Monitoring DNA Dynamics Using Spin-Labels with Different Independent Mobilities†

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ABSTRACT: The electron paramagnetic resonance (EPR) spectra of spin-labeled DNA duplexes, both bound to DEAE-Sephadex and free in solution, have been analyzed. The nitroxide spin-labels are covalently linked to a deoxyuridine residue using either a monoacetylene or diacetylene tether. This difference in tether length produces a dramatic difference in the independent mobility of the nitroxide relative to the DNA. In the case of the monoacetylene tether, the motion of the nitroxide has previously been shown to be tightly coupled to that of the DNA duplex. With the diacetylene tether, there is considerable independent motion of the probe. The diacetylene tether is intended to minimize the possibility of the nitroxide producing a perturbation of the dynamics of DNA. It is demonstrated here that, when coupled via the diacetylene tether, the nitroxide undergoes a rapid uniaxial rotation about the tether. A detailed analysis of the EPR spectrum of duplex DNA in solution, spin-labeled using the diacetylene tether, demonstrates that the motion of the nitroxide can be modeled in terms of this independent uniaxial rotation together with motion of the DNA which is consistent with the global tumbling of the duplex. As was previously found using the monoacetylene tether, there is no evidence of rapid, large-amplitude motions of the base pair in the EPR spectrum of a nitroxide coupled to duplex DNA via the diacetylene tether. This result confirms the small amplitudes of internal motion, local and collective, previously observed in duplex DNA with the monoacetylene-tethered nitroxide.

The rigidity of duplex DNA may play a large role in controlling the interaction of DNA with histones and with proteins involved in transcription and translation (Hogan & Austin, 1987; Koudelka et al., 1988; Fujimoto & Schurr, 1990; Schultz et al., 1991). Knowledge of the internal dynamics of DNA is important to give a complete understanding of the factors regulating expression of the genetic code. A number of recent theoretical and experimental results have led to increased understanding of the internal dynamics of DNA. Among these are the development of new spin-labeled probes which can be site-specifically incorporated into DNA and the application of these probes in electron paramagnetic resonance (EPR) studies of DNA dynamics. Any probe, such as a nitroxide spin-label, must be evaluated in terms of the extent to which it creates structural or dynamic perturbations and the extent to which the probe faithfully reports the dynamics of the DNA. These concerns will be addressed in this report in an attempt to explain the discrepancy in the dynamics reported by two different classes of spin-labels for DNA.

Three general strategies have been used to incorporate nitroxide spin-labels into duplex DNA for the purpose of studying DNA dynamics using EPR spectroscopy. Early studies employed spin-labeled versions of intercalators (Robinson et al., 1980a,b; Hurley et al., 1982). More recently, Bobst and co-workers have synthesized a wide variety of spin-labeled nucleosides incorporated into duplex DNA. All are characterized by a relatively flexible covalent tether between the nitroxide and the labeled base (Strobel et al., 1990; Kao & Bobst, 1985; Bobst et al., 1984). Bobst and co-workers have systematically varied the tether length and interpreted their results in terms of a "base disk" model. The motion of the nitroxide is modeled as axial rotational diffusion, with the symmetry axis of the diffusion tensor defined as being along the tether axis. Rotation about this axis is characterized by a time, τ_{para}, which is dependent on the tether length and ascribed to the independent motion of the nitroxide. Rotation about the two orthogonal axes perpendicular to the tether axis, characterized by a single parameter τ_{perp}, has been found to be independent of tether length (Bobst et al., 1984). Bobst and co-workers have consistently obtained a value of 4 ns for τ_{perp} in duplex DNA, and the lack of a dependence of τ_{perp} on the duplex length has led them to conclude that τ_{perp} is monitoring the local motion of the base to which the spin label is attached (Bobst et al., 1988). Implicit in the base disk model is the assumption that this local base motion is unrestricted.

Using a third approach, Spaltenstein et al. have employed a relatively rigid two-atom monoacetylenic tether to link a nitroxide-bearing ring to deoxyuridine. This spin-labeled residue, which is referred to as T* because it replaces thymidine in DNA, can then be incorporated into a desired oligomer using standard automated DNA synthesis procedures (Spaltenstein et al., 1988, 1989a,b). The nature of the tether ensures that there is only one degree of motional

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freedom of the probe relative to the base and thus minimizes independent probe motion. The EPR spectrum of a T*-labeled DNA duplex is quite sensitive to duplex length, and this length dependence has been successfully modeled in terms of the uniform rotational diffusion of the entire duplex. In the model of Hustedt et al. (1993a), all internal motions of the spin-labeled DNA duplex, including the collective motions of the DNA base pairs, the local motion of the labeled base, and the local motion of the label, are treated as being rapid on the EPR time scale, which is defined by subnanosecond correlation times at X-band. The total mean-squared amplitude of all internal motion contains both a length-dependent and a length-independent term, both of which are temperature-dependent. The length-independent amplitude was found to be small, approximately 10° at 20 °C, and the length-dependent amplitude could be fit to a $N^{1/2}$ dependence, where N is the number of base pairs, in accord with the theory of Schurr and co-workers (Wu et al., 1987; Wilcoxon & Schurr, 1983; Allison & Schurr, 1979).

