DNA polymerase (pol) β is a two-domain DNA repair enzyme that undergoes structural transitions upon binding substrates. Crystallographic structures indicate that these transitions include movement of the amino-terminal 8-kDa lyase domain relative to the 31-kDa polymerase domain. Additionally, a polymerase subdomain moves toward the nucleotide-binding pocket after nucleotide binding, resulting in critical contacts between α-helix N and the nascent base pair. Kinetic and structural characterization of pol β has suggested that these conformational changes participate in stabilizing the ternary enzyme-substrate complex facilitating chemistry. To probe the microenvironment and dynamics of both the lyase domain and α-helix N in the polymerase domain, the single native tryptophan (Trp-325) of α-helix N in the lyase domain (F25W/W325A) or near the end of α-helix N (L287W/W325A). Influences of substrate on the fluorescence anisotropy decay of these single tryptophan forms of pol β were determined. The results revealed that the segmental motion of α-helix N was rapid (~1 ns) and far more rapid than the step that limits chemistry. Binding of Mg²⁺ and/or gapped DNA did not cause a noticeable change in the rotational correlation time or angular amplitude of tryptophan in α-helix N. More important, binding of a correct nucleotide significantly limited the angular range of the nanosecond motion within α-helix N. In contrast, the segmental motion of the 8-kDa domain was “frozen” upon DNA binding alone, and this restriction did not increase further upon nucleotide binding. The dynamics of α-helix N are discussed from the perspective of the “open” to “closed” conformational change of pol β deduced from crystallography, and the results are more generally discussed in the context of reaction-cycle-regulated flexibility for proteins acting as molecular motors.

DNA polymerase (pol) β is a 39-kDa enzyme composed of two distinct domains of 8- and 31-kDa connected by a protease-hypersensitive hinge region. The amino-terminal 8-kDa domain possesses the 5′-deoxyribose phosphate lyase activity required to process the 5′-terminus in a DNA gap during base excision repair. The polymerase active site is part of the carboxyl-terminal 31-kDa domain (for a review see Ref. 1). The domain and subdomain organization of pol β is illustrated in Fig. 1, A and B. Although pol β appears to have evolved separately from other families of polymerases of known structure (2), it shares many general structural and mechanistic features with other polymerases. The polymerase domain is composed of three functionally distinguishable subdomains. The polymerase catalytic subdomain coordinates two divalent metal cations that assist the nucleotidyl transferase reaction. Two additional subdomains that have primary roles in duplex DNA binding and nascent base pair (nucleoside 5′-triphosphate and templating nucleotide) binding border the catalytic subdomain. These subdomains will be referred to as C (catalytic)-, D (duplex DNA binding)-, and N (nascent base pair binding)-subdomains to discern their intrinsic function. These would correspond to the palm, thumb, and fingers subdomains, respectively, according to the nomenclature that utilizes the architectural analogy to a right hand (3).²

Because of its small size, lack of accessory proteins, and excellent expression properties, pol β serves as an excellent model for study of the nucleotidyl transferase reaction, DNA synthesis fidelity, and protein-DNA interactions. Many revealing x-ray crystallographic structures of apoenzyme (4), binary DNA substrate complexes (substrate and product) (5, 6), and ternary substrate complexes (DNA and 2′-deoxynucleoside 5′-triphosphate) (6–8) have been solved.

Comparison of the structure of pol β in different liganded states indicates that there are numerous structural transitions that occur upon substrate binding. These transitions include repositioning of the amino-terminal lyase domain toward the carboxyl polymerase domain upon binding DNA. Additionally, a large, ~30°, rotation of α-helix M repositions α-helix N adjacent to the nascent base pair upon binding a dNTP to form a ternary complex (9). The repositioning of α-helix N upon binding a nucleotide results in an “open” (−dNTP) to “closed” (+dNTP) conformational transformation and forms an effective active site that coordinates two Mg²⁺ ions (see Fig. 1C). When bound to single-nucleotide gapped DNA, the overall architecture of pol β resembles a doughnut, which suggests that nucleotide access may be restricted (9, 10). Upon binding a correct dNTP and forming a closed complex, extensive contacts are observed between the polymerase and nascent base pair (6, 7). The structure of pol β bound to single-nucleotide gapped DNA indicates that the template strand is bent ~90° as it enters the polymerase active site, permitting polymerase stacking inter-
actions with one face of the nascent base pair and the DNA minor groove. This allows for a survey of the geometry of the nascent base pair to probe whether it conforms to Watson-Crick geometry. The structure of a "catalytic intermediate" ternary complex of pol β indicates that closure of the N-subdomain can be induced by metal-dNTP binding in the absence of the catalytic metal (8). Kinetic approaches to study nucleotide insertion and fidelity have indicated that rate-limiting conformational changes occur before and after chemistry, suggesting an induced-fit mechanism for nucleotide insertion and fidelity (see Ref. 11). The identity of the proposed conformational changes are unknown, but recent kinetic (8, 10, 12), structural (8), and modeling (11) studies with pol β indicate that it is not the large subdomain movements inferred from structural studies. However, the rate and function of this large structural transition remains unknown.

DNA polymerases are more than nucleotidyl transferase catalytic centers. Some DNA polymerases are processive and remain bound to the DNA substrate as they move along the template strand synthesizing DNA. DNA polymerase β is known to be processive on short gapped DNA but distributive on non-gapped DNA (13). Because the isolated polymerase domain (31-kDa domain) of pol β exhibits only distributive DNA synthesis (14), the 8-kDa lyase domain confers processive DNA synthesis to the intact enzyme. The dynamic description of processive DNA polymerases should include their catalytic properties (e.g. DNA and dNTP binding, conformational changes, and chemistry), as well as their movement (i.e. translocation). The force and velocity of RNA polymerase has been studied as a function of load (15), and it appears to function as a molecular motor. Recently, the vectorial nature of these motors has been modeled in terms of a "Brownian Ratchet" (16–18).

The requirements for rectification of Brownian motion seem to be as follows: 1) a cycle of flexibility and/or hinging motion followed by one or more rigid states in the biopolymer, 2) the coupling of nearly irreversible chemical events to selected portions of the cycle, and 3) a change in conformation or binding affinity associated with those events. The myosin-actin and dynein-tubulin systems are prototypes for this model (17, 18). Regulation of flexibility also plays a role in recognition; in particular, some protein-protein associations are mediated by "target-induced folding" of disordered domains (19–21). In those systems, target-dependent structure seems to be a compromise to achieve relatively tight binding without the stringent, and selective, needs of a "lock and key" interface.

