enzyme and the separation of polymerase and exonuclease sites. Collectively these distances permitted the construction of the model for the ternary complex whose overall dimensions are in satisfactory agreement with those obtained from the X-ray crystal structure from the binary KF·dTMP complex (Ollis et al., 1985).

Escherichia coli DNA polymerase I (pol I) is a multifunctional enzyme responsible for DNA repair and replication in vivo (Kornberg, 1980). The enzyme's polymerase activity, which catalyzes the template-directed extension of a primer DNA strand, and the separate 3′–5′ and 5′–3′ exonuclease activities are all found on a single 103-kDa polypeptide chain (Jovin et al., 1969). Treatment of pol I by subtilisin produces two peptides; the larger, 68 kDa, is named Klenow fragment and contains the polymerase and 3′–5′ exonuclease activities (Klenow et al., 1970; Brutlag et al., 1969).

Klenow fragment has been crystallized with bound dTMP, and X-ray crystallography has provided structural data at 3.3-Å resolution (Ollis et al., 1985). The crystal structure, determined in the absence of duplex DNA, shows that the protein consists of two distinct domains, the larger containing a deep cleft into which duplex DNA can be fit through model building. The polymerase activity is believed to reside at one end of the cleft, near the interdomain linkage, while the 3′–5′ exonuclease site is found on the smaller domain, where the dTMP is observed to bind. Further evidence for the location of the 3′–5′ exonuclease site has come from site-directed mutants (Derbyshire et al., 1987). A double mutant, D355A E357A, involving a metal binding site, yielded protein possessing normal levels of polymerase activity but whose exonuclease activity is reduced by about 5 orders of magnitude as compared to the wild-type enzyme. Cocrystals of duplex DNA KF have been difficult to obtain (Joyce & Steitz, 1987), prohibiting the direct observation of the separation between the polymerase and 3′–5′ exonuclease sites, although structures with model-built duplex DNA in the deep cleft of the large domain estimate this separation to be about 30 Å.

In order to determine distances between bound substrates in the ternary complex in solution, we have employed fluorescently modified substrates of Klenow fragment as well as labeled enzyme itself. We report here the use of donor–acceptor groups in resonance energy transfer studies to determine the separation between substrate binding sites and a known point on the enzyme.

Materials and Methods

Materials. Klenow fragment was purified from *E. coli* CJ333, which was kindly provided by Dr. C. M. Joyce. Polyacrylamide gel electrophoresis of the purified enzyme indicated at least 99% homogeneity. Protein concentrations were determined by using the extinction coefficient of $\epsilon = 6.32 \times 10^4$ cm^{-1} M^{-1} at 278 nm (Setlow et al., 1972). Polymerase activity was measured with the DE-81 filter paper assay (Bryant et al., 1983). Mansyl chloride, IAEDANS,[1] and IANBD were from Molecular Probes. 5′-Dimethoxytrityl 3′-(methoxyphosphoramidites) were from American Bionetics. Nucleotide triphosphates were from Sigma. [γ-^{32}P]ATP (>3000 Ci/mmol) was from New England Nuclear. Acetonitrile and triethylamine were from Aldrich. All other reagents were of the highest quality commercially available.

†This research was supported by a grant from the National Institutes of Health (2RO1GM13306-23).

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Sulfhydryl Modification of Klenow Fragment. Klenow fragment (580 μL, 78 μM) was added to 100 mM KP_i, pH 7.0, and 10 mM DTT (280 μL) and allowed to stand at 4 °C for 2 h, after which time it was dialyzed anaerobically (under argon) against 100 mM KP_i, pH 7.0 (3.0 L) at 4 °C for 18 h. The solution was transferred to a screw-capped tube with a rubber seal, and 3.0 mM IANBD in DMSO (364 μL) was added. This solution was kept at room temperature for 2 h and then cooled to 4 °C for 24 h. Following reaction with IANBD, the solution was dialyzed against 50 mM Tris-HCl, pH 7.4 (4 × 2.0 L), to remove any noncovalent label. The extent of label incorporated was calculated by UV absorbance at 278 nm, using $\epsilon = 6.32 \times 10^4$ cm^{-1} M^{-1} for Klenow fragment, and at 495 nm, using $\epsilon = 3.74 \times 10^4$ cm^{-1} M^{-1} for NBD (Haugland, 1975).

Synthesis of Oligonucleotides. Oligomers were synthesized on an Applied Biosystems DNA synthesizer, Model 380A, by using methoxyphosphoramidite chemistry. At a specified position in the primer 11mer, a modifiable phthalimide-protected 5-(aminopropyl)-2′-deoxyuridine 3′-(diisopropylmethoxyphosphoramidite) (Gibson et al., 1987) was used in place of the normal phosphoramidites. This oligomer was derivatized before purification. 5′-OH-tritylated oligomers were purified on a reverse-phase C-18 HPLC column (Altex). A gradient was run from 10% acetonitrile–90% 100 mM TEAA, pH 7.0 (solvent A), to 100% acetonitrile (solvent B) at 1% solvent B per minute following a 4-min isocratic step of solvent A. The collected oligomer was lyophilized and dissolved in 6% HOAc and kept at room temperature for 30 min to remove the trityl group. The deblocked oligomer was repurified by HPLC using the gradient conditions of the tritylated oligomer.

Mansyl Derivatization of Primer 11mer. To 400 μL of 100 μM primer 11mer (assumed $\epsilon_{260} = 10\,000$ cm^{-1} M^{-1} per base) in 200 mM TEAB, pH 11, was added 18 μL of 55 mM mansyl chloride in acetone to a final concentration of 2.5 mM. The reaction was performed in the dark. After 6 h, another 18 μL of the above acid chloride solution was added and the reaction alllowed to proceed for another 12 h. A precipitate formed that was removed by centrifugation, and the resulting supernatant was purified by reverse-phase C-18 HPLC as described above. The labeled oligomer was identified by the association of a UV absorbance at 340 nm (mansyl group) with the 260-nm absorbance of the oligonucleotide.

