Investigation of Complexes Formed between Gene 32 Protein from Bacteriophage T4 and Heavy-Atom-Modified Single-Stranded Polynucleotides Using Optical Detection of Magnetic Resonance†

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ABSTRACT: Optical detection of triplet-state magnetic resonance (ODMR) is employed to study the complexes formed between gene 32 protein (GP32), a single-stranded DNA-binding protein from bacteriophage T4, and the heavy-atom-derivatized polynucleotides poly(5-HgU) and poly(5-BrU). The triplet-state properties of some of the tryptophan (Trp) residues in the complexes are dramatically different from those in the free protein, in that they are subject to an external heavy-atom effect. Direct evidence for the presence of a heavy-atom effect, and hence a close-range interaction between mercurated or brominated nucleotide bases and Trp residues in the complex, is provided by the observation of the zero-field $|D| + |E|$ ODMR transition of Trp, which is not normally observed in the absence of a heavy-atom perturbation. The amplitude-modulated phosphorescence–microwave double-resonance (AM-PMDR) technique is employed to selectively capture the phosphorescence spectrum originating from the heavy-atom-perturbed Trp residue(s) in the GP32–poly(5-HgU) complex. Arguments based on our experimental results lead to the conclusion that the heavy-atom perturbation arises from aromatic stacking interactions between Trp and mercurated bases. Wavelength-selected ODMR measurements reveal the existence of two environmentally distinct and spectrally different types of Trp in GP32. One of these types is perturbed selectively by the heavy atom and hence undergoes stacking interactions with the heavy-atom-derivatized bases of the polynucleotide while the second type of Trp residue is unaffected. Both Trp types are buried in relatively polarizable, hydrophobic regions of the protein, which supports a model for the binding of GP32 to single-stranded polynucleotides in which the bases are inserted into hydrophobic regions of the protein.

Gene 32 protein (GP32)[1] from bacteriophage T4 belongs to a class of single-stranded DNA-binding proteins that bind selectively to single-stranded DNA or polynucleotide and hence act to destabilize double stranded DNA (Alberts & Strenglanz, 1977). The origin of the single-strand binding affinity in this class of proteins is not well understood. Stacking interactions between aromatic amino acid residues in the proteins and nucleotide bases in single-stranded DNA were suggested as one of the recognition processes involved in forming these complexes (Hélène et al., 1976; Köster et al., 1980). Extensive studies of model complexes formed between polynucleotides and aromatic amino acid-containing peptides have been reported in support of this idea (Hélène & Maurizot, 1981).

Recently, optically detected magnetic resonance (ODMR) spectroscopy has been utilized as a method for obtaining direct evidence for aromatic stacking interactions in biologically important protein–nucleic acid complexes (Cha & Maki, 1984b). ODMR spectroscopy (Clarke, 1982) provides detailed information about the properties of the triplet excited state, particularly when measurements are made in zero applied magnetic field. Because of the external heavy-atom effect (Kasha, 1952), time-resolved ODMR experiments can be performed in which heavy-atom-perturbed triplet-state responses can be selected from the longer lived background emission of unperturbed residues (Hershberger & Maki, 1980). A readily measureable heavy-atom effect occurs only if the perturber atom and target molecule are located within the van der Waals distance (Kasha, 1952). Thus, the heavy-atom perturbation acts as a probe for extremely close range interactions between the perturber and the perturbed molecule (Hélène et al., 1976; Cha & Maki, 1982, 1984a,b). In previous work (Cha & Maki, 1984b) the complex formed between *Escherichia coli* single-stranded DNA-binding protein (SSBP) and poly(5-HgU) was investigated by phosphorescence and ODMR spectroscopy. On the basis of the ODMR data, evidence was presented for the occurrence of stacking interactions between at least one tryptophan (Trp) residue in SSBP and the mercurated uridine bases in the polynucleotide. Their results suggest that stacking interactions are one of the processes that stabilize protein complexes with single-stranded polynucleotides (Hélène et al., 1976; Köster et al., 1980).