We remain cognizant that we are using dynamics measured in hundreds of picoseconds to a few nanoseconds to study cycles requiring at least hundreds of milliseconds; nevertheless, nanosecond flexibility is clearly relevant to an understanding of the conformational events in that cycle. Gangal et al. (22) have shown that the 1–3-ns rotational correlation times of fluorescein conjugated to engineered surface cysteines correlate strongly with the long-term structural disorder reported by crystallographic B-factors for the adjacent backbone elements. The measurement of segmental flexibility has a long history (see Ref. 23); in particular, emission anisotropy has a long history of revealing local motions (24, 25). In addition, time-resolved methods have the advantage of quantifying both the angular extent and the rate of these motions (24, 26, 27). In favorable cases, the fast motion of a segment on a loose "hinge" can be interpreted both in terms of the size of the independent segment and the angular range of facile hinging. Even in flexible coiled polymers, the fast term presents a measure of granularity and offers insight into the persistence of rigid segments (28). In all, nanosecond flexibility appears to be an important predictor of functional flexibility.

Wild-type pol β is comprised of 335 amino acids and includes a single tryptophan near the carboxyl terminus (Trp-325; see Fig. 1, A and B). The effects of metal binding on the tryptophan fluorescence and the hydrodynamic shape of wild-type rat pol β were characterized previously (29) by time-resolved fluorescence and analytical ultracentrifugation. In this study, we have strategically positioned tryptophan residues in the lyase domain (L297/W322A) or the N-subdomain (L297/W322A) by site-directed mutagenesis to monitor changes in flexibility of these structural elements upon binding substrates. We interpret changes in flexibility in the context of pol β function.

EXPERIMENTAL PROCEDURES

Materials—Wild-type and mutant derivatives of human recombinant pol β were overexpressed in Escherichia coli and purified as described (30). Oligonucleotide site-directed mutagenesis and activity measurements were performed as described previously (31). The enzyme concentration was determined from the absorbance at 280 nm using an extinction coefficient of 21,200 cm⁻¹ M⁻¹ (32). Acrylamide and melatonin were obtained from Sigma.

A 24-mer DNA substrate was prepared by annealing three high pressure liquid chromatography-purified oligonucleotides (Oligos Etc., Wilsonville, OR) to create a single-nucleotide gap at position 13. Each oligonucleotide was resuspended in 50 mM Tris-HCl, pH 7.4, and 1 mM EDTA, and the concentration was determined from their UV absorbance at 260 nm. The annealing reactions were carried out by incubating a solution of 10 μM primer with 11 μM of both the downstream and template oligonucleotides at 90 °C for 2 min followed by slow cooling to room temperature. The sequence of the gapped DNA substrate was as follows: upstream 12-mer primer, 5′-CCGGCTGATGCGC-3′; downstream 11-mer oligonucleotide, 5′-GGTGGTGCCCC-3′; 24-mer template, 5′-GGCCCCAGGGAACATCAGGG-3′. The 3′ end of the upstream primer was synthesized without a 3′-hydroxyl so it would not be extended with the addition of pol β and dNTP. The downstream primer was synthesized with a 5′-phosphate.

Absorption and Steady-State Fluorescence—Absorption spectra were recorded on a Hewlett Packard 8453 UV-VIS spectrophotometer (Palo Alto, CA), and fluorescence emission spectra were obtained on an SLM AB-2 (Thermo Spectronics, Rochester, NY) or SLM8000 (SLM Instruments, Urbana, IL) spectrofluorometer. The protein concentration was between 2.5 and 8 μM, and the sample absorbance at 285 nm was <0.1 with 3-mm path length. Excitation and emission monochromators were set at 4-nm band pass. Emission spectra were collected with excitation polarizer at 54.7° and emission polarizer at 0° to the vertical, and background fluorescence from a solvent blank was subtracted. Steady-state anisotropies were measured using the L-format method with Glan-Thomson calcite prism polarizers. Anisotropy (r) was defined from the intensity of the vertical (Iv) and horizontal ( Ih) measurements with a grating correction factor (G): A = [Iv - GIv]/[Ih + GIh]. G was calculated from the ratio of intensities (Ih/Iv) when the excitation light was polarized horizontally. Stern-Volmer quenching experiments were carried out as described previously (29). Temperature was controlled at 20 °C with a circulating water bath.

Circular Dichroism—Circular dichroism spectra were measured using an Applied Photophysics PSI-star-180 spectrometer using a 0.1-cm cell thermostated at 20 °C. All measurements were corrected for background signal.

Time-resolved Fluorescence Decay—Measurements were performed in 50 mM Tris-HCl, pH 7.4, and 0.1 M RCl at 20 °C with a protein concentration of 4.7 to 9.4 μM. To avoid tyrosine absorbance, samples were excited at 295 nm, and the absorbance at 280 nm was 0.1–0.2. The fluorescence intensity profiles were measured by the time-correlated single-photon counting technique as described (28). Time per channel was 85 ps, and 512 channels were recorded. Samples were excited at 295 nm using a synchronously pumped, cavity-dumped, frequency-doubled dye laser as excitation source, with a repetition rate of 4 MHz, a pulse width of 5 ps, and an average UV power under 200 microwatts. The Hamamatsu R2809 MCP photomultiplier operated with a 0.1-cm cell thermostated at 20 °C. All measurements were corrected for background signal.

\[ I(t) = \sum_{i} a_i e^{-t/\tau_i} \]  
(Eq. 1)
The relative amplitudes \( (\alpha_i) \) and decay constants \( (\tau_i) \) were extracted from the fit. The decay-associated spectra were obtained as described previously (29, 33).

For fluorescence anisotropy decay measurements, protein fluorescence at 340 nm was monitored through a film polarizer oriented parallel \( I_{\perp}(t) \) and/or perpendicular \( I_{\parallel}(t) \) to the vertical excitation polarization and alternatively recorded. Data were collected up to 20,000 counts in the maximum channel at 20 °C. For each sample, sixteen fluorescence intensity decays were obtained contemporaneously and summed to generate the anisotropy decay, \( r(t) \), as follows in Equation 2.

\[
r(t) = \frac{I_{\parallel}(t) - GI_{\parallel}(t)}{I_{\parallel}(t) + 2GI_{\parallel}(t)}
\]  

(Eq. 2)

The factor \( G \) was reduced to unity with a wedge depolarizer (Optics for Research, Caldwell, NJ) by placing the depolarizer at the entrance slit of the monochromator. The reference lamp profile and color shift used for convolution analysis was tested with a monoexponential standard of the fluorescence lifetime of 5.27 ns. The convolution was compared with the experimental fluorescence intensity decay by a nonlinear least-squares (reduced chi-squared; \( \chi^2 \)) minimization. The weighted residuals and autocorrelation function of the weighted residuals were calculated to estimate the reliability of each fit.