The relatively small amplitude of internal motions observed with T* contrasts with the results of Bobst and co-workers, where the EPR spectra are clearly dominated by rapid, large-amplitude, local motion. The question then arises as to whether the flexible tethers employed by Bobst and co-workers (Strobel et al., 1990; Kao & Bobst, 1985; Bobst et al., 1984) lead to an overestimation of the local base motion in duplex DNA or whether the relatively rigid tether of T* in some way restricts the local base motion. Space-filling models of T* incorporated into duplex B-DNA suggest that the nitroxide resides in the major groove where steric interactions restrict the motion of the nitroxide about the acetylenic tether without disrupting the DNA structure. The observed sensitivity of the EPR spectra of T*-labeled DNA to duplex length indicates that there is no large-amplitude local nitroxide motion. This was confirmed in a detailed quantitative analysis (Hustedt et al., 1993a). The lack of any large structural disruption was confirmed by the observation that the UV-monitored thermal denaturation profile and the circular dichroism spectrum of the self-complementary sequence 5′-d[CGCGAAT(T*)CGCG] were similar to those of the unlabeled sequence (Spaltenstein et al., 1988). The presence of the T* spin-label appeared to cause a slight increase in the melting temperature, T_m. A similar increase in T_m was obtained by Froehler et al. (1992) using a C-5 propyne derivative of deoxyuridine in studies of DNA/RNA duplex melting. At present, it is unclear whether these observed increases in T_m are due to entropic or enthalpic effects. If the apparent increase in T_m due to the presence of an acetylenic moiety at C-5 (on the order of 2 °C) were due solely to an increase in base stacking energy, it remains unclear what effect this might have on the internal dynamics of the duplex, particularly the collective modes. The additional possibility remains that the steric hindrance of the nitroxide motion may also restrict the motion of the base to which it is attached. This would cause the T* probe to underestimate the internal motion in native DNA. To test this possibility, an analogue of T* using a longer diacetylenic tether was synthesized by Kirchner et al. (1990) and incorporated into duplex DNA. The longer tether of T** is intended to allow for unrestricted rotation of the nitroxide about the tether axis and to minimize the possibility of the probe hindering the internal dynamics of DNA. It should also be noted that T** appears to have a much smaller

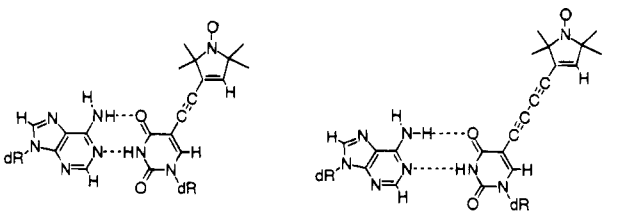

FIGURE 1: Structures of T* (left) and T** (right) hydrogen-bonded to adenine as in duplex DNA.

influence on the thermal denaturation profile of 5′-d[CGC-GAAT(T**)CGCG] than T* (Kirchner, 1991). This result is consistent with the notion that T** should produce even less of a perturbation than whatever small influence T* may have on the structure or dynamics of duplex DNA.

In this report, a detailed analysis of the EPR spectra of T** incorporated into duplex DNA is presented. This analysis is performed to determine whether or not there are additional internal dynamics not observed using T*. The EPR spectrum of a T*-labeled 48-base-pair duplex bound to DEAE-Sephadex is used as a reference point in this study. As is evident from this spectrum, binding to DEAE-Sephadex eliminates all global tumbling of the duplex. The EPR spectra of duplex 5′-d[CGCGAAT(T*)CGCG] and 5′-d[CGCGAAT(T**)CGCG], both bound to DEAE-Sephadex and free in solution, are then analyzed. The EPR spectrum of 5′-d[CGCGAAT(T**)CGCG] bound to DEAE-Sephadex can be fit by treating the motion of the nitroxide as a rapid uniaxial rotation about the diacetylenic tether. The best-fit parameters obtained are consistent with the structure of T**. The EPR spectrum of the T**-labeled 12-base-pair duplex free in solution can be simulated by considering the rapid independent rotation of the nitroxide and the global tumbling of the duplex. These two motional modes alone are sufficient to explain the data. There is no need to invoke additional, large-amplitude local base motion.

MATERIALS AND METHODS

The spin-labels, T* and T** (see Figure 1), were prepared as described by Spaltenstein et al. (1989b) and Kirchner et al. (1990), respectively, using the naturally abundant [^{14}N,H_{13}]-nitroxide. Oligomers were prepared on an Applied Biosystems 6800 DNA synthesizer. After synthesis, all oligomers were sized on denaturing polyacrylamide electrophoretic gels and purified on a Sephadex column. The sample buffer in all cases was 0.01 M phosphate adjusted to pH 7, 0.1 mM EDTA, and 0.1 M NaCl. The samples were placed in 50-μL capillary tubes. The EPR spectra were recorded digitally on an EPR spectrometer as previously described (Mailer et al., 1985). All spectra were measured at 0 °C. The DEAE-Sephadex was prepared by incubation for 3 h at 37 °C and then 18 h at room temperature in 1 M NaCl, 0.1 M phosphate, and 1 mM EDTA. The suspension was then centrifuged, the supernatant was removed, and the pellet was resuspended in the sample buffer 5 times.

The nonlinear least-squares fits to the data were performed as recently described by Hustedt et al. (1993b) with the following modifications. For the case of a nitroxide undergoing infinitely rapid uniaxial rotation, the EPR spectrum can be fit using motionally averaged **A**- and **g**-tensors as given by Van et al. (1974):

$$\langle A_{xx}\rangle = {}^1/_2[A_{zz}(1-\cos^2\theta) + A_{yy}(1+\cos^2\theta) \quad (1)$$

$$+ (A_{xx} - A_{yy})(1 - \sin^2\theta\cos^2\psi)]$$

$$\langle A_{yy}\rangle = \langle A_{xx}\rangle$$

$$\langle A_{zz}\rangle = A_{zz}\cos^2\theta + A_{yy}(1-\cos^2\theta) + (A_{xx} - A_{yy})\sin^2\theta\cos^2\psi$$