In the present study, complexes formed between GP32 from bacteriophage T4 and heavy-atom-modified single-stranded polynucleotides are investigated. Unlike other single-stranded binding proteins, GP32 is known to form long linear aggregates in which the binding sites are aligned (Carroll et al., 1975). In vitro studies of GP32 have shown that binding to single-stranded DNA (ssDNA) is highly cooperative (Alberts & Frey, 1970; Jensen & von Hippel, 1976), with each GP32 molecule binding to between eight and ten nucleotide residues (Alberts & Frey, 1970; Anderson & Coleman, 1975; Jensen et al., 1976; Kelly & von Hippel, 1976; Prigodich et al., 1984). Electron microscopy studies on GP32–ssDNA complex (Delius et al., 1972) confirmed the previously reported observation (Alberts & Frey, 1970) that the bound protein greatly extends

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[1] Abbreviations: AM-PMDR, amplitude-modulated phosphorescence–microwave double resonance; *D* and *E*, triplet-state zero-field-splitting parameters; GP32, gene 32 protein from bacteriophage T4; ODMR, optically detected magnetic resonance; poly(5-BrU), brominated poly(uridylic acid); poly(5-HgU), mercurated poly(uridylic acid); SSBP, single-stranded DNA binding protein from *Escherichia coli*; Trp, tryptophan; Tyr, tyrosine.

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the folded DNA chain and also revealed that the complexes appear as uniform ropelike structures having diameters of 50–70 Å with a 4.6-Å separation between nucleotides. GP32 plays a prominent role in normal T4 metabolism and is required for DNA replication (Epstein et al., 1963; Huberman et al., 1971; Hibner & Alberts, 1980), repair (Wu & Yeh, 1973; Krish & van Houwe, 1976), and genetic recombination (Tomizawa et al., 1966; Kozinski & Felgenhauer, 1967). In addition, GP32 regulates its own synthesis at the translational level by specific association to its mRNA after stoichiometric binding of the *E. coli* ssDNA (von Hippel et al., 1982). The amino acid sequence reveals five Trp and eight tyrosine (Tyr) residues (Williams et al., 1980, 1981). These aromatic residues are thought to interact with DNA bases to stabilize the association between the protein and the DNA. To obtain direct evidence for the presence of these stacking interactions, GP32 is complexed with poly(5-HgU) in this present work, and ODMR results indicate that at least one Trp residue is involved in a stacking interaction with the mercurated uridine bases of poly(5-HgU).

Since 5-bromouridine may be readily incorporated into cellular DNA as a thymine analogue (Bick & Davidson, 1974; Kihlman & Kronborg, 1975; Little, 1976; Rydberg, 1977), a bromine-modified polynucleotide was introduced as another heavy-atom-derivatized substrate (Cha & Maki, 1984a; Maki & Cha, 1984). Poly(5-BrU) was synthesized and complexed with GP32. This polynucleotide provides what should be a more natural model for the interaction of GP32 with single-stranded polynucleotides than does poly(5-HgU). Furthermore, a comparison of the effects produced by two perturbing external heavy atoms, bromine and mercury, is provided.

Materials and Methods

GP32 was a generous gift from Dr. Bruce Alberts. Additional GP32 was purchased from P-L Biochemicals in 20 mM Tris-HCl (pH 8.0) buffer containing 1 mM EDTA, 1.0 mM dithiothreitol, and 100 mM NaCl in a 50% glycerol solution. Poly(5-HgU) and poly(U) were obtained from P-L Biochemicals and were used without further purification. According to the manufacturer, greater than 70% of the uridine bases in the poly(5-HgU) supplied are mercurated covalently at the 5-position. Poly(5-BrU) was prepared by direct action of bromine on poly(U) in a modification of the procedure used to prepare poly(5-BrC) (Deubel & Leng, 1974). Poly(U) (5.6 μmol) was dissolved in 50 μL of H_2O and formamide (1 mL) was added. The H_2O was removed under vacuum and 350 μL of 21.0 mM tri-*n*-butylamine and 150 μL of 98.2 mM bromine, each dissolved in formamide, were then added. The resulting yellow solution was left in the dark for 2 h at room temperature. One milliliter of 10 mM sodium cacodylate buffer (pH 7.0) was added, and the solution was shaken several times with ether (1 mL). The aqueous fraction was dialyzed against 1 mM sodium cacodylate buffer (pH 7.0) containing 0.1 mM EDTA for 3 days at 4 °C. The UV spectrum of the resulting solution was recorded, and a shift in λ_{max} relative to that of poly(U) (poly(U), λ_{max} at 260 nm; poly(5-BrU), λ_{max} at 278 nm) was observed in accordance with that reported earlier for poly(5-BrU) prepared by enzymatic synthesis (Michelson et al., 1962). Stock solutions of poly(5-HgU) and poly(U) were prepared by dissolving the polynucleotide in 1 mM, pH 7.0, cacodylate buffer containing 0.1 mM EDTA. All other chemicals were of the highest commercially available grade.