The fluorescence data were analyzed by the “sum and difference” method described previously (34). The general procedure was to fit the experimental anisotropy decays to the sum of two exponential functions, shown in Equation 3, where \( \beta_{fast} \), \( \beta_{slow} \), \( \phi_{fast} \), and \( \phi_{slow} \) were the variable parameters.

\[
r(t) = \beta_{fast} e^{\phi_{fast}t} + \beta_{slow} e^{\phi_{slow}t}
\]  

(Eq. 3)

The pre-exponential \( \beta \) terms directly relate to the amplitude of rotational motion, i.e. are measures of the extent to which the emission is depolarized by each rotational component, thereby measuring the restriction upon diffusion of the residue reporter (35).

RESULTS

Site-specific Introduction of Tryptophan in pol β—In wild-type pol β, the carboxyl-terminal Trp-325 is located on the surface of the enzyme just after the last helix (αO) (Fig. 1B). X-ray structures of rat and human pol β indicate that one side of the indole ring is exposed to solvent, and the opposite face makes van der Waals contact with His-285 and Ile-323 (6, 7). Those observations were in excellent agreement with our earlier characterization (29) of the fluorescence properties of Trp-325 in rat pol β. To probe dynamic aspects of other regions of pol β, Trp-325 was replaced with alanine, and tryptophan residues were substituted at other strategic positions. In the L287W/W325A double mutant (from this point on, referred to simply as L287W), the tryptophan was introduced near the end of α-helix N in the N-subdomain (Fig. 1). Residue 287 was selected for tryptophan substitution because of its solvent-exposed environment and minimal contact with substrates as deduced from crystal structures with and without substrates (6). Crystallographic characterization of pol β in various liganded states indicates that numerous structural transitions occur upon binding substrates. The most prominent conformational event occurs upon binding of the correct nucleotide resulting in an open to closed transition where α-helix N tilts toward the active site intimately contacting the nascent base pair. The characteristics of L287W thus enabled us to use it as a probe for the effects of substrate binding on the dynamics of α-helix N.

For study of the 8-kDa lyase domain, we substituted tryptophan for Phe-25 (i.e. P25W/W325A; from this point on, referred to simply as F25W). The amino-terminal lyase domain possesses lyase, single-stranded DNA binding, and 5’-phosphate binding activities (1). Several of the key residues for these activities have been characterized (36–39). Trp-25 is located on the first helix of the protein (αA; see Fig. 1, A and B) and is predicted to be partially solvent-exposed. The crystallographic structure of the pol β ternary complex indicates that there are no direct substrate contacts with this residue, and residue 25 is located about 10 Å from the lyase active site (i.e. Lys-72) (6). The structure of the apoenzyme form of rat pol β (4), or in complex with non-gapped DNA (7), indicates that the lyase domain is distant from the carboxyl-terminal subdomain (i.e. N-subdomain), in contrast to its position when bound to gapped DNA (6). The open and closed conformations of the amino-terminal lyase domain suggest that this domain is flexible in solution. The dynamic properties of the lyase domain in full-length enzyme are unknown even though the NMR structure of the 8-kDa lyase domain has been solved (37).

![FIG. 1. DNA polymerase and DNA substrate organization and structure. A](image)
characterization of polymerase conformation and catalytic activity—the purity of the recombinant proteins was accessed by SDS-PAGE and found to be greater than 99% (Fig. 2, inset). To access the effect of the mutations on the protein conformation, we measured circular dichroism spectra for wild-type, F25W, and L287W proteins (Fig. 2). The spectra indicate that the mutations had no effect on overall protein folding. The catalytic activity of wild-type enzyme and the mutant derivatives were assessed on a single-nucleotide gapped DNA substrate utilizing Mg\(^{2+}\) as the divalent cation. The single (W325A) or double mutants (F25W/W325A and L287W/W325A) exhibited minimal effects on catalytic activity; \(k_{cat}\) for wild-type enzyme was 0.22 ± 0.05 compared with 0.20 ± 0.06, 0.24 ± 0.06, and 0.13 ± 0.02 for W325A, F25W, and L287W, respectively. The DNA binding affinity of the mutant proteins for gapped DNA substrates was also not affected by the mutation as assessed by analytical ultracentrifugation (data not shown).

steady-state fluorescence of wild-type and mutant proteins—the fluorescence emission spectra for F25W, L287W, and wild-type proteins were measured in the absence and presence of substrates. The emission spectra for F25W, L287W, and wild-type proteins are shown in Fig. 3A. The emission maximum for F25W was ~340 nm, whereas it was 348 nm for the L287W and wild-type proteins indicating solvent-exposed tryptophan microenvironments in these later instances. For F25W, the emission maximum suggests that Trp-25 is less exposed to solvent as compared with other tryptophans. The relative integrated intensities of F25W and L287W were about 2- and 3-fold weaker than that of Trp-325 in wild-type pol β, respectively. The steady-state anisotropy values for F25W, L287W, and wild-type pol β were 0.13 ± 0.01, 0.14 ± 0.003, and 0.16 ± 0.005, respectively, indicating a more flexible tryptophan in F25W and L287W than the wild-type protein. The steady-state parameters for wild-type human pol β were similar to those of rat enzyme determined previously (29).

The effects of Mg\(^{2+}\) and DNA binding on steady-state fluorescence were also determined. The binding of 10 mM Mg\(^{2+}\) resulted in a minimal fluorescence decrease for wild-type (2.7%; see Fig. 3B) and L287W proteins (7.8%; see Fig. 3D). In contrast, Mg\(^{2+}\) binding to F25W resulted in a significant decrease of fluorescence intensity (24.8%; see Fig. 3C). The fluorescence quenching detected with F25W suggests metal-induced structural changes around Trp-25. Metal soaking experiments with pol β crystals indicated that Mg\(^{2+}\) could bind to the hairpin of the first Helix-hairpin-Helix motif (40). This metal binding motif is also found in the D-subdomain and is proposed to facilitate DNA binding (5). Our data suggest that the Trp-25 probe in the lyase domain senses metal binding, even though it is not proximate to these hairpin residues. Further binding of gapped DNA and dTTP resulted in 25.8% (F25W), 32.3% (L287W), and 29.3% (wild-type) fluorescence decreases as compared with proteins without substrates. Notably, DNA binding resulted in ~7 nm of blue shift in the emission maximum of F25W (Fig. 3C), further indicating that the environment around Trp-25 is altered with DNA binding. Although Phe-25 of wild-type protein is not implicated in DNA binding as deduced from crystallographic structures, its location adjacent to a positively charged groove may indicate a role in DNA binding. The longer DNA molecule used here (24 bp) may interact with a larger binding surface than that observed in crystallographic structures utilizing shorter (16 bp) DNA molecules (6).