with similar equations for the **g**-tensor elements. The angles θ and ψ determine the orientation of the rotation axis relative to the nitroxide reference frame (see Figure 2A). The powder pattern fitting procedure described by Hustedt et al. (1993b) has been modified such that, for a given set of rigid-limit **A**- and **g**-tensors, a spectrum can be fit using the motionally averaged **A**- and **g**-tensors by adjusting the angles θ and ψ. For fitting linear continuous-wave EPR spectra to isotropic or axial rotational diffusional models, the rapid simulation routines developed by Freed and co-workers (Schneider & Freed, 1989) have been incorporated into the nonlinear least-squares analysis program. A similar approach has been reported by Shin and Freed (1989). In this case, the fitting parameters are the characteristic rotation times, either $\tau = 1/6D$ for isotropic (Brownian) diffusion or $\tau_{para} = 1/6D_{para}$ and $\tau_{perp} = 1/6D_{perp}$ for axial rotational diffusion, an intrinsic line width parameter Γ [ω_0^{int} in the notation of Schneider and Freed (1989)], and an angle which determines the orientation of the rotational diffusion tensor relative to the nitroxide **A**- and **g**-tensors. The program developed by Freed and co-workers uses only a single angle to determine this orientation. Normally, this is the angle θ of Figure 2A, which determines the orientation of the unique axis of an axial or uniaxial rotational diffusion tensor in the $x-z$ plane of the nitroxide (ψ is fixed to 0°). A circular permutation of the elements of the **A**- and **g**-tensors can be performed such that the z-axis of the nitroxide is fixed to be perpendicular to the unique rotation axis ($\theta = 90°$) and the angle fit is ψ. To assess the quality of a fit, the correlation coefficient between the data and the fit, R, was calculated as described by Hustedt et al. (1993a).

RESULTS

Figure 3 shows the EPR spectrum of a T*-labeled 48-base-pair duplex bound to DEAE-Sephadex. The EPR spectrum of a 24-base-pair duplex, with the same sequence as the core of the 48-base-pair duplex, also bound to DEAE-Sephadex (data not shown) is virtually identical to that of the 48-base-pair duplex. In contrast, a dramatic difference in the EPR spectra of the same 24- and 48-base-pair duplexes is seen for the duplexes free in solution due to the difference in their global rotational diffusion (Hustedt et al., 1993a). It is evident that binding to DEAE-Sephadex eliminates the uniform rotational motion of the DNA. The effect of binding to DEAE-Sephadex on the collective and local motions of the DNA and the independent probe motion is unknown. However, it is clear from the lack of significant motional broadening in the spectrum shown in Figure 3 that, as is seen from T*-labeled duplexes in solution, the amplitudes of these internal motions are not large. In Figure 3, the EPR spectrum of the 48-base-pair duplex bound to DEAE-Sephadex has been fit to a powder pattern to determine the best-fit **A**- and **g**-tensors (Hustedt et al., 1993b) which are used below as the effective rigid-limit **A**- and **g**-tensors.

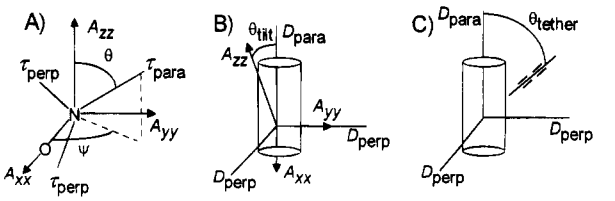

FIGURE 2: (A) Axial rotational diffusion tensor of a nitroxide spin-label oriented in the frame of the nitroxide. (B) Orientation of the nitroxide axis system with respect to the rotational diffusion tensor of a DNA helix. The helix axis is aligned with the unique axis of the diffusion tensor (D_{para}). (C) Orientation of the tether axis with respect to the DNA helix axis.

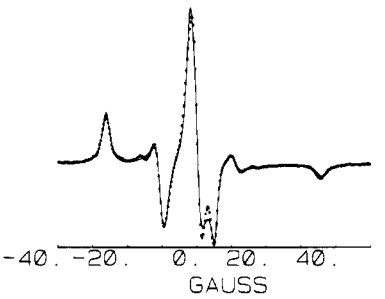

FIGURE 3: EPR spectrum (dots) of 5′-d[CCCGATCAGTCTGG-CGCGGATAAT(T*)CGTGCCGGCGTATCCTGCCAACG] combined with ≈25% excess of the unlabeled complementary strand to form duplex DNA and bound to DEAE-Sephadex. The powder pattern fit (solid line) determined the **A**- and **g**-tensor elements (g_{xx} = 2.00735, g_{yy} = 2.00607, g_{zz} = 2.00308, A_{xx} = 7.16 G, A_{yy} = 10.98 G, and A_{zz} = 31.18 G) and the manifold- (m_I-) dependent line widths [$\Gamma(m_I = +1)$ = 1.37 G, $\Gamma(m_I = 0)$ = 1.31 G, and $\Gamma(m_I = -1)$ = 2.16 G). R = 0.9941.

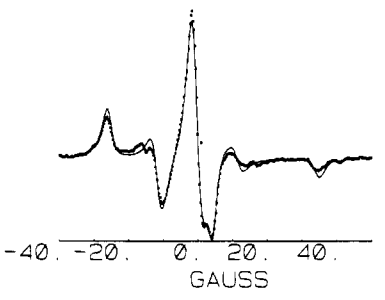

FIGURE 4: EPR spectrum (dots) of duplex 5′-d[CGCGAAT(T*)-CGCG] bound to DEAE-Sephadex, fit (solid line) to an isotropic rotational diffusion model using **A**- and **g**-tensors obtained in Figure 3. Best-fit isotropic rotational correlation time, τ = 177 ns, Γ = 1.55 G. R = 0.9928.