NBD Derivatization of Primer 11mer. To 400 μL of 50 μM primer 11mer (assumed $\epsilon_{260} = 10\,000$ cm^{-1} M^{-1} per base) in 400 mM TEAB and 30% acetonitrile was added 50 μL of 100 mM NBD-F in acetonitrile to a final concentration of 14 mM. After 12 h at room temperature in the dark, another 50-μL aliquot of NBD-F was added and the reaction allowed to proceed for another 6 h. The reaction mixture was diluted to 4 mL by the addition of triethylamine in H_2O, pH 11. The NBD-labeled 11mer was purified by reverse-phase C-18 HPLC under the same conditions used for the mansyl-labeled 11mer. The labeled oligomer was identified by the association of a UV absorbance at 486 nm (NBD group) with the 260-nm absorbance of the oligonucleotide.

Formation and Elongation of Oligonucleotide Duplexes. Oligonucleotide duplexes were prepared by the addition of equimolar amounts of complementary primer 11mer (unlabeled, mansyl labeled, or NBD labeled) and template 20mer in 50 mM Tris-HCl, pH 7.4. Due to the base composition of the template, the primer can be lengthened in discrete steps by the sequential addition of 30 μM dNTPs.

Synthesis of AEDANS-S-dUMP. The method of Ho et al. (1978) was used to synthesize bis(2′-deoxyuridine 5′-phosphate) 5,5-disulfide [bis(5-thio-dUMP)], which was obtained in 79% yield after purification on DEAE-Sephadex A-25. The oxidized bis(monophosphate) eluted at 0.18 M TEAB from a 0.56 cm × 22 cm column washed with a 5 to 300 mM TEAB gradient. The UV spectrum has an absorption maximum at 272 nm (pH 8.0), which upon addition of DTT changes to 333 nm.

The monophosphate disulfide (15 μmol) was dried from an ethanol solution as the triethylammonium salt and dissolved into 750 μL of degassed 0.5 M KP_i, pH 7.5. DTT (15 μmol) in 15 μL of KP_i was added and the solution kept under argon. After 30 min at room temperature, 100 μL of IAEDANS in the same buffer (20 μmol) was added and the solution kept at room temperature in the dark for 2 h. DTT (5 μmol, 5 μL) was added and the solution allowed to stand at room temperature in the dark. After 3 h, the reaction mixture was diluted into 25 mL of 10 mM TEAB and applied to a 2.4 cm × 60 cm DE-52 column. After being washed with 500 mL of 10 mM TEAB, a gradient of 10 to 500 mM TEAB eluted the monophosphate adduct AEDANS-S-dUMP at 150 mM TEAB. The yield was 13.8 μmol (95%).

Synthesis of AEDANS-S-dUTP. Pyrophosphorylation of the bis(5-thio-dUMP) (297 μmol) was achieved by the morpholidate method (Moffatt, 1964). The triphosphate was purified on a DEAE-cellulose column (2.4 cm × 33 cm) with a 200 to 500 mM TEAB gradient (1.8 L per side) and eluted as the oxidized disulfide at 360 mM TEAB. The yield was 64 μmol (21%).

The triphosphate disulfide (15 μmol) was dried from an ethanol solution as the triethylammonium salt under a stream of argon and then taken up in 750 μL of degassed 50 mM KP_i, pH 7.5, and DTT (15 μmol). The solution was kept under argon. After 20 min at room temperature, 94 μL of IAEDANS in the same buffer (18.7 μmol) was added. After 2 h, the solution was diluted to 10 mL and applied to a 1.4 cm × 20 cm DEAE-cellulose column. After being washed with 50 mL of 50 mM TEAB, a gradient of 50 to 500 mM TEAB eluted the triphosphate adduct AEDANS-S-dUTP at 200 mM TEAB. The yield was 9 μmol (60%). Phosphorus assay (Eaton et al., 1976) gave 3.0 phosphates per chromophore at 337 nm.

Determination of Quantum Yield. Quantum yields of donor fluorophores bound to Klenow fragment were determined at 25 °C by reference to a standard of quinine sulfate (Parker & Rhees, 1960). The quantum yields of 11*/20 elongated stepwise by the addition of dNTPs were also evaluated by this method. The Q value of quinine sulfate in 0.05 N H_2SO_4 was assumed to be 0.55 (Melhuish, 1961).

Solutions of donor fluorophores were adjusted to give absorbancy values (<0.01) approximately equivalent to that of quinine sulfate, when measured at 365 nm. Quantum yields were calculated by the relation

$$Q_1 = 0.55(A_2S_1/A_1S_2) \quad (1)$$

[1] Abbreviations: AEDANS-S-dUMP, 2′-[[[[[2-[(5-sulfonaphthalen-1-yl)amino]ethyl]amino]carbonyl]methyl]thio]deoxyuridine 5′-monophosphate; AEDANS-S-dUTP, 2′-[[[[[2-[(5-sulfonaphthalen-1-yl)-amino]ethyl]amino]carbonyl]methyl]thio]deoxyuridine 5′-triphosphate; ANS, aminonaphthalenesulfonate; dNMP, deoxynucleotide monophosphate; dNTP, deoxynucleotide triphosphate; DTT, dithiothreitol; IAEDANS, 5-[[[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid; IANBD, 4-[*N*-[(iodoacetoxy)ethyl]-*N*-methylamino]-7-nitrobenz-2-oxa-1,3-diazole; HPLC, high-pressure liquid chromatography; mansyl, 6-(*N*-methylanilino)naphthalene-2-sulfonate; NBD, 4-amino-7-nitrobenz-2-oxa-1,3-diazole; TEAA, triethylammonium acetate; TEAB, triethylammonium bicarbonate; Tris, tris(hydroxymethyl)aminomethane; DNA, deoxyribonucleic acid; DMSO, dimethyl sulfoxide; NBD-F, 4-fluoro-7-nitrobenz-2-oxa-1,3-diazole.

where Q_1 is the quantum yield of the donor fluorophore, A is the UV absorbance at 365 nm (A_1 = donor, A_2 = quinine sulfate), and S is the integrated fluorescence emission (S_1 = donor, S_2 = quinine sulfate). All fluorescence emission spectra were recorded on an SLM 8000C spectrofluorometer.