Stern-Volmer Quenching of Wild-type and Mutant Proteins—Effects of substrate binding on the microenvironment of the tryptophan residues were probed by collisional quenching experiments using acrylamide as a quencher (see Table I and Fig. 4). In Fig. 4A, Stern-Volmer plots for F25W, L287W, and wild-type proteins in the absence and presence of Mg\(^{2+}\), single-nucleotide gapped DNA, and dTTP are compared. The data, in the absence of substrates, were linear yielding Stern-Volmer constants (\(K_{sv}\)) of 17.34 ± 0.24 M\(^{-1}\), 20.97 ± 0.57 M\(^{-1}\), and 16.31 ± 0.12 M\(^{-1}\) for F25W, L287W, and wild-type enzymes, respectively. From these results, the relative accessibility of the tryptophans to the quencher was determined to be as follows: wild-type < F25W < L287W. In the presence of substrates (Mg\(^{2+}\), single-nucleotide gapped DNA, and dTTP), \(K_{sv}\) of the respective tryptophans decreased 50.2% (8.64 ± 0.06

![Circular dichroism spectra for wild-type and mutant pol β.](image-url)
Table I: Acrylamide quenching and fluorescence parameters for wild-type and single tryptophan mutants of pol β

|        | Substratesa | $K_{sv}$ | $k_a^b$ | $(k_a^b - k_q/k_q^°)$ |
|--------|-------------|----------|---------|-----------------------|
| Wild-type | Mg$^{2+}$, DNA, dTTP | 16.31 ± 0.12 | 2.09 ± 0.02 | 15.8 |
| F25W   | Mg$^{2+}$, DNA, dTTP | 13.91 ± 0.27 | 1.76 ± 0.03 | 14.7 |
| L287W  | Mg$^{2+}$, DNA, dTTP | 17.34 ± 0.24 | 4.67 ± 0.06 | 46.7 |
|        | Mg$^{2+}$, DNA, dTTP | 20.97 ± 0.57 | 6.11 ± 0.17 | 52.2 |
|        | Mg$^{2+}$, DNA, dTTP | 21.41 ± 0.33 | 6.16 ± 0.16 | 56.1 |
|        | Mg$^{2+}$, DNA, dTTP | 19.60 ± 0.25 | ND | ND |
|        | Mg$^{2+}$, DNA, dTTP | 16.68 ± 0.18 | ND | ND |
|        | Mg$^{2+}$, DNA, dTTP | 14.76 ± 0.54 | 4.48 ± 0.16 | 26.7 |
|        | Mg$^{2+}$, DNA, dTTP | 14.74 ± 0.26 | 4.49 ± 0.08 | 26.5 |

a The substrate concentrations were 10 mM Mg$^{2+}$, single-nucleotide gapped DNA was in 2-fold excess relative to enzyme, and 50 μM dTTP.
b $k_a = K_{sv}/\tau_m$, where $\tau_m$ (mean lifetime) is taken from Table II.
c Not determined.

For Trp-25, the relative small reduction of $K_{sv}$ for wild-type enzyme is also consistent with the location of its lone tryptophan (Trp-325) on the outer surface of the N-subdomain near the carboxyl terminus. However, the 14.7% decrease of $K_{sv}$ suggests that substrate binding can influence the accessibility of Trp-325, even though this residue is located on the side of the N-subdomain away from the active site. In the presence of substrates, the relative accessibility of the tryptophans probes is in the order of Trp-25 < Trp-325 < Trp-287. For Trp-287, there was 30% reduction in $K_{sv}$ without a change in the emission maximum. The smaller $K_{sv}$ is consistent with the structural location of residue 287 near the template backbone in the closed liganded ternary complex, whereas it is solvent-exposed in the absence of substrates (open complex). A tryptophan modeled at this position suggests it would be situated 5–6 Å from the template backbone. The relatively small decrease in $K_{sv}$ for Trp-287 with the 7-nm blue shift in the emission maximum (Fig. 3C) is consistent with the role of this residue in DNA binding.
though substrates yielded a decrease in $K_{sv}$, the wild-type (Trp-235) and L287W tryptophan environments appear to still be highly accessible to the quencher ($K_{sv}$ of 13.91 and 14.74 $\text{m}^{-1}$, respectively).

Because of the sensitive location of the tryptophan introduced on $\alpha$-helix N of the N-subdomain (Trp-237) near the templating base, the microenvironment of Trp-237 was studied in the presence of different substrate combinations (see Fig. 4B and Table I). Based on these results, $\text{Mg}^{2+}$ alone, or $\text{Mg}^{2+}$ and dTTP, did not afford significant protection of this tryptophan from acrylamide quenching (Table I). In contrast, binding of the gapped DNA alone resulted in $-21\%$ decrease in accessibility ($K_{sv} = 16.66 \text{ m}^{-1}$) suggesting that most of the protection was coming from DNA itself. The binding of the correct nucleotide, dTTP, had no effect on tryptophan accessibility. This is consistent with ternary complex structures of pol $\beta$ indicating that Leu-287 is exposed to solvent (6, 7). The linearity of the Stern-Volmer plots (Fig. 4), without a hint of curvature, suggests that the quenching mechanism for these proteins is dynamic and that the tryptophan microenvironments are homogeneously accessible.

In Table I, the bimolecular quenching rate constants ($k_q$) were calculated from the mean fluorescence lifetimes ($\tau_{av}$, Table II) and $K_{sv}$. The quenching rate constants for the all the proteins appear to be near diffusion-controlled ($10^9$-$10^{10} \text{ m}^{-1} \text{s}^{-1}$). The relative decreases in $k_q$ in the presence of substrates (last column in Table I) are very close to the reductions observed in $K_{sv}$ indicating that the observed decreases in tryptophan accessibility are primarily because of a decrease in dynamic encounters. The similar quenching parameters for L287W ($K_{sv}$ and $k_q$) in the presence and absence of the correct nucleotide suggest that the closure of the N-subdomain upon correct nucleotide binding has no effect on acrylamide accessibility for Trp-287 on $\alpha$-helix N.

**Time-resolved Fluorescence Decay**—The tryptophan fluorescence lifetime decays of wild-type and mutant proteins were measured in the presence and absence of substrates (Table II). The fluorescence lifetimes of wild-type and L287W can be well represented by the sum of two exponential decays (reduced $\chi^2 = 1.04$–1.30). In the absence of substrates, the lifetimes of Trp-235 (wild-type) were 0.95 and 8.14 ns, and those of Trp-287 were 1.42 and 5.02 ns. These lifetime parameters for the human wild-type enzyme are similar to those of rat pol $\beta$ (1.27 and 8.44 ns) measured previously (29). Thus, despite the 14-amino acid difference between the human and rat enzymes, the microenvironments of Trp-235 from these two sources appear to be similar, and the unusually long lifetime component characteristic of rat pol $\beta$ is also observed with the human enzyme. For the Trp-25 mutant, fits with a two-exponential model showed systematic deviations and high $\chi^2$ values. Three-exponential models fit the F25W decay data adequately (reduced $\chi^2 = 0.99$–1.24).