Figure 4 shows the EPR spectrum of the self-complementary sequence 5′-d[CGCGAAT(T*)CGCG], in the duplex form, bound to DEAE-Sephadex. In comparison to the EPR spectrum of the 48-base-pair duplex under the same conditions (Figure 3), the spectrum in Figure 4 shows a slight collapse of the high- and low-field z-turning points and a broadening of various spectral features, both of which are due to increased motion. The slightly increased motion seen for the 12-base-pair duplex relative to the 24- and 48-base-pair duplexes may be due to some sequence dependence in the internal motions of the DNA or probe or to less rigid binding of the shorter duplex to the DEAE-Sephadex, which would depend on the number of DEAE binding sites per length of DNA duplex. In Figure 4, the EPR spectrum of the 12-base-pair duplex bound to DEAE-Sephadex has been fit, using the **A**- and **g**-tensors determined in Figure 3,

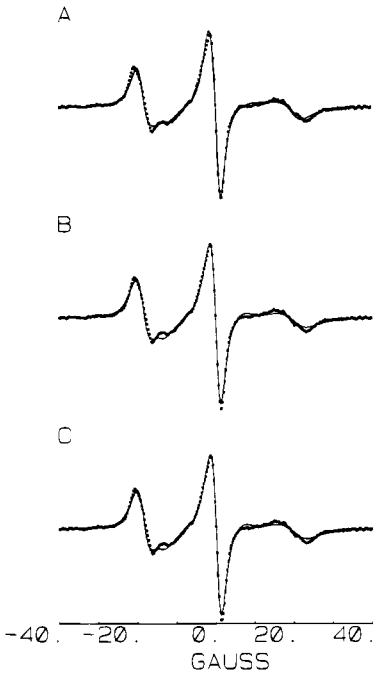

FIGURE 5: EPR spectrum of duplex 5′-d[CGCGAAT(T**)CGCG] bound to DEAE-Sephadex. The data (dots) are the same in all three panels; the different fits (solid lines) were obtained using the rigid-limit **A**- and **g**-tensors obtained in Figure 3. (A) Fit assuming infinitely fast uniaxial rotational diffusion. The best-fit orientation of the rotation axis relative to the **A**- and **g**-tensors is given by $\theta = 90°$ and $\psi = 52°$. Manifold-dependent line widths obtained are $\Gamma(m_I = +1) = 2.42$ G, $\Gamma(m_I = 0) = 1.73$ G, and $\Gamma(m_I = -1) = 4.21$ G. $R = 0.9929$. (B) Fit to an axial diffusion model (ψ fixed to 0°). Best-fit parameters are $\tau_{para} = 1.7$ ns, $\tau_{perp} = 28$ ns, $\theta = 86°$, and $\Gamma = 0.42$ G. $R = 0.9933$. (C) Fit to an axial diffusion model (θ fixed to 90°). Best-fit parameters are $\tau_{para} = 1.7$ ns, $\tau_{perp} = 29$ ns, $\psi = 8°$, and $\Gamma = 0.42$ G. $R = 0.9933$.

assuming isotropic rotational diffusion. This is not considered to be a realistic model of the motion in this case, but the large value of the best-fit isotropic rotational correlation time obtained, $\tau = 177$ ns, demonstrates that the spin-label is nearly immobile on the linear EPR time scale.

The EPR spectrum of the self-complementary sequence 5′-d[CGCGAAT(T**)CGCG], in the duplex form, bound to DEAE-Sephadex is shown in Figure 5. As is seen from a comparison of this spectrum with that in Figure 4, there is a dramatic change in the motion of the nitroxide on going from the monoacetylene to the diacetylene tether as demonstrated by the dramatic reduction in the overall width of the spectrum. It is also evident from the asymmetry of the lines that the spectrum in Figure 5 is that of a spin-label undergoing rapid, highly anisotropic rotation. As an initial model of the independent probe motion in T**, we assume unrestricted, infinitely fast rotation about a single axis. The fit overlaid on the data in Figure 5A is a powder pattern for motionally averaged **A**- and **g**-tensors calculated from the rigid-limit **A**- and **g**-tensors obtained in Figure 3 using eq 1. The parameters varied in the least-squares fit are the angles θ and ψ and the Lorentzian line widths for the low-, center-, and high-field manifolds. The best-fit angles, $\theta = 90°$ and $\psi = 52°$, are consistent with rotation about the tether axis. The diacetylene tether is linear and in the plane of the five-membered nitroxide ring. Thus, the tether is perpendicular to the z-axis of the nitroxide ($\theta = 90°$) and roughly bisects the $x-y$ plane of the nitroxide (see Figure 1). The estimated angle between the tether axis and the N−O bond axis (the nitroxide x-axis) for similar nitroxides is 30−40° (Lajzerowicz-Bonneteau, 1976).

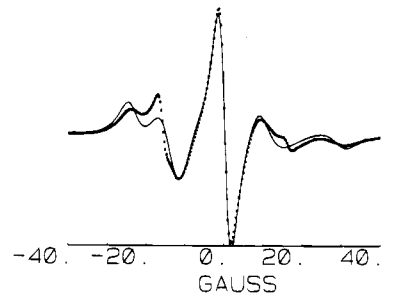

FIGURE 6: EPR spectrum (dots) of duplex 5′-d[CGCGAAT(T*)-CGCG] free in solution. The fit (solid line) was generated using the rigid-limit **A**- and **g**-tensors obtained in Figure 3 and assuming $^1/_6D_{perp} = 14.4$ ns, $^1/_6D_{para} = 7.5$ ns, and $\theta_{tilt} = 20°$ [see Hustedt et al. (1993a)]. Best-fit $\Gamma = 0.90$ G. $R = 0.9819$.