GP32–poly(5-HgU), GP32–poly(5-BrU), and GP32–poly(U) complexes were prepared by mixing stock solutions of GP32 and the appropriate polynucleotide. A slight excess of β-mercaptoethanol was added to GP32–poly(5-HgU) and served as a blocking reagent for Hg (Cha & Maki, 1982). Under these conditions the complex is readily dissociated by the addition of salt (Le Doan et al., 1984), indicating the absence of covalent bonding between the protein and the mercurated polynucleotide. The complexes were incubated at 37 °C for 10 min in 30% glycerol, which serves as a cryogenic solvent for low-temperature work. A 15-μL sample was sufficient to fill a Suprasil quartz sample tube (1 mm i.d.). This was contained within a microwave helix terminating a coaxial transmission line and was immersed in liquid helium for spectroscopic measurements.

The apparatus and experimental procedures for measurements of phosphorescence and ODMR responses have been described previously (Cha & Maki, 1982, 1984a,b; Ghosh et al., 1984). Phosphorescence spectra were recorded at 4.2 K, and phosphorescence decay measurements were made at 77 K where rapid spin–lattice relaxation effectively maintains equal triplet sublevel populations, producing a single exponential decay. All ODMR measurements were performed at 1.2 K, in order to quench spin–lattice relaxation, while changes in phosphorescence intensity were monitored at a particular emission wavelength during microwave sweeps through the magnetic resonance transitions in zero applied magnetic field. The microwave sweep rate was adjusted so that passage through the ODMR transition was slow compared with the lifetime of the longer lived triplet state sublevel involved in the transition. Accurate transition frequencies were determined by averaging the responses of sweeps in both directions in order to compensate for rapid passage effects.

The heavy-atom-perturbed short-lived triplet sublevels were probed by various time-resolved ODMR methods at pumped-liquid-helium temperatures of ca. 1.2 K. The sublevel lifetimes were obtained from an analysis of the phosphorescence transient induced by a microwave pulse or rapid passage through a zero-field magnetic resonance transition (Winscom & Maki, 1971).

The phosphorescence spectrum of the heavy-atom-perturbed triplet state was obtained by using the amplitude-modulated phosphorescence–microwave double-resonance (AM-PMDR) method, which is described by Olmsted and El-Sayed (1974). The experimental details of this measurement as carried out in our laboratory are described elsewhere (Davis & Maki, 1982; Cha & Maki, 1984a,b). Modulation of the Trp $|D| + |E|$ transition between the normally dark sublevels induces the phosphorescence spectrum of the perturbed Trp residues only when lock-in detection at the modulation frequency is employed.

Wavelength-selected ODMR was carried out as described earlier (von Schütz et al., 1974) in order to determine heterogeneity in the Trp phosphorescence from GP32. The ODMR transition was observed while different wavelengths throughout the 0,0-band phosphorescence region of the Trp emission were monitored with the use of narrow detection slits. In this manner, Trp residues emitting from dissimilar protein environments may be revealed by discontinuities that occur in the plots of ODMR frequency vs. detection wavelength (von Schütz et al., 1974; Kwiram et al., 1978; Davis & Maki, 1984).