Binding of substrates to wild-type and mutant proteins had a minimal effect on their decay parameters. For example, there were less than 7 and 5% decreases in the mean lifetimes of F25W and L287W, respectively, in the presence of all substrates (Table II). This is in contrast to the 15 and 36% substrate-induced quenching observed in the steady state for F25W and L287W, respectively (Fig. 3). The wild-type enzyme

![Fig. 4. Acrylamide quenching of tryptophan in pol $\beta$. Measurements were performed at 20°C in a reaction mixture with 2.0 $\mu$m enzyme in 50 mM Tris-HCl, pH 7.4, and 0.1 mM KCl. Samples were excited at 296 nm, and emission was recorded at 350 nm. Ligand concentrations were as follows: 10 mM $\text{Mg}^{2+}$, 4 mM gapped DNA, and 50 mM dTTP. A, Stern-Volmer acrylamide quenching of wild-type (dotted line) and solid lines), F25W (open and dotted lines), and L287W (closed and dashed lines) enzymes in the absence (open) and presence (filled) of ligands. B, Stern-Volmer acrylamide quenching of L287W in the presence (dotted lines) and absence (solid line) of various substrates as follows: $\blacklozenge$, $+\text{Mg}^{2+}$; $\blacklozenche$, $+\text{Mg}^{2+}$/dTTP; $\blacklozench$, $+\text{gapped DNA}$; and $\blacklozench$, $+\text{gapped DNA}$/dTTP.

| Table II | Fluorescence lifetime decay parameters for wild-type and single tryptophan mutants of pol $\beta$ |
|---------|-------------------------------------------------|
| pol $\beta$ | Substrates* | $\alpha_1$ | $\alpha_2$ | $\tau_1$ | $\tau_2$ | $\chi^2$ |
| Wild-type | $\text{Mg}^{2+}$ | 0.71 | 0.63 | 8.14 | 0.29 | 0.95 | 7.81 | 1.13 |
|           | $\text{Mg}^{2+}$/dTTP | 0.70 | 0.63 | 8.01 | 0.30 | 0.74 | 7.72 | 1.11 |
|           | $\text{Mg}^{2+}$/gapped DNA | 0.75 | 0.63 | 8.08 | 0.25 | 0.90 | 7.81 | 1.14 |
| F25W     | $\text{Mg}^{2+}$ | 0.77 | 0.63 | 8.18 | 0.23 | 1.03 | 7.92 | 1.04 |
|           | $\text{Mg}^{2+}$/dTTP | 0.41 | 0.63 | 1.93 | 0.35 | 4.66 | 3.71 | 1.20 |
|           | $\text{Mg}^{2+}$/gapped DNA | 0.42 | 0.63 | 1.81 | 0.42 | 4.51 | 3.69 | 1.24 |
| L287W    | $\text{Mg}^{2+}$ | 0.55 | 0.63 | 1.83 | 0.36 | 4.41 | 3.90 | 1.23 |
|           | $\text{Mg}^{2+}$/dTTP | 0.45 | 0.63 | 1.83 | 0.36 | 4.41 | 3.90 | 1.23 |
|           | $\text{Mg}^{2+}$/gapped DNA | 0.48 | 0.63 | 1.87 | 0.47 | 2.40 | 3.47 | 0.99 |
|           | $\text{Mg}^{2+}$/gapped DNA | 0.24 | 0.63 | 5.02 | 0.74 | 1.42 | 3.43 | 1.11 |

* The substrate concentrations were 10 mM $\text{Mg}^{2+}$, single-nucleotide gapped DNA was in 2-fold excess relative to enzyme, and 50 $\mu$m dTTP.

$b$ $\tau_{av} = \sum_i n_i \tau_i / \sum_i n_i$, where $\sum_i n_i = 1.$
exhibited the smallest change (<2%) in the mean lifetime induced by substrate binding (Table II). Hence, the steady-state intensity losses are predominantly static. The decay-associated spectra for L287W in the absence of substrates are shown in Fig. 5. The two lifetime components of L287W are in solvent-exposed environments based on the emission maximum (350 nm). The longer lifetime component contributes about 59% to total fluorescence. The decay-associated spectra for L287W in the presence of substrates are similar to those without substrates (not shown) suggesting a similar Trp-287 microenvironment when the N-subdomain is open or closed.

**Time-resolved Fluorescence Anisotropy Decay**—Fluorescence anisotropy decays were collected for the F25W, L287W, and wild-type proteins at 340 nm in the presence and absence of substrates. The fitted parameters ($\beta_{\text{fast}}$, $\phi_{\text{fast}}$, $\beta_{\text{slow}}$, $\phi_{\text{slow}}$, and $\chi^2$) values are presented together with initial anisotropy ($r_0$) in Table III. In all cases, the anisotropy decays could be fit best by a sum of two exponentials. Single exponential fits gave more $\chi^2$ for all cases, and addition of a third exponential term led to no significant improvement in $\chi^2$.

For wild-type protein, the fast component of Trp-325 has a rotational correlation time of 0.8 ns contributing 23% to total depolarization. Substrate binding did not alter the fast component ($\beta_{\text{fast}}$ and $\phi_{\text{fast}}$) but increased the slower correlation time (27 and 37 ns in the absence and presence of substrates, respectively; see Table III). This increase in the longer correlation time is consistent with the binding of the single-nucleotide gapped DNA (14.45-kDa). Nucleotide (dTTP) binding did not cause changes in the rotational parameters.