The EPR spectrum of 5′-d[CGCGAAT(T**)CGCG] bound to DEAE-Sephadex has also been fit using an axial rotational diffusion model. The parameters optimized are τ_{para} (which governs the rate of rotation about the tether axis), τ_{perp} (which governs the rate of rotation about the two perpendicular axes), and a single angle determining the orientation of the tether axis to the nitroxide **A**- and **g**-tensors. The fit shown in Figure 5B is obtained by varying the major tilt angle, θ, between the tether axis and the nitroxide z-axis. The optimal fit is obtained for $\tau_{para} = 1.7$ ns, $\tau_{perp} = 28$ ns, and $\theta = 86°$. The results are again consistent with a highly anisotropic rotational diffusion, with the fast rotation axis perpendicular to the nitroxide z-axis. Assuming this to be the case, the elements of the **A**- and **g**-tensors can be circularly permuted such that the nitroxide z-axis is constrained to remain perpendicular to the fast rotation axis ($\theta = 90°$). In the fit shown in Figure 5C, the angle varied is the minor tilt angle, ψ. There is no significant improvement in the fit, relative to that of Figure 5B, with the best-fit parameters $\tau_{para} = 1.7$ ns, $\tau_{perp} = 29$ ns, and $\psi = 8°$.

The fit to the EPR spectrum of 5′-d[CGCGAAT(T**)-CGCG] bound to DEAE-Sephadex which was obtained assuming infinitely rapid rotation about a single axis (Figure 5A) is quite good. The values of θ and ψ are entirely consistent with the structure of T** and rapid rotation about the diacetylene tether axis. The fits assuming a finite rate of rotation are also consistent with this model. The motion is highly anisotropic ($\tau_{perp}/\tau_{perp} \approx 18$), and the axis of rapid ($\tau_{para} \approx 1.7$ ns) rotation is perpendicular to the nitroxide z-axis ($\theta \approx 90°$). In this case, the value of $\psi \approx 8°$ is significantly different from what one would expect, $\psi \approx 35°$. However, the effect of ψ on EPR spectra is rather small at X-band (Schneider & Freed, 1989). In the absence of a known angle relating the tether axis to the DNA helix (see below), the motions governed by τ_{perp} cannot be related to the DNA frame.

Figure 6 shows the EPR spectrum of the 5′-d[CGCGAAT-(T*)CGCG] duplex free in buffer at 0 °C. The simulation overlaid on the data was obtained using the **A**- and **g**-tensors obtained in Figure 3 and diffusion coefficients obtained from the hydrodynamic theory of Tirado and de la Torre (1980) for a rigid cylinder assuming a duplex length of 12 × 3.4 Å and a hydrodynamic radius of 12 Å (Hustedt et al., 1993a). The angle between the z-axis of the nitroxide and the helix axis, θ_{tilt} (Figure 2B), has been taken to be 20° as determined by Hustedt et al. (1993a).

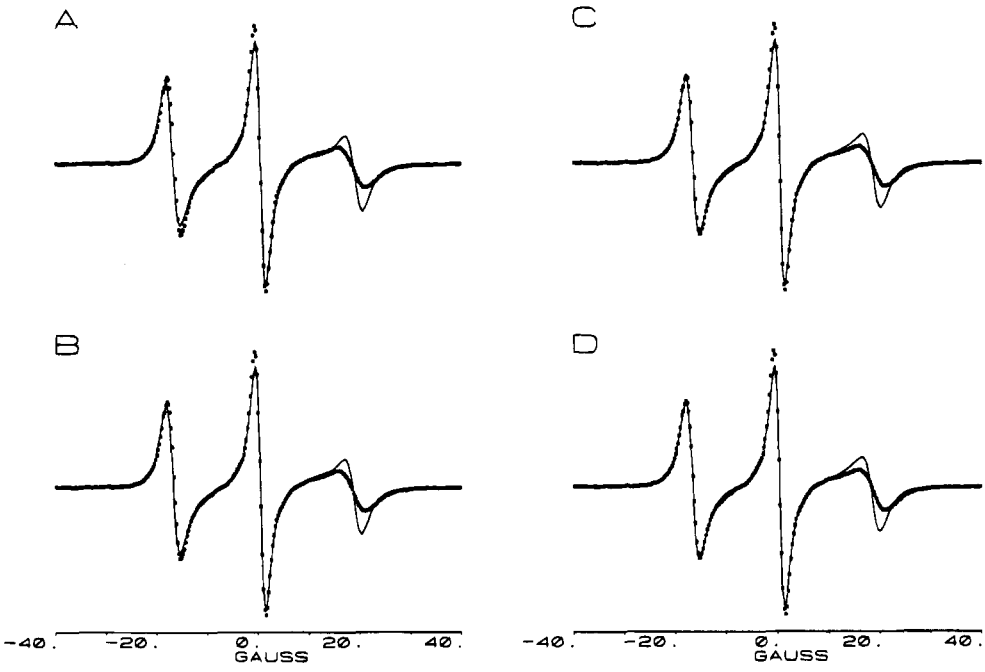

FIGURE 7: EPR spectrum of duplex 5′-d[CGCGAAT(T**)CGCG] free in solution. The data (dots) are the same in all four panels; the fits (solid lines) were generated using motionally averaged **A**- and **g**-tensors, calculated from eq 1 for $\theta = 90°$ and $\psi = 52°$ using the rigid-limit tensors obtained in Figure 3. The global tumbling was modeled assuming $1/6D_{perp} = 14.4$ ns, $1/6D_{para} = 7.5$ ns, and θ_{tether} = (A) 0° ($R = 0.9792$), (B) 30° ($R = 0.9824$), (C) 60° ($R = 0.9851$), or (D) 90° ($R = 0.9851$). In all cases, the best-fit Γ was ≈1.2 G.