Results and Discussion

Phosphorescence Spectra and Lifetime Determination

(A) GP32 and Its Complexes with Poly(5-HgU) and Poly(U). The phosphorescence spectra of GP32, the GP32–poly(5-HgU) complex and the GP32–poly(U) complex are shown in parts a–c of Figure 1, respectively. The phos-

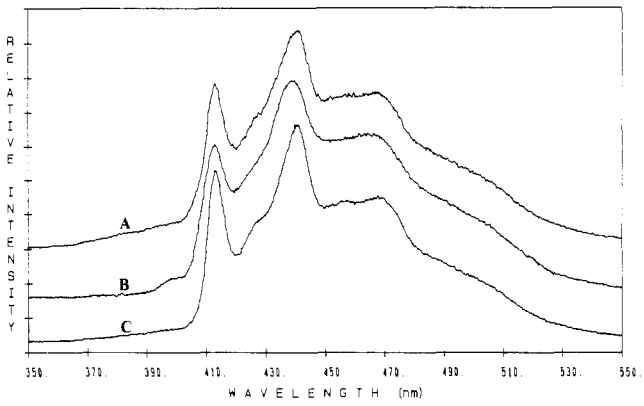

FIGURE 1: Phosphorescence spectra: (a) GP32 (2.1×10^{-5} M), (b) complex of GP32 (4.0×10^{-5} M) and poly(5-HgU) (8.0×10^{-4} M), (c) complex of GP32 (2.1×10^{-5} M) and poly(U) (4.4×10^{-4} M). All spectra were obtained at 4.2 K with excitation at 295 nm with 16-nm band-pass and emission slits of 3 nm. The solvent was 30% glycerol by volume in aqueous cacodylate buffer (1 mM, pH 7.0) containing 0.1 mM EDTA.

Table I: Phosphorescence Lifetimes of GP32 and Its Complexes with Poly(5-BrU) and Poly(5-HgU)

sample	T (K)	λ_{em} (nm)	lifetime components
GP32[a]	77	413.0	6.1 s (93%), 2.7 s (7%)
GP32–poly(U)[a]	77	413.0	5.4 s (100%)
GP32–poly(5-BrU)[a]	77	413.0	5.6 s (87%), 0.66 s (13%)
poly(5-BrU)[b]	77	450.0	3.0 ms (96%), 34 ms (4%)
GP32–poly(5-HgU)	77	413.5	5.4 s (89%)[c]

[a] Excitation wavelength is 295 nm. [b] Excitation wavelength is 337 nm with a pulsed N_2 laser. [c] Obtained by linear least-squares fitting with the observation of a short component of ca. 1 ms (11%) in the decay profile, which was too short to analyze accurately.

phorescence spectra of GP32 is due mainly to Trp, but a small contribution from Tyr is apparent below ca. 400 nm. The 0,0-band of Trp is quite red-shifted and peaks at 413.4 nm, which is characteristic of Trp buried in a hydrophobic region of the protein (Purkey & Galley, 1970; Hershberger et al., 1980). The complex of GP32 with poly(5-HgU) shows a somewhat broadened phosphorescence spectrum. Similar effects of poly(5-HgU) binding have been observed earlier in the *E. coli* SSBP–poly(5-HgU) complex (Cha & Maki, 1984b). From Figure 1c it is seen that binding of poly(U) produces little change in the Trp emission, but the Tyr phosphorescence is quenched. Decay measurements on the GP32–poly(5-HgU) complex reveal a dramatic shortening of the Trp triplet lifetime (Table I) similar to that observed previously (Le Doan et al., 1984). GP32 and the GP32–poly(U) complex each exhibit a normal unperturbed Trp lifetime (Table I), with no short components. The short Trp lifetime component observed in the GP32–poly(5-HgU) complex gives strong evidence for a heavy-atom perturbation by mercury in van der Waals contact with Trp (Anderson & Maki, 1980). The extent to which the Trp lifetime is reduced ($\leq$1 ms) provides strong evidence for an above-the-plane approach of the mercury atom relative to Trp in the complex between GP32 and poly(5-HgU). Previous model studies using CH_3Hg^+ as an edge-on perturber of benzimidazole (Svejda et al., 1978) yielded a triplet-state lifetime greater by 2 orders of magnitude than that observed for the GP32–poly(5-HgU) complex. On the other hand, similar Trp phosphorescence lifetimes (between 1 and 10 ms) were observed for CH_3Hg^+–Trp complexes in which the Hg atom is known to be located above the indole plane. Since the Hg atom of 5-HgU is blocked with β-mercaptoethanol, steric considerations