For Trp-25, 30% of the depolarization originated from the fast component (0.78–0.98 ns) in the absence of substrates. In contrast to the significant effects that Mg$^{2+}$ binding had on the emission spectra (Fig. 3C), metal binding had little effect on the nanosecond mobility of Trp-25 suggesting that this divalent cation only produces static quenching without affecting the dynamics of Trp-25. The longer rotational correlation times are much smaller than those expected for rigid motion of a 39-kDa protein alone (10.76 ns) or complexed with DNA (21.89 ns). It is possible to estimate the rotational correlation time of a rigid hydrated sphere from the Stokes-Einstein equation, shown in Equation 4, where $M_w$ is the molecular mass of F25W, $\nu$ is the calculated partial specific volume (0.739 cm$^3$/g of human pol β at 20 °C), $h$ is the degree of hydration (assuming 0.3 cm$^3$/g of protein), $\eta$ is the solution viscosity (~1.02 centipoise at 20 °C), $R$ is the gas constant (8.314 × J mol$^{-1}$ K$^{-1}$), and $T$ is the absolute temperature (293.15 K).

$$\phi_{\text{calc}} = M_w \nu (v + h) \eta RT \quad \text{(Eq. 4)}$$

From Equation 4, the predicted rotational correlation time for pol β is ~16.3 ns. Further, the asymmetric nature of pol β can only yield correlation times that exceed those for a sphere of the same size (29). Hence, the lower $\phi_{\text{slow}}$ in F25W (11.34 ns) indicates segmental motion of the amino-terminal lyase domain. Most importantly, DNA binding increased $\phi_{\text{slow}}$ and decreased the depolarization amplitude of the fast component ($\beta_{\text{fast}}$) suggesting that both the fast (local) movement of Trp-25 and the 8-kDa lyase domain are restricted in the presence of DNA and are not restricted further upon nucleotide binding.

The anisotropy decay of unliganded Trp-287 can be best described with two rotational correlation times of 0.93 and 21.57 ns (Table III). However, in contrast to tryptophan in wild-type enzyme or F25W, the contribution from the fast component ($\beta_{\text{fast}}$) is noticeably higher (44%), indicating a significant angular contribution from the fast component to the total depolarization. The fast correlation time for L287W could be attributed to segmental motion involving several residues in α-helix N. Alternatively, it may represent an unusually slow local motion of Trp-287; i.e., the internal rotation of the indole ring about the $C_{\text{C}a}$-$C_{\beta}$ and $C_{\text{C}a}$-$C_{\beta}$ bonds corresponding to the $\chi_1$ and $\chi_2$ dihedral angles of the tryptophan side chain (28, 41). The rotational motion of tryptophan side chains in intact proteins typically yield correlation times of 50–400 ps (42–47). Because some of these determinations

![Fig. 5. Decay-associated spectra for L287W pol β. Spectra were determined in 50 mM Tris-HCl, pH 7.4, and 0.1 mM KCl at 20 °C in the absence of substrates with excitation at 295 nm.](image)

**Table III**

| pol β | Substrates | $\beta_{\text{fast}}$ | $\phi_{\text{fast}}$ | $\beta_{\text{slow}}$ | $\phi_{\text{slow}}$ | $r_0$ | $\chi^2$ |
|-------|------------|-----------------------|----------------------|----------------------|----------------------|------|---------|
| Wild-type | Mg$^{2+}$ | 0.06 | 0.77 | 0.17 | 24.86 | 0.23 | 1.20 |
| | Mg$^{2+}$, DNA | 0.06 | 0.62 | 0.20 | 27.26 | 0.26 | 1.10 |
| F25W | Mg$^{2+}$, DNA, dTTP | 0.06 | 0.74 | 0.22 | 37.54 | 0.28 | 1.25 |
| | Mg$^{2+}$ | 0.08 | 0.98 | 0.19 | 11.34 | 0.27 | 1.08 |
| | Mg$^{2+}$, DNA | 0.08 | 0.78 | 0.19 | 10.76 | 0.28 | 1.23 |
| L287W | Mg$^{2+}$, DNA, dTTP | 0.04 | 0.78 | 0.23 | 24.64 | 0.27 | 1.19 |
| | Mg$^{2+}$ | 0.12 | 0.93 | 0.15 | 21.57 | 0.27 | 1.10 |
| | Mg$^{2+}$, DNA | 0.12 | 0.99 | 0.13 | 20.97 | 0.25 | 1.25 |
| | Mg$^{2+}$, DNA, dTTP | 0.05 | 0.87 | 0.20 | 35.90 | 0.25 | 1.19 |
| | Mg$^{2+}$, DNA, dCTP | 0.13 | 0.98 | 0.16 | 32.45 | 0.29 | 0.96 |

* The substrate concentrations were 10 mM Mg$^{2+}$, single-nucleotide gapped DNA was in 2-fold excess relative to enzyme, and 50 µM dNTP (correct, dTTP, or incorrect, dCTP).

$^a$ $r_0 = \beta_{\text{fast}} + \beta_{\text{slow}}$. 

**Legend:**

Table III: Time-resolved fluorescence anisotropy decay parameters for wild-type and single tryptophan mutants of pol β.
probably include averaging with slower segmental contributions, we consider 400 ps an upper estimate for this process. Thus, indole side-chain motions are too fast to correspond to a correlation time of 0.93 ns. This correlation time is more reasonably assigned to \( \alpha \)-helix N tilting in the solitary enzyme.

In the presence of \( \text{Mg}^{2+} \) and DNA, the slower correlation time increased to \( \approx 33 \text{ ns} \) because of DNA binding. Most interesting, further addition of the correct nucleotide, dTTP, resulted in a dramatic decrease in the amplitude of the fast component (44 to 20%) with a minor decrease in the correlation time (0.87 ns). If the short correlation time reflected local side chain rotation only, we would expect the amplitude to remain stable. This is reasonable when we consider that other indicators of the local tryptophan environment indicate that dTTP binding has no effect on fluorescence quenching (Table I), emission maximum (Fig. 3D), and lifetime parameters (Table II). Further, crystallographic structures suggest that Leu-287 would not be affected (solvent exposure) by nucleotide binding (6). The significant reduction of angular freedom induced by correct nucleotide binding can only be observed on the tryptophan located on \( \alpha \)-helix N (L287W) that interacts with the nascent base pair in the closed conformation. Because the conformational transitions of pol \( \beta \) are benchmarked with crystallographic structures (6), our data suggest that the reduced motion of Trp-287 represents rigidity of \( \alpha \)-helix N in the vicinity of the nascent base pair subsequent to closing of the N-subdomain. In contrast to the results observed for binding the correct nucleotide, the binding of the incorrect nucleotide, dCTP, did not result in a restrained Trp-287 mobility (Table III). This may primarily be because of the fact that DNA polymerases generally bind the incorrect nucleotide with a lower affinity than the correct nucleotide so that the ternary complex with an incorrect nucleotide is not populated significantly.

Although the binding affinity for the incorrect nucleotide (dCTP) is increased about 2-fold for the L287W mutant relative to wild-type enzyme, the mutant also binds the incorrect nucleotide with low affinity (\( K_{D} = 360 \pm 40 \text{ nM} \)). To avoid inner filter effects, we are unable to use higher concentrations of the incorrect nucleotide. The dynamics of Trp-287 in binary (DNA) and ternary (DNA, dNTP) pol \( \beta \) complexes are illustrated in Fig. 6.