K_D Determination of AEDANS-S-dUTP and AEDANS-S-dUMP. To a 1.0-mL 50 mM Tris-HCl, pH 7.4, solution containing either 500 nM AEDANS-S-dUTP and 3 mM $MgCl_2$ or 135 nM AEDANS-S-dUMP and 2 mM $Zn(OAc)_2$ were added aliquots of concentrated Klenow fragment. The fluorescence emission, excited at 340 nm, was measured following each addition. K_D was calculated by a nonlinear least-squares fitting program, NLIN (SAS User's Guide, 1985), of the fluorescence emission increase as a function of added enzyme.

Anisotropy Measurements. Anisotropy measurements were made at 25 °C on an SLM 8000C spectrofluorometer using the T-format method and an excitation wavelength of 340 nm. Filters used were 408-nm cutoff and were purchased from SLM. Measurements were made on a 1.0-mL solution containing either 110 nM 11*/20 (mansyl labeled), 500 nM NBDKF, 500 nM AEDANS-S-dUTP, or 500 nM AEDANS-S-dUMP in 50 mM Tris-HCl, pH 7.4, 25 °C. Following initial measurements, Klenow fragment was added to all samples, excluding NBDKF, to obtain bound anisotropy values. In the case of the 11*/20, dNTPs were added sequentially so that differing primer lengths could be observed.

Resonance Energy Transfer Measurements. For all energy-transfer experiments, four samples were required: 1, DY; 2, DA; 3, XA; 4, XY. A is the acceptor, X is a nonabsorbing, nonfluorescent donor analogue, and Y is an equivalent acceptor analogue. In constructing the DA sample, the number of assemblies having donors but lacking acceptors should be minimized as these assemblies have donors unable to participate in the transfer process. For distance measurements to cysteine 907 from substrate binding sites, assemblies containing acceptor fluorophores were maximized by the use of NBD-labeled Klenow fragment containing >0.95 equiv of NBD. For measurement to the NBD-labeled oligonucleotide duplex, the NBD-labeled duplex was used at a concentration 100 times its K_D to ensure saturation with the acceptor fluorophore. All measurements were taken at 25 °C with an SLM 8000C spectrofluorometer with excitation and emission slits of 8 nm. All samples had a final volume of 1.0 mL.

Sample preparation for the following distances:

(A) Sulfhydryl to DNA Duplex Primer Terminus Site (11/20).* All samples (see 1–4 above) contained 50 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 300 nM oligonucleotide duplex (labeled or unlabeled), and 400 nM enzyme (labeled or unlabeled). Samples were excited at 330 nm. Oligonucleotide duplexes were stepwise elongated by the addition of 3.3 μL of a 10 mM dNTP solution.

(B) Sulfhydryl to Triphosphate Binding Site. All samples contained 50 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 500 nM triphosphate (AEDANS-S-dUTP or dTTP), and 2 μM enzyme (labeled or unlabeled). From the calculated K_D of 2.2 μM, the emission due to unbound AEDANS-S-dUTP was subtracted from all samples containing this fluorescent analogue. Samples were excited at 340 nm.

(C) Sulfhydryl to Monophosphate Binding Site. All samples contained 50 mM Tris-HCl, pH 7.4, 2 mM $Zn(OAc)_2$, 500 nM monophosphate (AEDANS-S-dUMP or dTMP), and 3 μM enzyme (labeled or unlabeled). As the emission from enzyme-bound AEDANS-S-dUMP is increased 17-fold over that of the unbound species, the fluorescence emission from the fraction of unbound AEDANS-S-dUMP at 3 μM enzyme was ignored. Samples were excited at 335 nm.

(D) Monophosphate Binding Site to DNA Primer Terminus. All samples contained 50 mM Tris-HCl, pH 7.4, 2 mM $Zn(OAc)_2$, 500 nM oligonucleotide duplex 11*/20 (NBD labeled or unlabeled), 500 nM monophosphate (AEDANS-S-dUMP or dTMP), and 2 μM enzyme (labeled or unlabeled). Samples were excited at 340 nm.

(E) Monophosphate Binding Site to DNA (17/20).* All samples contained 50 mM Tris-HCl, pH 7.4, 500 nM oligonucleotide duplex 11*/20 (NBD labeled or unlabeled), and 2 μM enzyme. dCTP, dATP, and dGTP were added to 30 μM to extend the 11*/20 (NBD labeled or unlabeled) to 17*/20. Following the elongation, $Zn(OAc)_2$ was added to 2 mM and monophosphate (AEDANS-S-dUMP or dTMP) was added to 500 nM.

Spectra. Corrected emission spectra of all samples were taken with an excitation wavelength characteristic of the donor AEDANS and an emission range including the fluorescence of both AEDANS and the acceptor NBD: excitation = 330–340 nm; emission = 350–650 nm. The emission spectrum of sample XY, containing both a nonfluorescent donor and acceptor analogue, was subtracted from the emission spectrum of samples DA (energy-transfer sample) and DY (donor with acceptor analogue) to correct for any background fluorescence. The emission spectra from sample XA (donor analogue with acceptor) was further subtracted from the DA emission to correct for the emission that arises from the acceptor due to direct excitation by the wavelength of light chosen to excite the donor. The acceptor, NBD, has a small absorption at 340 nm that will cause direct excitation of the fluorophore, as opposed to that which arises via energy transfer. The two resulting emission spectra, DA − XY − XA (DA′) and DY − XY (DY′), were integrated to determine the efficiency of energy transfer:

$$E = 1 - (Q_{DA'}/Q_{DY'}) \quad (2)$$

The spectra's emissions were integrated from 350 to 490 nm and these values used to calculate the ratio ($Q_{DA'}/Q_{DY'}$) and the value of E. The integrations measure the amount of donor quenching due to energy transfer and are performed from the beginning of the donor's emission to that point at which the acceptor's emission begins (>490 nm), as the inclusion of acceptor emission causes error in the calculation of E.