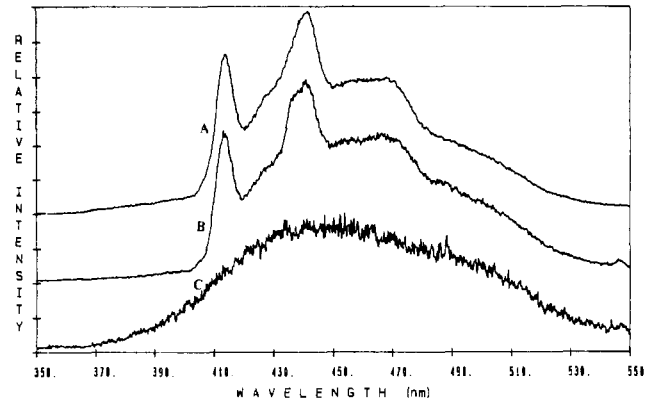

FIGURE 2: Phosphorescence spectra: (a) GP32 (2.5×10^{-5} M), (b) complex of GP32 (2.5×10^{-5} M) and poly(5-BrU) (2.5×10^{-4} M), (c) poly(5-BrU) (1.34×10^{-4} M in aqueous 50% glycerol–cacodylate buffer obtained at 77 K). Except for spectrum c the solvent and temperature are the same as given in Figure 1.

dictate that the only conceivable geometry that allows van der Waals contact between Hg and the π-electrons of Trp is a stacking interaction.

(*B*) *Poly(5-BrU) and Its Complex with GP32.* The unstructured phosphorescence spectrum of poly(5-BrU) presented in Figure 2c originates at ca. 380 nm and exhibits a maximum at about 450 nm. When the percentage of glycerol used in the solvent matrix is decreased, however, a red-shifted phosphorescence spectrum is observed, which suggests that stacking interactions occur between uridine bases. Phosphorescence red shifts observed in polynucleotides have been attributed previously to base stacking interactions (Rahn et al., 1966). Poly(5-BrU) exhibits a very short phosphorescence lifetime of ca. 3 ms at 77 K as determined from decay measurements employing pulsed N_2 laser excitation (Table I).

Figure 2b shows the phosphorescence spectrum of the GP32–poly(5-BrU) complex. Unlike that in GP32–poly(5-HgU) complex, the 0,0-band is well resolved, but similar quenching of Tyr emission is observed. Phosphorescence lifetime measurements on the GP32–poly(5-BrU) complex reveal the presence of a ca. 600-ms component (Table I), which does not occur either in GP32 or in poly(5-BrU). Furthermore, as one monitors the decay at different wavelengths through the Trp 0,0-band of the complex, the relative intensity of the short component is found to increase as the monitored wavelength moves to the red. This increase in relative intensity suggests the existence of short-lived Trp residue(s) whose emission is shifted to the red relative to that of unperturbed Trp, an effect previously observed in Br-atom-perturbed Trp (Maki & Cha, 1984). Direct evidence for the presence of a heavy-atom-perturbed Trp residue in this complex is based on ODMR results, which are presented in the following section. However, a narrow, unshifted Trp spectrum remains when GP32 is complexed with both poly(5-HgU) and poly(5-BrU). This may be explained by the presence of two types of Trp in GP32; one type remains unperturbed by nucleotide binding, while the other undergoes a heavy-atom perturbation. Evidence for this interpretation is presented in a later section.