**DISCUSSION**

Fluorescence characterization of structure-based site-specific introduction of tryptophan can be an instructive approach in investigating not only its microenvironment but also the segmental dynamics of its structural support. In this study, we have used the wild-type protein with its lone tryptophan (Trp-25) and two engineered tryptophan mutants (F25W and L287W) where Trp-325 had been altered to alanine (W325A) (Fig. 1, A and B). These tryptophan residues were used to probe the effects of substrate binding on the lyase (Trp-25) and polymerase (Trp-287 and Trp-325) domains.

With regard to the amino-terminal lyase domain, \( \text{Mg}^{2+} \) significantly quenched Trp-25 (25%; see Fig. 3C). The other tryptophan probes also sensed metal binding to a smaller extent (<8%). Despite the metal-induced steady-state fluorescence quenching observed for Trp-25, metal binding had almost no effect on Trp-25 flexibility (Table III). The 8-kDa lyase domain is connected to the 31-kDa polymerase domain through a protease-sensitive hinge (Fig. 1B). The anisotropy decay parameters for Trp-25 strongly support the conclusion that the lyase domain is highly mobile in solution as evidenced from the much faster rotational correlation time (11 ns) than expected for an ideal sphere (≈16 ns). To define the contribution of the lyase domain, we attempted to collect additional data but failed to...
uncover a single segmental correlation time corresponding to that anticipated for an isolated spherical lyase domain motion (3 ns). We believe that $\phi_{\text{slow}}$ here includes contributions averaged from both the lyase domain and intact enzyme. In this view, the 8-kDa lyase domain motion in solution probably occurs on a time scale intermediate between 3 and 8 ns.

To probe dynamics within the polymerase domain, we studied the tryptophan in wild-type enzyme (Trp-325) and a tryptophan engineered into $\alpha$-helix N of the N-subdomain (Trp-287). Crystalllographic studies indicate that Trp-325 is located on the surface, in a loop after $\alpha$-helix O (Fig. 1B), with one side of the indole ring making van der Waals contact with His-285 and Ile-232 and the other side exposed to solvent (solvent-accessible area $= 79 \text{Å}^2$). This is consistent with partial shielding of this tryptophan from acrylamide quenching. Leu-287 is situated near the nascent base pair and is more solvent-exposed than Phe-25 or Trp-325. Modeling tryptophan residues at these positions, by selecting the best tryptophan rotamer from a library, suggests that the solvent-accessible surface of Trp-287 ($\sim 160 \text{Å}^2$) would be much greater than Trp-25 ($\sim 42 \text{Å}^2$) or Trp-325. Because of the more exposed environment of Trp-287, the indole ring is expected to experience more rapid ring flipping motions as compared with Trp-325. In this case, the rapid anisotropy decay of Trp-287 is far too long (0.93 ns) to correspond to isolated indole ring flipping ($<300$ ns). Based on Stern-Volmer quenching data, the binding of the correct nucleotide does not influence the accessibility of Trp-287. If the rapid 1-ns component simply represented fast indole ring rotation for this tryptophan, then it would be expected that the amplitude of the short component would remain the same as in the unliganded protein, because the microenvironment of this tryptophan is not influenced by nucleotide binding (see Figs. 2–4 and Tables I–III). The significant reduction in the amplitude of the rapid anisotropy decay of Trp-287 upon nucleotide binding (14 to 22%) indicates an indirect restriction of this tryptophan. An indirect restriction of Trp-287 could originate from segmental motion in $\alpha$-helix N that is constrained upon nucleotide binding. After formation of the closed conformation, $\alpha$-helix N interacts with the nascent base pair, and these additional interactions are expected to limit segmental motion in this helix.

The flexibility of $\alpha$-helix N may play important roles in DNA synthesis. The analysis described above indicates that there is a strong reduction of the amplitude of the $\alpha$-helix N angular motion in the closed conformation after binding a nucleotide. The cone angles for the motion of $\alpha$-helix N in the open and closed states are calculated to be 39 and 21°, respectively (Fig. 6). The larger angular motion of $\alpha$-helix N in the open state may facilitate selection of the correct incoming nucleotide from the pool of structurally similar competing molecules. When a correct nucleotide binds in the pol $\beta$ active site and forms Watson-Crick hydrogen bonds with the templating base, $\alpha$-helix N can probe the geometry of the nascent base pair. Optimum interactions (van der Waals and electrostatic) are expected to occur between the correct nascent nucleotide and $\alpha$-helix N thereby restricting segmental motions of this critical helix. The closed conformation is expected to signal correct geometry to initiate further critical conformational changes and/or chemistry (10, 11). In contrast, if an incorrect nucleotide binds to the pol $\beta$ active site, then the interactions with $\alpha$-helix N and the nascent base pair (templating nucleotide and incorrect incoming nucleotide) are not optimum, and the nucleotide can diffuse rapidly out of the active site before chemistry can occur. An illustration of these events and their postulated relationship to polymerase “selectivity” is presented in Fig. 7.

The restricted motion of $\alpha$-helix N observed upon binding a correct nucleotide may also play a role in the motor function of the polymerase to facilitate directional translocation. DNA polymerase translocation (5’ $\rightarrow$ 3’) along the growing DNA duplex during processive DNA synthesis is kinetically assumed to be rapid; i.e., translocation is much more rapid than nucleotide insertion. Additionally, the importance of these motions to correct nucleotide selection could imply that these events are coupled so that translocation would not occur until the correct nucleotide is inserted. The rapid segmental motions observed here are much more rapid than the kinetic step that limits nucleotide insertion, 3s $\rightarrow$ 1s (31), indicating that this segmental motion does not represent the rate-limiting step for DNA synthesis (10). Indirect experimental approaches had predicted previously (8, 10–12) that the open to closed structural transition observed with $\alpha$-helix N upon binding a correct nucleotide was too rapid to be rate-limiting for nucleotide insertion.

The anisotropy of Trp-25 also indicate a loss in angular extent or “freezing” of segmental mobility in the lyase 8-kDa domain. Interestingly, unlike L287W, this loss of segmental motion occurs immediately upon DNA binding (Table III) and is not restricted further upon nucleotide binding. Overall, the native Trp-325 is unperturbed by substrate binding, whereas our site-selected reporters (Trp-25 and Trp-287) yielded information about local segmental motions that responded to catalytic cycle-dependent conformational changes, albeit in very different ways. Site-directed reporters like tryptophan and analogs have the potential to detect flexibility changes with functional importance in complexes.

Acknowledgment—We are grateful to Jennifer Myers for help with preparation of this manuscript.