The efficiency of energy transfer between a donor and an acceptor chromophore is related to their apparent distance, R, by the equation (Wu & Stryer, 1972):

$$R = (1/E - 1)^{1/6} R_0 \quad (3)$$

R_0, called the "Förster's critical distance", corresponds to the distance allowing a transfer efficiency of 50%. R_0 is related to the spectral properties of both donor and acceptor by the relation (Stryer & Haugland, 1967)

$$R_0 = (9.79 \times 10^3)(K^2 n^{-4} Q_D J_{DA})^{1/6} \quad (\text{Å}) \quad (4)$$

where Q_D represents the donor quantum yield in the absence of acceptor and n is the refractive index of the medium. The overlap integral J_{DA} represents the degree of resonance between donor and acceptor dipoles, and its value is obtained by integration of the overlapping area of the donor emission spectrum F_D and the acceptor absorption spectrum ϵ_A according to the equation (Fairclough & Cantor, 1978):

$$J_{DA} = [F_D(\lambda)\epsilon_A(\lambda)\lambda^4\, d\lambda]/[F_D(\lambda)\, d\lambda] \quad (M^{-1}\, cm^3) \quad (5)$$

K^2, the orientation factor for a dipole–dipole interaction, can have a value between 0 and 4. If both the donor and acceptor rotate freely in a time that is short compared to the excited-

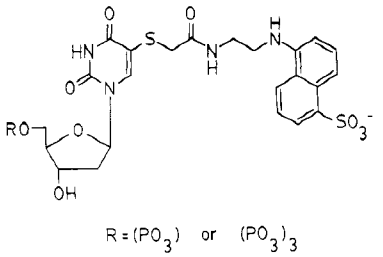

FIGURE 1: Structure of AEDANS-S-dUMP and AEDANS-S-dUTP.

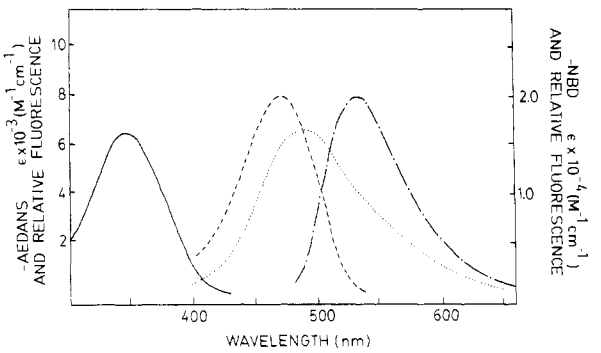

FIGURE 2: Absorption and emission spectra of donor -S-AEDANS (absorption, —; emission, ···) and of an acceptor NBD derivative of Klenow fragment (absorption, - - -; emission, -·-) carrying an NBD group at cysteine 907. The emission spectrum of -S-AEDANS was recorded with an excitation at 340 nm and that of labeled Klenow at 470 nm.

state lifetime of the donor, then K^2 is equal to $^2/_3$. From the observed transfer efficiency, an apparent distance, R, can be calculated on the assumption that K^2 is equal to $^2/_3$. The actual distance, R', can be related to the apparent distance by

$$R' = \alpha R_{(2/3)} = (1.5K^2)^{1/6} R_{(2/3)} \quad (6)$$

where α is the sixth root of the ratio of the actual value of K^2 to the assumed value of $^2/_3$. Since K^2 can be between 0 and 4, the potential range of α is 0–1.35. A range of values for α can be calculated for a donor rotating very rapidly within a cone of semiangle θ (Stryer, 1978) measured by picosecond anisotropy decays.

Measurements of Donor Cone Semiangles (θ). Picosecond anisotropy decay measurements on donor fluorophores were obtained with an Ar^+-pumped dye laser (Coherent, Inc., Palo Alto, CA) whose emission was 315 nm. Channel widths used were 7.04, 35.96, and 87.92 ps for 1024 channels. Data sets consisted of 1024 decay points. A_0, the emission anisotropy of the donor in the absence of any rotational motion, and A_1, the initial value observed at a time short compared to the excited lifetime, were used to calculate the donor cone semiangle θ given (Kawato et al., 1977)

$$A_1/A_0 = (\cos^2\theta)(1 + \cos\theta)^2/4 \quad (7)$$

Results

Sulfhydryl to Deoxynucleotide Triphosphate Binding Site. In order to observe the interaction of deoxynucleotide triphosphates with Klenow fragment, a fluorescent derivative, AEDANS-S-dUTP, was synthesized (Figure 1). Preliminary to resonance energy transfer experiments, it was necessary to examine the spectroscopic properties of the interacting components of our system. In fact, transfer of excitation energy requires an overlapping of the emission spectrum of the donor and the absorption spectrum of the acceptor. In Figure 2, the emission spectrum of AEDANS-S-dUTP and the absorption

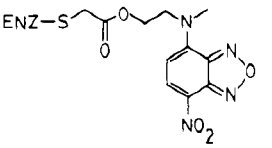

FIGURE 3: NBD-labeled Klenow fragment. The residue labeled is cysteine 907.

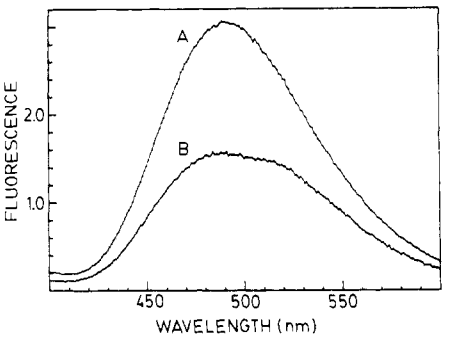

FIGURE 4: Energy transfer between AEDANS-S-dUTP and NBD-labeled Klenow fragment. Spectrum A is the emission spectrum of AEDANS-S-dUTP bound to unlabeled Klenow fragment. Spectrum B is the emission spectrum of AEDANS-S-dUTP bound to NBD-labeled Klenow fragment. Notice the emission in spectrum B at 520 nm due to the emission from NBD-labeled Klenow fragment following excitation due to energy transfer from AEDANS-S-dUTP.

spectrum of NBD-labeled Klenow fragment are shown. There is evidence for partial overlapping of the two spectra, making their use in resonance energy transfer experiments feasible. Similar conclusions can be drawn for the mansyl donor fluorophore and the acceptor NBD (spectra not shown). The donor fluorophore ANS was chosen due to its compact size, relative to other fluorophores, and its spectral overlap with the fluorophore chosen to label Klenow fragment NBD.

Labeled Klenow fragment is depicted in Figure 3 and shows the small relative size of the fluorophore. A small fluorophore was chosen so as not to disrupt the enzyme's structure by its introduction. Polymerase assays, measuring the incorporation of [γ-^{32}P]dATP into poly[d(AT)], showed no difference in the rate of incorporation of the radiolabel between labeled and unlabeled Klenow fragment, showing that the IANBD modification did not affect the polymerase activity of the enzyme.