ODMR Spectra

(*A*) *GP32 and Its Complex with Poly(5-HgU).* The $|D| - |E|$ and $2|E|$ microwave slow passage ODMR signals of Trp in GP32 observed at 413 nm are shown in Figure 3. The transition frequencies are characteristic of a Trp residue buried in a hydrophobic region of the protein rather than exposed to the polar solvent (Hershberger et al., 1980). The line widths (Figure 3) also are considerably narrower than those typically

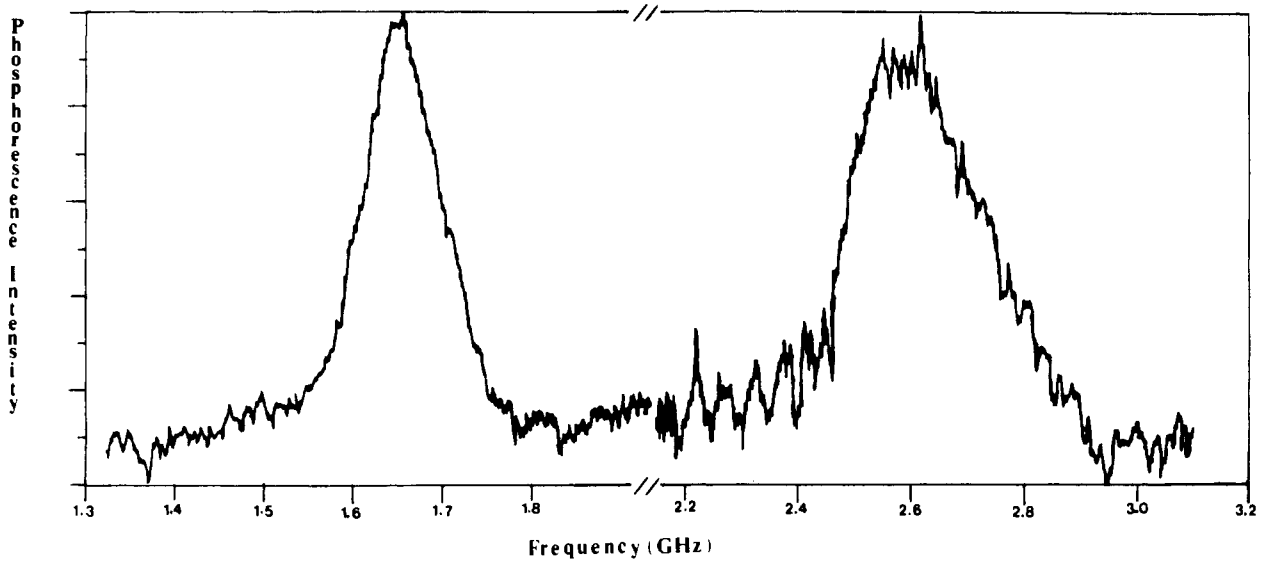

FIGURE 3: $|D| - |E|$ (lower frequency) and $2|E|$ (higher frequency) ODMR transitions of Trp in GP32 (5.0×10^{-5} M). The sweep rates were 16.7 and 26.7 MHz s^{-1}, respectively. Signal averaging was performed for 64 scans. The temperature was 1.2 K, and phosphorescence was monitored at 413 nm with 3-nm slits.

Table II: Tryptophan Zero-Field ODMR Frequencies and ZFS Parameters[a]

sample	$\|D\| - \|E\|$ (GHz)	$2\|E\|$ (GHz)	$\|D\| + \|E\|$ (GHz)	$\|D\|$ (GHz)	$\|E\|$ (GHz)
Lys-Trp-Lys[b]	1.72	2.49	c	2.96	1.24
Lys-Trp-Lys[b] + poly(5-HgU) + 2-ME	1.69	2.61	4.17	2.97	1.30
SSBP[b]	1.65	2.55	c	2.92	1.27
SSBP[b] + poly(5-HgU) + 2-ME	1.64	2.60	4.15	2.94	1.30
GP32[d]	1.658	2.56	c	2.94	1.28
GP32[e]	1.631	2.58	c	2.92	1.29
GP32[f-h] + poly(5-HgU) + 2-ME	1.7	2.5	4.4	2.9	1.2
GP32[g-i] + poly(5-BrU)	1.65	2.59	4.16	2.94	1.30