REFERENCES

1. Beard, W. A., and Wilson, S. H. (2000) Mutat. Res. 460, 231–244
2. Holm, L., and Sander, C. (1990) Trends Biochem. Sci. 20, 345–347
3. Oliis, D. L., Brick, P., Hamko, R., Xuong, N. G., and Steitz, T. A. (1985) Nature 313, 762–766
4. Sawaya, M. R., Pelletier, H., Kumar, A., Wilson, S. H., and Kraut, J. (1994) Science 264, 1930–1935
5. Pelletier, H., Sawaya, M. R., Wolfe, W., Wilson, S. H., and Kraut, J. (1996) Biochemistry 35, 12742–12761
6. Sawaya, M. R., Prasad, P., Wilson, S. H., Kraut, J., and Pelletier, H. (1997) Biochemistry 36, 11205–11215
7. Pelletier, H., Sawaya, M. R., Kumar, A., Wilson, S. H., and Kraut, J. (1994) Science 264, 1891–1893
8. Awolt, J. W., Gong, W., Zheng, X., Shwaltzer, A. K., Liu, J., Dunlap, C. A., Lin, Z., Paxson, C., Tsai, M.-D., and Chan, M. K. (2001) Biochemistry 40, 5368–5375
9. Beard, W. A., and Wilson, S. H. (1998) Chem. Biol. 5, R7–R13
10. Vande Berg, B. J., Beard, W. A., and Wilson, S. H. (2001) J. Biol. Chem. 276, 3408–3416
11. Yang, L., Beard, W. A., Wilson, S. H., Brydey, S., and Schlack, T. (2002) J. Mol. Biol. 317, 679–699
12. Dunlap, C. A., and Tsai, M.-D. (2002) Biochemistry 41, 11226–11235
13. Singhal, R. K., and Wilson, S. H. (1993) J. Biol. Chem. 268, 15906–15911
14. Kumar, A., Abbott, J., Karwuya, E. M., and Wilson, S. H. (1999) Biochemistry 38, 7156–7159
15. Wang, M. D., Schnitzer, M. J., Yin, H., Landick, R., Gelles, J., and Block, S. M. (1998) Science 283, 902–907
16. Yin, H., Artsimovitch, I., Landick, R., and Gelles, J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13124–13129
17. Brokaw, C. J. (2000) Cell Motil. Cytoskeleton 47, 108–119
18. Brokaw, C. J. (2001) Biophys. J. 81, 1333–1344
19. Spolar, R. S., and Record, M. T. (1994) Science 263, 777–784
20. Shen, F., Triezenberg, S. J., Hensley, P., Porter, D., and Knutson, J. R. (1996) J. Biol. Chem. 271, 4827–4837
21. Kussie, P. H., Gorina, S., Marechal, E., Elenbaas, B., Moreau, J., Levine, A. J., and Pavletich, N. P. (1996) Science 274, 948–953
22. Gangul, M., Cox, S., Lew, J., Clifford, T., Garrod, M. A., Ashchabar, M., Taylor, S. S., and Johnson, D. A. (1998) Biochemistry 37, 13728–13735
23. Tanford, C. (1967) Physical Chemistry of Macromolecules, pp. 432–456, John Wiley & Sons, Inc., New York
24. Wahl, P., Pauletto, J., and Le Pecq, J. B. (1973) Proc. Natl. Acad. Sci. U. S. A. 65, 417–421
25. Beard, W. A., and Anderson, R. S. (1989) Biochemistry 8, 361–371
26. Munro, I., Pech, I., and Stryer, L. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 56–60
27. Yguerabide, J. (1972) Methods Enzymol. 26, 498–576
28. Tether, Kazu, O., Priyai, O., and Knutson, J. R. (2000) Biochemistry 39, 1879–1889
29. Kim, S. J., Lewis, M. S., Knutson, J. R., Porter, D. K., Kumar, A., and Wilson,
30. Beard, W. A., and Wilson, S. H. (1995) Methods Enzymol. 262, 98–107
31. Beard, W. A., Shuck, D. D., Yang, X.-P., DeLauder, S. F., and Wilson, S. H. (2002) J. Biol. Chem. 277, 8235–8242
32. Casas-Finet, J. R., Karpel, R. L., Maki, A. H., Kumar, A., and Wilson, S. H. (1991) J. Mol. Biol. 221, 693–709
33. Knutson, J. R., Walbridge, D. G., and Brand, L. (1982) Biochemistry 21, 4671–4679
34. Brand, L., Knutson, J. R., Davenport, L., Beechem, J. M., Dale, R. E., Walbridge, D. G., and Kowalczyk, A. A. (1985) in Spectroscopy and the Dynamics of Molecular Biological Systems (Dale, R. E., ed) Academic Press, London
35. Chuang, T. J., and Eisenthal, K. B. (1972) J. Chem. Phys. 57, 5994–5997
36. Prasad, R., Casas-Finet, J. R., Karpel, R. L., and Wilson, S. H. (1994) in Techniques in Protein Chemistry V (Crabb, J. W., ed) pp. 359–369, Academic Press, San Diego, CA
37. Liu, D. J., Prasad, R., Wilson, S. H., Derose, E. F., and Mullen, G. P. (1996) Biochemistry 35, 6188–6200
38. Prasad, R., Beard, W. A., Chyan, J. Y., Maciejewski, M. W., Mullen, G. P., and Wilson, S. H. (1998) J. Biol. Chem. 273, 11121–11126
39. Deterding, L. J., Prasad, R., Mullen, G. P., Wilson, S. H., and Tomer, K. B. (2000) J. Biol. Chem. 275, 10463–10471
40. Pelletier, H., and Sawaya, M. R. (1996) Biochemistry 35, 12778–12787
41. Semisotnov, G., Zikherman, K., Kasatkin, S., Pritsyan, O., and Anufrieva, E. (1981) Biopolymers 20, 2287–2309
42. She, M., Dong, W. J., Umeda, P. K., and Cheung, H. C. (1997) Biophys. J. 73, 1042–1055
43. Sterch, E. M., Grinstead, J. S., Campbell, A. P., Daggett, V., and Atkins, W. M. (1999) Biochemistry 38, 5065–5075
44. Kungl, A. J., Visser, N. V., van Hoek, A., Visser, A. J., Billich, A., Schilk, A., Gstacl, H., and Auer, M. (1998) Biochemistry 37, 2778–2786
45. Nishimoto, E., Yamashita, S., Szabo, A. G., and Imoto, T. (1998) Biochemistry 37, 5599–5607
46. Kemple, M. D., Buckley, P., Yuan, P., and Prendergast, F. G. (1997) Biochemistry 36, 1678–1688
47. Clayton, A. H., and Sawyer, W. H. (2000) Biophys. J. 79, 1066–1073
48. Davies, J. F., Almassy, R. J., Hostomelska, Z., Ferre, R. A., and Hostomsky, Z. (1994) Cell 76, 1123–1133