Figure 7 shows the EPR spectrum of the 5′-d[CGCGAAT-(T**)CGCG] duplex free in buffer at $T = 0$ °C. The simulations were calculated using the **A**- and **g**-tensors, motionally averaged by rotation about the tether axis, which were obtained from 5′-d[CGCGAAT(T**)CGCG] bound to DEAE-Sephadex in Figure 5A. For rapid rotation about the tether axis, the z-axes of the motionally averaged, effective **A**- and **g**-tensors are aligned with the tether axis. Simulations are shown for various values of the angle between the tether axis and the helix axis ($\theta_{tether} = 0°$, 30°, 60°, and 90°) (Figure 2C). There is very little sensitivity to this angle and all of the simulations are reasonable fits to the data. The anisotropies of the nitroxide **A**- and **g**-tensors used in this simulation have been substantially reduced by the rapid motion about the diacetylenic tether. Furthermore, a 12-base-pair duplex has a length-to-diameter ratio of approximately 2. As a result, the diffusion tensor governing the global tumbling of a 12-base-pair duplex is not highly anisotropic ($D_{para}/D_{perp} \approx 2$). It is therefore not surprising that the simulations have little sensitivity to the relative orientation of the effective nitroxide tensors to the DNA rotational diffusion tensor.

In Figure 8, the EPR spectrum of duplex 5′-d[CGCGAAT-(T**)CGCG] is fit to an axial rotational diffusion model, which is equivalent to the base disk model of Bobst and co-workers (Strobel et al., 1990). The value of τ_{para} obtained, 0.6 ns, is consistent with values obtained by Kao and Bobst (1985) for more flexible two-atom (0.7 ns) and four-atom (0.3 ns) tethers. Furthermore, the best fit to the data is obtained when the nitroxide z-axis is constrained to be perpendicular to the tether axis ($\theta = 90°$) as expected on the basis of the structure of the T** label. On the other hand, the value of τ_{perp} obtained, 9 ns, is slightly more than double that obtained by Bobst and co-workers, 4 ns, using a variety of different spin-labeled nucleic acids incorporated into much longer DNA duplexes (Strobel et al., 1990; Pauley et al., 1987; Kao & Bobst, 1985; Bobst et al., 1984). Given

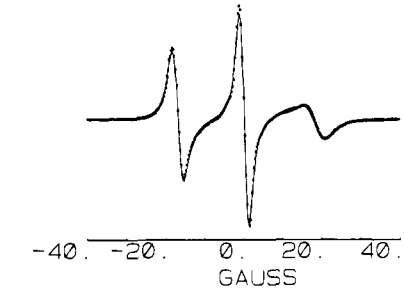

FIGURE 8: EPR spectrum of duplex 5′-d[CGCGAAT(T**)CGCG] free in solution (same data as in Figure 7). The fit (solid line) has been generated for an axial rotational diffusion model. Best-fit parameters are $\tau_{para} = 0.6$ ns, $\tau_{para} = 9$ ns, $\theta = 90°$ (fixed), $\psi = 38°$, and $\Gamma = 0.56$ G. $R = 0.9851$.

the relatively low ratio of D_{para} to D_{perp} for a 12-base-pair duplex, the global tumbling of the duplex can be reasonably modeled, to a first approximation, using a single isotropic τ. This effective τ should fall within the range determined by D_{para} and D_{perp}. For $\theta_{tether} = 0°$, it is expected that $\tau \approx 1/6D_{para}$, while for $\theta_{tether} = 90°$, it is expected that $\tau \approx 1/6D_{para}$ (see Figure 2C). The value of τ_{perp} obtained for duplex 5′-d[CGCGAAT(T**)CGCG], 9 ns, is within the range of the characteristic times which govern the global rotation of the duplex about the helix axis, $1/6D_{para} = 7.5$ ns, and the global end-to-end tumbling of the duplex, $1/6D_{perp} = 14.4$ ns. Thus, the value of τ_{perp} obtained with the T** label is determined by the uniform motion of the duplex. There is no evidence of a rapid, large-amplitude local motion of the labeled base in the EPR spectrum of duplex 5′-d[CGCGAAT(T**)-CGCG].

DISCUSSION

The EPR spectrum of the 48-base-pair T*-labeled duplex bound to DEAE-Sephadex has been used as the starting point for this study. This spectrum has been fit to a powder pattern

spectrum to determine what were subsequently used as the effective rigid-limit **A**- and **g**-tensors. The EPR spectrum of the 12-base-pair T*-labeled sequence 5′-d[CGCGAAT-(T*)CGCG] bound to DEAE-Sephadex in Figure 4 shows some increased motion relative to that of the 48-base-pair duplex in Figure 3. This spectrum was simulated using a phenomenological isotropic rotational correlation time of 177 ns. The additional motion of the 12-base-pair duplex may be due to a sequence-dependent effect or the reduced number of binding sites on the shorter sequence. While there is some slight increase in motion for the 12-base-pair T*-labeled duplex relative to the 48-base-pair sequence, for all other spectra considered here there is a substantial increase in the motion.