Previous titration of samples of AEDANS-S-dUTP with Klenow fragment (Allen et al., 1989) has shown that the dNTP analogue binds to the enzyme with a K_D of 2.2 μM. This value was used to subtract the emission signal due to free AEDANS-S-dUTP (57% unbound at 500 nM AEDANS-S-dUTP, 2 μM enzyme) from that of the enzyme-bound species in the energy-transfer samples so that the spectra observed are due solely to enzyme-bound AEDANS-S-dUTP.

Comparison of enzyme-bound dTTP and AEDANS-S-dUTP (Figure 4) enabled the calculation of the energy-transfer efficiency between the bound donor triphosphate and labeled enzyme acceptor. Values of E of 0.30, Q_D = 0.12, and J_{DA} = 4.8 × 10^{-14} cm^6/mol enabled the determination of the apparent distance, $R_{(2/3)}$, separating these sites of 36 Å. The measured cone semiangle of 38° for AEDANS-S-dUTP·KF gave a range for the actual distance separating these sites of 28–41 Å.

Sulfhydryl to Deoxynucleotide Monophosphate Binding Site. Upon being bound to Klenow fragment, the emission intensity of AEDANS-S-dUMP (Figure 1) increases 17-fold and the peak maximum shifts 28 nm, from 497 to 469 nm (Figure 5). A nonlinear fit of the titration curve found K_D = 0.9 μM. The fluorescence emission increase observed upon AEDANS-S-dUMP binding to Klenow fragment is decreased

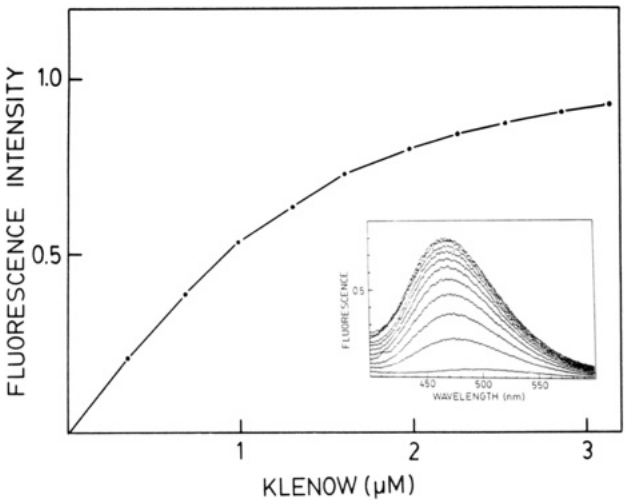

FIGURE 5: Titration of AEDANS-S-dUMP with Klenow fragment. Aliquots of Klenow fragment were added to a 135 nM solution of AEDANS-S-dUMP, and the fluorescence emission increase from the ANS fluorophore was followed at an excitation of 340 nm.

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                    11*/20
        3' AGCGTCGGCAGGTTCCCAAA 5'
11*     5' TCGCAGCCGUC          3'

        +dCTP
12*     5' TCGCAGCCGUCC         3'

        +dATP
14*     5' TCGCAGCCGUCCAA       3'

        +dGTP
17*     5' TCGCAGCCGUCCAAGGG    3'

        +dTTP
20*     5' TCGCAGCCGUCCAAGGGTTT 3'
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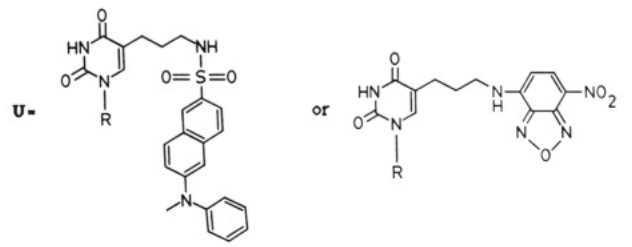

FIGURE 6: Labeled oligonucleotide duplex (11*/20). The duplex was formed by the annealing of mansyl- (A) or NBD- (B) labeled primer 11mer to a complementary template 20mer. The labeled primer 11mer can be elongated to a 12-, 14-, 17-, and 20mer by the stepwise addition of dCTP, dATP, dGTP, and dTTP.

upon addition of a large excess of dTMP, indicating that the AEDANS-S-dUMP is binding in the monophosphate site of the enzyme.

Due to the large differences in the quantum yields of the free and bound AEDANS-S-dUMP, the signal arising from free AEDANS-S-dUMP was negligible and was not subtracted from the energy-transfer samples. Comparison of enzyme-bound dTMP and AEDANS-S-dUMP yielded a transfer efficiency of 0.88 between the donor and acceptor fluorophores. This measured efficiency along with the Q_D = 0.15 and J_{DA} = 4.1 × 10^{-14} cm^6/mol yielded an apparent distance separating the labeled sulfhydryl from the monophosphate binding site of 23 Å. The range of actual distances, calculated from a cone semiangle of 30° for AEDANS-S-dUMP, was 17–26 Å.

Table I: Resonance Energy Transfer Parameters for Distance Measurements from NBD-Labeled Sulfhydryl to Various Positions in the Elongated Oligonucleotide Duplex 11*/20[a]

primer length	Q_D	J_{DA} (cm^6/mol)	$R_{0(2/3)}$ (Å)	$R_{(2/3)}$ (Å)	R' (Å)
11*	0.10	3.41 × 10^{-14}	29	34	24–37
12*	0.062	3.41 × 10^{-14}	27	32	23–35
14*	0.048	3.41 × 10^{-14}	26	32	24–36
17*	0.024	3.58 × 10^{-14}	23	31	24–36
20*	0.023	3.58 × 10^{-14}	23	33	25–38

[a] Q_D = donor quantum yield; J_{DA} = spectral overlap integral between donor and NBD acceptor fluorophore.