[a] Measurements are made by selecting the peak of the Trp 0,0 band with a monochromator at 3-nm resolution, except where indicated. [b] From Cha and Maki (1984b). [c] Signal is not observed. [d] Measurements are made at 410 nm with a monochromator at 2-nm resolution. [e] Measurements are made at 415 nm with a monochromator at 2-nm resolution. [f] Uncertainty is ±0.1 GHz for this sample. [g] Transitions are in heavy-atom-perturbed Trps. [h] Sweep rates were 2.0, 5.0, and 77 GHz s^{-1} for $|D| - |E|$, $2|E|$, and $|D| + |E|$, respectively. [i] Sweep rates were 0.16, 0.24, and 0.46 GHz s^{-1} for $|D| - |E|$, $2|E|$, and $|D| + |E|$, respectively.

observed for solvent-exposed Trp sites with their large heterogeneity in local environments. The Trp zero-field splittings were measured by monitoring the emission to the blue and to the red of that presented in Figure 3 with the use of narrow slits and are presented in Table II. The corresponding transitions in the GP32–poly(5-HgU) complex observed under rapid sweep conditions are also presented in Table II. The signals are easily observed but are quite broad. Similar broadening was observed in the SSBP–poly(5-HgU) complex (Cha & Maki, 1984b), and is probably due to the formation of a heterogeneous distribution of perturbed complexes. The microwave sweep rate used in obtaining the ODMR transitions for the complex was 2 orders of magnitude greater than that used for the protein itself. These signals originate from a heavy-atom-perturbed triplet state since the long-lived triplet state of unperturbed Trp does not respond to the rapid sweep conditions used. The $|D| + |E|$ slow passage signal is not observed for GP32 under either fast or slow sweep conditions since the triplet sublevels connected by this transition decay nonradiatively in the absence of a heavy-atom perturbation. The $|D| + |E|$ ODMR transition is observed in the GP32–poly(5-HgU) complex, however (Figure 4a). The appearance of this transition may be considered diagnostic of a heavy-atom effect. In order to obtain a more accurate estimate of the lifetime of the perturbed Trp in the complex, a transient microwave pulse measurement (see Materials and Methods) was performed. These results are shown in Figure 4b. A lifetime on the order of 1 ms can be estimated for the more radiative sublevel from an analysis of the transient response. A similar lifetime was observed earlier for the SSBP–poly(5-HgU) complex (Cha & Maki, 1984b). These results provide confirming evidence of a heavy-atom effect and the presence of aromatic stacking interactions in both protein complexes.

(B) Poly(5-BrU) and Its Complex with GP32. Poly(5-BrU) exhibits a distinctive ODMR slow-passage transition at ca. 7.2 GHz, which serves to characterize the triplet state of 5-BrU (Cha & Maki, 1984a; Maki & Cha, 1984). The rapid microwave sweep rate that was required to produce this signal with optimal intensity indicates a very short-lived triplet state in poly(5-BrU).

The $|D| - |E|$ and $2|E|$ Trp ODMR transitions of the GP32–poly(5-BrU) complex are presented in Table II. Comparison of microwave sweep rates used in obtaining these signals (0.16 GHz s^{-1} and 0.24 GHz s^{-1} for the $|D| - |E|$ and $2|E|$, respectively) optimally with those required for the uncomplexed protein (see Figure 3) indicates that the triplet lifetime of the Trp responsible for these signals in the complex is approximately 1 order of magnitude shorter than that of unperturbed Trp. The appearance of the $|D| + |E|$ transition in the GP32–poly(5-BrU) complex (Figure 5a) provides further evidence that we are observing ODMR signals of heavy-atom-perturbed Trp. The fast passage response induced by this transition (Figure 5b) exhibits a lifetime which is on the order of 200–300 ms. Comparison of Figures 4b and 5b reveals that the lifetime of Hg-perturbed Trp is 2 orders of