In an analysis of the EPR spectra of T*-labeled DNA duplexes as a function of temperature and duplex length, Hustedt et al. (1993a) modeled all of the internal motions of the spin-labeled DNA in terms of motionally averaged **A**- and **g**-tensors. Rigid-limit **A**- and **g**-tensors were obtained from the EPR spectrum of a 48-base-pair duplex in 51% (w/w) sucrose at 0 °C. The total mean-squared amplitude of all internal motions, $\langle\phi^2\rangle$, can be obtained by the extent to which the z-component of the **A**-tensor is collapsed by the motion:

$$\langle\phi^2\rangle = \frac{A_{zz} - \langle A_{zz}\rangle}{A_{zz} - 0.5(A_{xx} + A_{yy})} \qquad (2)$$

From the values of the rigid-limit tensor elements previously obtained (A_{xx} = 6.27 G, A_{yy} = 10.10 G, A_{zz} = 32.21 G) by Hustedt et al. (1993a) and the value of $\langle A_{zz}\rangle$ obtained here for the 48-base-pair duplex bound to DEAE-Sephadex, one obtains a mean-squared amplitude of internal motion of $\langle\phi^2\rangle$ = 0.0430 radians^2 which gives a root-mean-squared amplitude of 11.9°. A similar result, 12.3°, is obtained using the **g**-tensor. Hustedt et al. (1993a) determined that the root-mean-squared amplitude of internal motion for the same 48-base-pair duplex in buffer was 13.0° at 0 °C. These results suggest that binding to DEAE-Sephadex does not significantly alter the amplitude of the internal motions in duplex DNA.

The simulations of the EPR spectrum of 5′-d[CGCGAAT-(T**)CGCG] bound to DEAE-Sephadex in Figure 5 demonstrate that the nitroxide is undergoing essentially uniaxial rotational diffusion about the tether axis. The diacetylenic tether of T** is sufficiently long to allow free rotation of the nitroxide. There is no evidence in the EPR spectrum for rapid, large-amplitude motions about an axis perpendicular to the tether axis which would correspond to internal motions of the DNA. It is important to note that the uniaxial rotation alone has not sufficiently collapsed the EPR spectrum such that there would no longer be any sensitivity to additional motional modes.

The simulation of the EPR spectra of T*-labeled duplexes free in solution have been discussed in detail previously (Hustedt et al., 1993a). These spectra have been shown to be sensitive to the length-dependent global tumbling of the duplex, the length-dependent internal bending modes of the duplex, and local motion of the nitroxide and the T* base. Here, the EPR spectrum of 5′-d[CGCGAAT(T*)CGCG] in buffer shown in Figure 6 has been fit considering only the global tumbling of the duplex. The simulation slightly underestimates the motion of the nitroxide, which is presumably enhanced by the additional, small-amplitude internal modes not considered here. Hustedt et al. (1993a) have suggested that the root-mean-squared amplitude of internal motion in a similar 12-base-pair sequence is ≈11° at 0 °C. However, it has been argued that the presence of the nitroxide within the major groove may lock out the local motion of the base (Strobel et al., 1990). The T** label was designed to allow free rotation of the nitroxide spin-label about the tether axis and to minimize the possibility of steric hindrance of the internal DNA base motions (Kirchner et al., 1990).

In Figure 7, reasonable simulations to the EPR spectrum of 5′-d[CGCGAAT(T**)CGCG] in buffer were obtained by modeling the motion of the nitroxide in terms of the uniform tumbling of the duplex DNA and an infinitely rapid, uniaxial rotation of the nitroxide about the diacetylenic tether. The line widths of these simulations, paticularly those of the high-field manifold, are somewhat narrower than those of the data. This suggests that the rate of the nitroxide motion is overestimated in these simulations, presumably because the rotation about the diacetylenic tether has been assumed to be infinitely fast.

The T** label is similar to the many spin-labeled nucleic acids reported by Bobst and co-workers (Strobel et al., 1990; Kao & Bobst, 1985; Bobst et al., 1984) in that the nitroxide is coupled to the nucleotide by a long tether which allows for considerable independent motion of the probe. It was predicted that the diacetylenic tether of T** would allow rapid, independent rotation of the nitroxide about the tether axis. This prediction has been confirmed by the excellent fits to the EPR spectra of a T**-labeled 12-base-pair duplex, both bound to DEAE-Sephadex (Figure 5) and free in solution (Figure 7), assuming rapid rotation about the diacetylenic tether. These results suggest that the EPR spectra of T**-labeled duplex DNA would be excellent candidates for analysis in terms of the base disk model of Bobst and co-workers (Strobel et al., 1990). In the base disk model the motion of the nitroxide is modeled as axial rotational diffusion. The unique element of the diffusion tensor is ascribed to the independent probe motion, and the two orthogonal elements of the diffusion tensor are then ascribed to DNA dynamics. According to the results of Bobst and co-workers, the value of τ_{para}, which governs the independent motion of the nitroxide about the tether axis, decreases with increasing tether length, consistent with longer tethers being less restrictive (Kao & Bobst, 1985). The value of τ_{perp} = 4 ns, which governs rotation about two axes orthogonal to the tether axis, obtained by Bobst and co-workers is independent of the tether length and is far too small to be related to the global tumbling of the large duplexes, from 300 (Pauley et al., 1987) to 20 000 (Strobel et al. 1990) base pairs, employed. This has led Bobst and co-workers to the conclusion that there is rapid, large-amplitude local base motion in duplex DNA.

An excellent fit to the EPR spectrum of duplex 5′-d[CGCGAAT(T**)CGCG free in solution has been obtained using an axial diffusion model (Figure 8). The best-fit values of τ_{para} = 0.6 ns, θ = 90°, and ψ = 38° are all consistent with a rapid rotation of the nitroxide about the diacetylenic tether axis. The best-fit value of τ_{perp} = 9 ns is within the range (7.5−14.4 ns) of what one would expect if τ_{perp} is a measure of the uniform tumbling of the DNA duplex. In all of the analyses of the EPR spectra of duplex 5′-d[CGCGAAT(T**)CGCG] presented here, both bound to

DEAE-Sephadex and free in solution, there has been no need to invoke large-amplitude, rapid local base motions to fit the data.