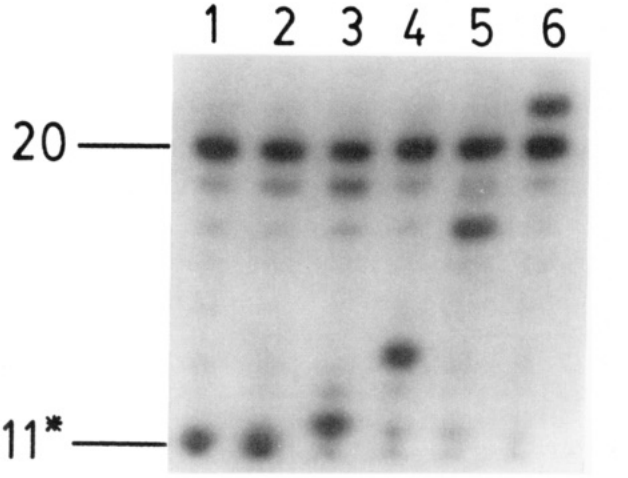

FIGURE 7: Elongation of mansyl-labeled 11*/20. Both the 11*mer and 20mer were 5′-OH-labeled with ^{32}P. Lane 1: 11*mer and 20mer. Lane 2: 11*/20 duplex elongated with Klenow, no dNTPs. Lanes 3–6: 11*/20 duplex with Klenow fragment and dNTPs. Lane 3: dCTP. Lane 4: dC, dATP. Lane 5: dC, dA, dGTP. Lane 6: dC, dA, dG, dTTP. Note the 20*mer has a lessened mobility relative to the unlabeled 20mer due to the added fluorophore.

Sulfhydryl to DNA Binding Site. In order to determine the distance separating the DNA binding site from the labeled sulfhydryl of Klenow fragment, an oligonucleotide duplex system was used in which a fluorophore is located one base removed from the 3′-OH of the primer strand (Figure 6). The fluorescently labeled primer oligonucleotide (11*mer) is annealed to a complementary 20mer to produce a fluorescent (11*/20) oligonucleotide duplex. The fluorophore's position relative to the 3′-OH of the terminus of the primer strand can be changed by the polymerase-catalyzed addition of the appropriate dNTP to the primer terminus of a Klenow-bound 11*/20 duplex (Figures 6 and 7). Through this method, the distance of the mansyl fluorophore used in these experiments from the labeled sulfhydryl of Klenow fragment could be varied as a function of the fluorophore's position within the duplex.

Previous titration of mansyl-labeled 11*/20 with Klenow fragment has shown that the labeled duplex interacts with the enzyme with a K_D of 0.1 nM, negating the need to subtract the emission signal of free 11*/20 from the energy-transfer samples. The results from the transfer experiments are shown in Table I and Figure 8. The distances observed at the five positions in the oligonucleotide duplex did not change appreciably even though the fluorophore was moved through

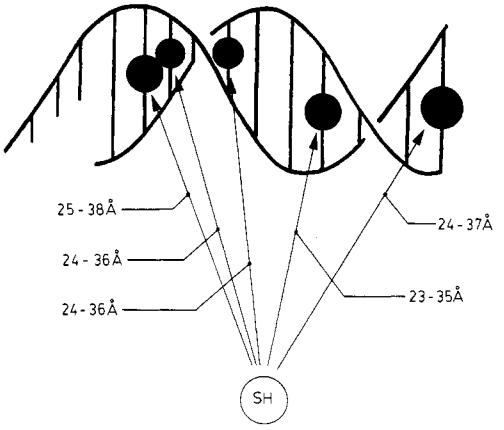

FIGURE 8: Distances from labeled sulfhydryl at cysteine 907 to various positions within mansyl-labeled 11*/20.

almost a complete turn of DNA (≅36 Å).

Deoxynucleotide Monophosphate Binding Site to DNA Primer Terminus (11/20).* The introduction of an energy-transfer acceptor fluorophore NBD into the oligonucleotide duplex 11*/20 enabled the distance determination between donor AEDANS-S-dUMP (Figure 1), bound in the monophosphate binding site, and acceptor NBD-labeled 11*/20 (Figure 6). By use of the Q_D determined for AEDANS-S-dUMP of 0.15 and a J_{DA} of 3.2×10^{-14} cm^6/mol with the absorption spectrum of NBD-labeled 11*/20 duplex, an R_0 value of 31 Å was calculated for this donor–acceptor pair. Comparison between Klenow fragment bound AEDANS-S-dUMP with bound NBD-labeled 11*/20 and unlabeled 11/20 gave a transfer efficiency of 0.80 and an apparent distance separating these two sites of 25 Å. The range of actual distances was 19–28 Å, calculated from the donor cone angle of 30°. The results of distances measured between various binding sites on the enzyme are summarized in Figure 9.

Deoxynucleotide Monophosphate Binding Site to DNA (17/20).* The addition of dCTP, dATP, and dGTP to a sample containing NBD-labeled 11*/20 and Klenow fragment allowed the extension of the primer 11*mer to a 17*mer, creating NBD-labeled 17*/20 in which the acceptor fluorophore has been moved away from the primer terminus (Figure 6). Comparison between Klenow fragment bound AEDANS-S-dUMP with bound NBD-labeled 17*/20 and unlabeled 17/20 gave a transfer efficiency of 0.29 and an apparent distance separating these fluorophores of 36 Å. The actual distances separating these sites were increased from 19–28 Å, in the case of the 11*/20, to 26–41 Å.

Anisotropy Measurements of Substrates. Anisotropy and polarization values for bound and unbound substrates are reported in Table II. In all cases, substrate binding to Klenow fragment caused an increase in the measured anisotropy as the rotational mobility of the substrate was decreased.

Discussion

Experimental Approach. Transfer measurements between fluorophores were used to locate substrate binding sites on Klenow fragment from a known position on the enzyme, cysteine 907. Klenow fragment fortunately possesses a single sulfhydryl that when modified with IANBD showed no detectable loss in the enzyme's polymerase activity. The modified cysteine serves as an energy-transfer acceptor for the aminonaphthalenesulfonate donor fluorophores, used in the dNMP and dNTP analogues, and the mansyl fluorophore, used in the oligonucleotide duplex. Spectral characteristics of these donor–acceptor fluorophores along with the quantum yield of the enzyme-bound donors gave $R_{0(2/3)}$ values of 23–32 Å, that distance at which there is 50% transfer between donor–acceptor groups. The small differences between the R_0 values and the actual measured distances facilitated the measurement of the transfer efficiency by the donor quenching method as $R \ll R_0$ gives total quenching and $R \gg R_0$ yields little or no quenching.