The significant difference between T** and the many spin-labeled nucleic acids employed by Bobst and co-workers (Strobel et al., 1990; Pauley et al., 1987; Kao & Bobst, 1985; Bobst et al., 1984) is the fact that the diacetylenic tether of T** highly restricts the independent motion of the nitroxide to rotation about the tether axis. This is precisely what is required by the base disk model, which assumes that the independent motion of the nitroxide is about a single axis. It is likely, then, that the separation of motional modes into those attributed to the independent motion of the nitroxide (governed by τ_{para}) and those attributed to the DNA (governed by τ_{perp}) is more valid in the case of T** than for other spin probes. In this case, values of τ_{perp} obtained using T** should give a more accurate estimate of the rates of DNA rotation.

It has been suggested (Strobel et al., 1990) that the relatively small amplitudes of internal DNA motion observed with T* (Hustedt et al., 1993a) are a result of the nitroxide, coupled via a monoacetylene tether, hindering the natural base motion in duplex DNA. The T** label was developed to test this possibility. It has been demonstrated in this work that the nitroxide of T** does rotate freely about the diacetylenic tether. Therefore, the nitroxide moiety cannot significantly hinder the internal motion in duplex DNA. It could be argued that the rigidity of the diacetylenic tether itself is responsible for restricting the internal DNA motion; however, this seems unlikely. Space-filling models give no evidence for such an interaction. The EPR spectra of a T**-labeled DNA duplex have been successfully simulated by considering only the rapid, independent rotation of the nitroxide about the diacetylenic tether and the rigid-body tumbling of the duplex. There has been no need to invoke additional large-amplitude internal DNA motions to fit the data. This result confirms that, as previously observed using T* (Hustedt et al., 1993), there are no very large amplitude local base motions in DNA. We conclude that the presence of the T* probe does not significantly perturb the internal dynamics of DNA and that T* is a faithful reporter of internal DNA motion.

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REFERENCES

Allison, S. A., & Schurr, J. M. (1979) *Chem. Phys. 41*, 35−59.

Bobst, A. M., Kao, S.-C., Toppin, R. C., Ireland, J. C., & Thomas, I. E. (1984) *J. Mol. Biol. 173*, 63−74.

Bobst, A. M., Pauly, G. T., Keyes, R. S., & Bobst, E. V. (1988) *FEBS Lett. 228*, 33−36.

Froehler, B. C., Wadwani, S., Terhorst, T. J., & Gerrard, S. R. (1992) *Tetrahedron Lett. 33*, 5307−5310.

Fujimoto, B. S., & Schurr, J. M. (1990) *Nature 344*, 175−178.

Hogan, M. E., & Austin, R. H. (1987) *Nature 329*, 263−266.

Hurley, I., Osei-Gyimah, P., Archer, S., Scholes, C. P., & Lerman, L. S. (1982) *Biochemistry 21*, 4999−5009.

Hustedt, E. J., Spaltenstein, A., Kirchner, J. J., Hopkins, P. B. & Robinson, B. H. (1993a) *Biochemistry 32*, 1774−1787.

Hustedt, E. J., Cobb, C. E., Beth, A. H., & Beechem, J. M. (1993b) *Biophys. J. 64*, 614−621.

Kao, S.-C., & Bobst, A. M. (1985) *Biochemistry 24*, 5465−5469.

Kirchner, J. J. (1991) Ph.D. Thesis, University of Washington, Seattle, WA.

Kirchner, J. J., Hustedt, E. J., Robinson, B. H., & Hopkins, P. B. (1990) *Tetrahedron Lett. 31*, 593−596.

Koudelka, G. B., Harbury, P., Harrison, S. C., & Ptashne, M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4633−4637.

Lajzerowicz-Bonneteau, J. (1976) in *Spin Labeling: Theory and Applications* (Berliner, L. J., Ed.) Vol. 1, pp 239−249, Academic Press, New York.

Mailer, C., Danielson, J. D. S., & Robinson, B. H. (1985) *Rev. Sci. Instrum. 56*, 1917−1925.

Pauley, G. T., Thomas, I. E., & Bobst, A. M. (1987) *Biochemistry 26*, 7304−7310.

Robinson, B. H., Forgacs, G., Dalton, L. R., & Frisch, H. L. (1980a) *J. Chem. Phys. 73*, 4688−4692.

Robinson, B. H., Lerman, L. S., Beth, A. H., Frisch, H. L., Dalton, L. R., & Auer, C. (1980b) *J. Mol. Biol. 139*, 19−44.

Schneider, D. J., & Freed, J. H. (1989) in *Biological Magnetic Resonance, Volume 8, Spin Labeling: Theory and Applications* (Berliner, L. J., & Reuben, J., Ed.) pp 1−76, Academic Press, New York.

Schultz, S. C., Shields, G. C., & Steitz, T. A. (1991) *Science 253*, 1001−1007.

Shin, Y.-K., & Freed, J. H. (1989) *Biophys. J. 55*, 537−550.

Spaltenstein, A., Robinson, B. H., & Hopkins, P. B. (1988) *J. Am. Chem. Soc. 110*, 1299−1301.

Spaltenstein, A., Robinson, B. H., & Hopkins, P. B. (1989a) *Biochemistry 28*, 9484−9495.

Spaltenstein, A., Robinson, B. H., & Hopkins, P. B. (1989b) *J. Am. Chem. Soc. 111*, 2303−2305.

Strobel, O. K., Keyes, R. S., & Bobst, A. M. (1990) *Biochemistry 29*, 8522−8528.

Tirado, M. M., & de la Torre, J. G. (1980) *J. Chem. Phys. 73*, 1986−1993.

Van, S. P., Birrel, G. B., & Griffith, O. H. (1974) *J. Magn. Reson. 15*, 444−459.

Wilcoxon, J., & Schurr, J. M. (1983) *Biopolymers 22*, 2273−2321.

Wu, P., Fujimoto, B. S., & Schurr, J. M. (1987) *Biopolymers 26*, 1463−1488.

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