Table II: Anisotropy Values of Unbound and Klenow Fragment Bound Fluorescent Substrate Analogoues[a]

substrate	anisotropy, no enzyme	anisotropy, added enzyme
11*/20	0.0776 ± 0.0010	0.2089 ± 0.0012
AEDANS-S-dUTP	0.0744 ± 0.0014	0.1724 ± 0.0018
AEDANS-S-dUMP	0.0188 ± 0.0008	0.0783 ± 0.0010
NBDKF	0.1847 ± 0.0012	

[a] Measurements were taken at an excitation of 340 nm.

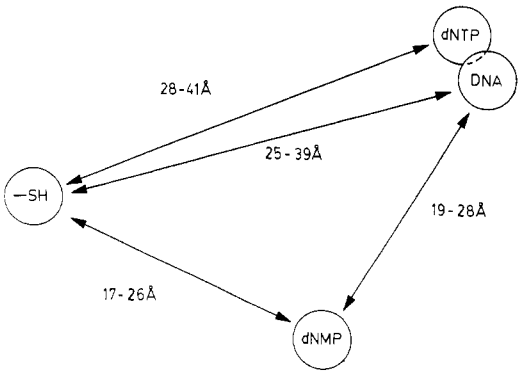

FIGURE 9: Separation distances between substrate binding sites on Klenow fragment and a known residue, cysteine 907 on the enzyme.

In the measurement of the separation distances, the apparent distances were initially calculated by using a value of $K^2 = {}^2/_3$, the value for donors and acceptors that randomize by rotational diffusion prior to energy transfer. Since this case is rarely true in biological systems not involving metal ions (Co^{2+}, Tb^{3+}, Eu^{3+}), a range of actual distances were calculated by using the donor cone semiangle, θ, measured by picosecond anisotropy decays (Millar, Allen, and Benkovic, to be published). The determined θ values were used to determine a range of α values that gave a range of distances separating the fluorophores (eq 6). It should be noted that this method assumes that the energy acceptor has no rotational freedom. This is obviously not true for the acceptor NBDKF (Table II) as the anisotropy is low. Mobility of the acceptor leads to a narrower range of α values, due to a larger relative cone semiangle, and thus a smaller range of actual distances. The actual separation distances, R' (Å), reported here are maximum ranges due to uncertainties in K^2 and actually narrow toward $R_{0(2/3)}$ owing to some degree of rotational mobility of the acceptor.

Sulfhydryl to DNA Distances. The distance measured between the labeled sulfhydryl and the mansyl-labeled 11*/20 oligonucleotide duplex was 24–37 Å. The mansyl fluorophore was not placed at the 3′-OH terminus, but was placed one base removed due to the nature of the phosphoramidite chemistry used to synthesize the oligonucleotides. Movement of the fluorophore away from the 3′-OH primer terminus by the addition of dNTPs (Figure 6) produced no great changes in the measured distances even though the fluorophore was moved a distance of almost a complete turn of DNA. The measured distances, summarized in Figure 7, place the labeled sulfhydryl in the center of the observed DNA positions and support the

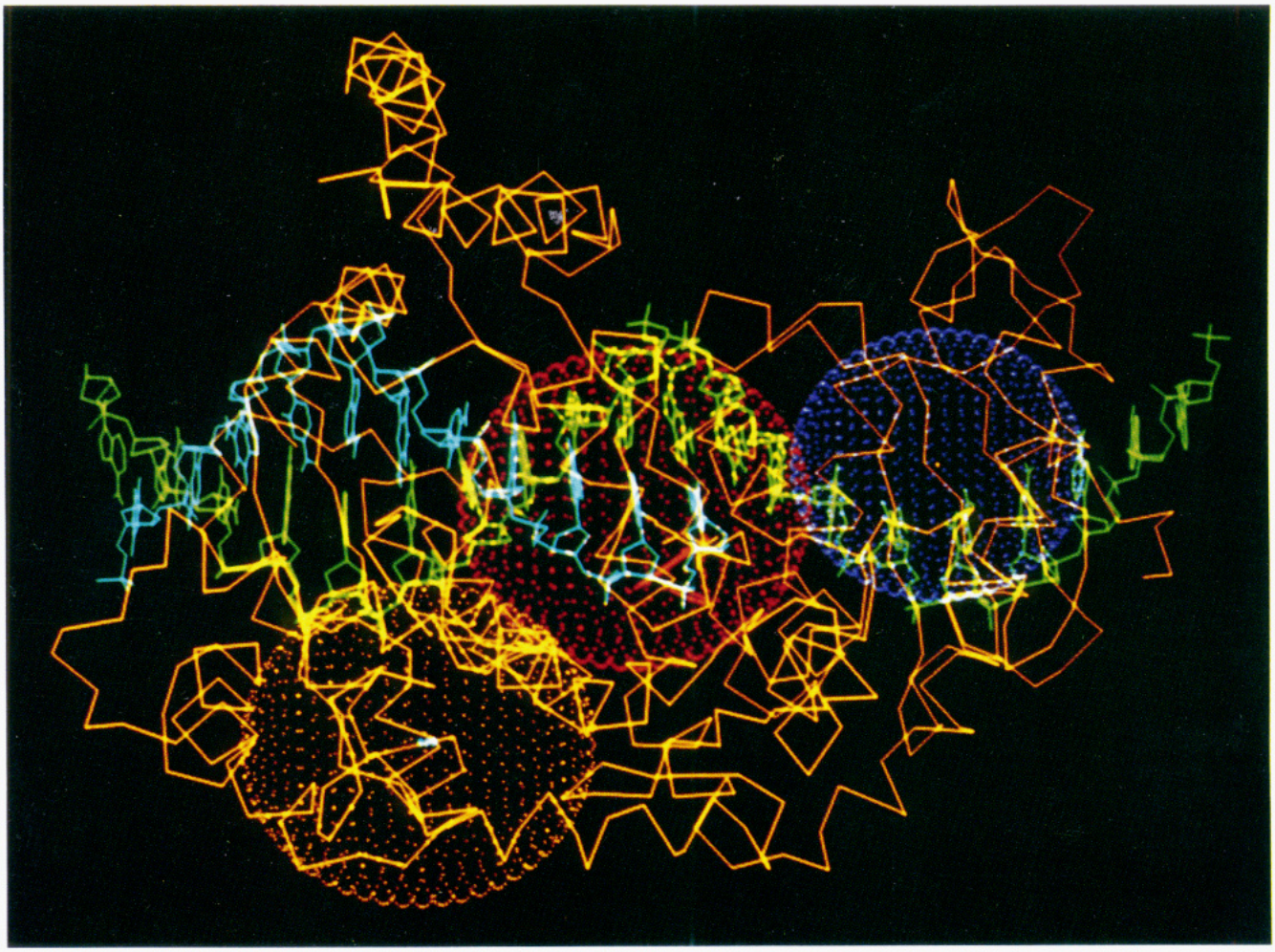

FIGURE 10: Klenow fragment with bound duplex DNA (11*/20). Dot spheres (radius = 12 Å) show possible placement of the fluorophores attached to the substrate analogues (orange = labeled sulfhydryl, violet = labeled dNMP, and red = labeled primer terminus and dNTP). This view illustrates the placement of the sulfhydryl in the center of the first turn of duplex DNA (see Figure 8).

placement of the DNA duplex within the deep cleft found in the large domain of the Klenow fragment crystal structure (Figure 10).

Sulfhydryl to Triphosphate Distance. The measured distance separating the labeled sulfhydryl from the triphosphate binding site was 28–41 Å, very close to that value separating the sulfhydryl from the mannsyl-labeled 11*/20. It is presumed that the dNTP is bound in close proximity to its site of incorporation, so it is expected that the distances separating the DNA primer terminus and dNTP from the sulfhydryl are similar. Previously, it has been shown that AEDANS-S-dUTP binds to Klenow fragment (Allen et al., 1989) and can be incorporated into an oligonucleotide duplex at a dTTP-dependent position, indicating that the triphosphate analogue is binding to the enzyme at its dNTP binding site.

Sulfhydryl to Monophosphate Distance. A deoxynucleotide monophosphate analogue AEDANS-S-dUMP was prepared that bound at the monophosphate binding site of Klenow fragment with a K_D = 0.9 μM. The emission from this monophosphate analogue is increased upon binding to the enzyme and is blue-shifted 28 nm, indicating that the fluorophore resides in a more hydrophobic environment relative to that of solution. The emission increase upon binding of AEDANS-S-dUMP to Klenow fragment is unaffected by the oligonucleotide duplex and dNTP, but is decreased upon addition of dNMP, indicating that the AEDANS-S-dUMP is being bound by the enzyme at its monophosphate binding site. The measured separation between the labeled sulfhydryl and monophosphate binding site was 17–26 Å, and since the emission spectra were unchanged upon the addition of the oligonucleotide duplex (11/20), this suggests that this separation distance remains unchanged upon binding of the DNA duplex.

Monophosphate to DNA Primer Terminus (11/20) and to 17*/20.* With the polymerase activity residing on the large domain and the exonuclease activity localized on the small domain (Derbyshire et al., 1987), it has been postulated that, for a base to be removed by the exonuclease activity of the enzyme, the DNA duplex must slide 8 base pairs toward the exonuclease site and melt out about 4 bases (Steitz & Joyce, 1988). Direct evidence for this separation has been shown by using biotinylated oligonucleotide duplexes, and the requirement of a 3–4 base separation for exonuclease activity to occur has been illustrated by using site-specific cross-linked DNA (Cowart et al., 1989). Further evidence for the physical separation of the polymerase and exonuclease sites has been shown here through the use of resonance energy transfer between donor AEDANS-S-dUMP and acceptor NBD-labeled 11*/20 and 17*/20. The observed separation distance of 19–28 Å between the monophosphate site and DNA primer terminus appears to be in agreement with the proposed editing mechanism. Polymerization of the NBD-labeled 11*/20 by 6 base pairs causes a decrease in the transfer efficiency from AEDANS-S-dUMP from 0.80 to 0.29 and an increase in the separation distance from the monophosphate site to 36 Å. The increase in distance from the monophosphate site upon po-

lymerization is consistent with the placement of the DNA duplex within the cleft on the large domain (Figure 10). Polymerization would move the labeled base through the cleft and away from the monophosphate/exonuclease site located on the small domain.

Conclusions

The measurements of distances within binary dNTP, dNMP DNA and ternary DNA·dNMP·Klenow complexes are in satisfactory agreement with those measured directly from the X-ray crystallographic structure. However, owing to the nature of this method, the separation distances reported here are the distances separating the donor and acceptor fluorophores. In all cases, the fluorophore is attached to the 5-position of a uracil ring (in the case of DNA and nucleotide derivatives), or to a sulfhydryl, via a tether arm. The tether arm thus places the fluorophore an unknown distance away from the anchor site of the moiety used. The exact position of these contacts with the enzyme is unknown. The tether arms, fully extended, would place the fluorophore approximately 10–15 Å away from the respective binding site, although the actual distance is probably less. In the case of labeled duplex DNA, since the 5-position of the uridine ring faces into the major groove, the fluorophore (mansyl or NBD) probably resides within, and is bounded by, the major groove. Nevertheless, from the monophosphate·DNA separation distances, there appears to be no compressing of the structure by folding it around an axis separating the polymerase and exonuclease domains. Separation distances between monophosphate and 11*/20, 17*/20 duplex DNA support the model for DNA movement between the polymerase and exonuclease sites as opposed to the movement of these sites toward each other. A model-built complex of duplex DNA and Klenow fragment (Figure 10) using the dimensions from the resonance energy transfer measurements is in accord with the duplex occupying primarily the polymerase site as opposed to the exonuclease site. Placement of the primer terminus of the DNA was done to agree with fluorescence footprinting experiments (Allen et al., 1989) and from photo-cross-linking experiments using the 11*/20 system described here but in which the fluorophore has been replaced by an aryl azide. The latter labels tyrosine 766 (Catalano et al., 1989). The placement of the DNA duplex in the enzyme is also in agreement with Figure 8, which shows that the labeled sulfhydryl is positioned in the center of the first turn of duplex DNA extending away from the primer terminus.

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