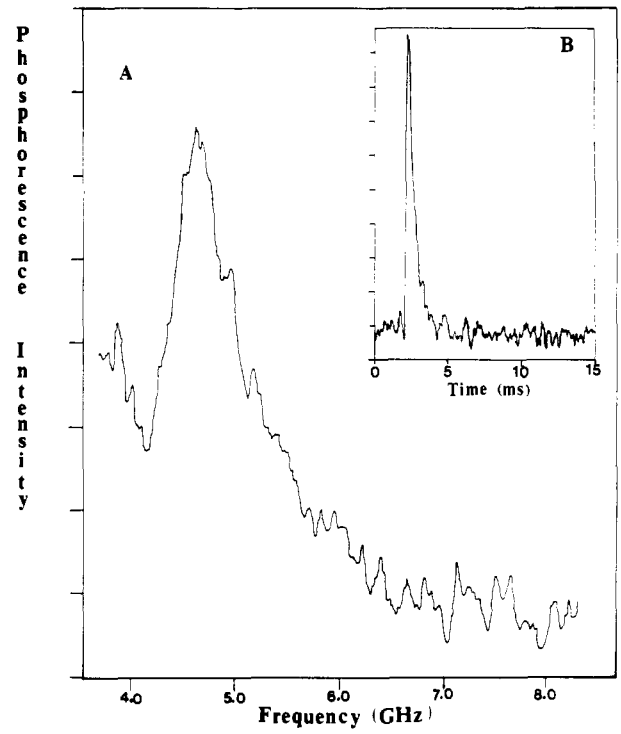

FIGURE 4: (a) Slow-passage $|D| + |E|$ ODMR signal of heavy-atom-perturbed Trp in the GP32–poly(5-HgU) complex. The scan rate was 77 GHz s^{-1}, and 6800 scans were signal averaged. (b) Phosphorescence transient resulting from a short (ca. 0.1 ms) pulse of microwaves centered at 4.4 GHz, which was frequency modulated over a range of 200 MHz at 2.0 KHz. For both parts, the phosphorescence was monitored at 470 nm with 3-nm bandwidth at 1.2 K.

magnitude shorter than that of a Br-perturbed one. This is not unexpected since the heavy-atom effect increases markedly with the atomic number of the perturber atom.

AM-PMDR Spectrum of GP32 Complex with Poly(5-HgU)

The amplitude-modulated phosphorescence–microwave double-resonance technique is used to obtain the phosphorescence associated with a particular ODMR transition, and thus of a particular triplet-state environment. In this case, the $|D| + |E|$ signal is modulated so that the phosphorescence associated with the perturbed Trp is isolated selectively. Figure 6 shows the AM-PMDR spectrum obtained for the GP32–poly(5-HgU) complex when modulating the $|D| + |E|$ transition. The resulting spectrum is effectively the phosphorescence of the heavy-atom-perturbed Trp. Comparison with Figure 1b shows that the apparent broadening of the phosphorescence upon binding poly(5-HgU) to GP32 is the result of an underlying heavy-atom-perturbed Trp component. This interaction results in the broadening of the slow-passage ODMR signals as well. Similar results have been obtained for the SSBP–poly(5-HgU) complex (Cha & Maki, 1984b). It was not possible to perform similar AM-PMDR measurements on the complex with poly(5-BrU) since the lifetime reduction of the triplet state is not sufficient to allow modulation. The broadness of the phosphorescence spectrum of the heavy-atom-perturbed Trp as revealed by the AM-PMDR measurements has two likely origins. First, there could be a heterogeneous distribution of Trp-mercurated base complexes, which would lead to a smearing out of the triplet-state electronic energies in the sample. This heterogeneity would also be reflected in broad ODMR signals as we observe. A second contributing factor could be the introduction of vibronic spin–orbit couplings by the Hg atom inducing radiative intensity into otherwise weak vibronic transitions. The additional vibronic intensity could lead to a loss in resolution, as observed. This mechanism in itself would not greatly affect ODMR line widths, however, but it could be operative along with the heterogeneity of the complexes.

Wavelength-Selected ODMR

In order to characterize further the Trp sites in GP32, and in its complex with poly(5-HgU), wavelength-selected ODMR measurements were conducted on the phosphorescence 0,0-band region of GP32 and of the GP32–poly(5-HgU) complex. The results are presented in Figure 7. The plot of the $|D| - |E|$ transition vs. wavelength for GP32 (Figure 7, solid circles) reveals a sudden change of slope at ca. 412.5 nm, suggesting

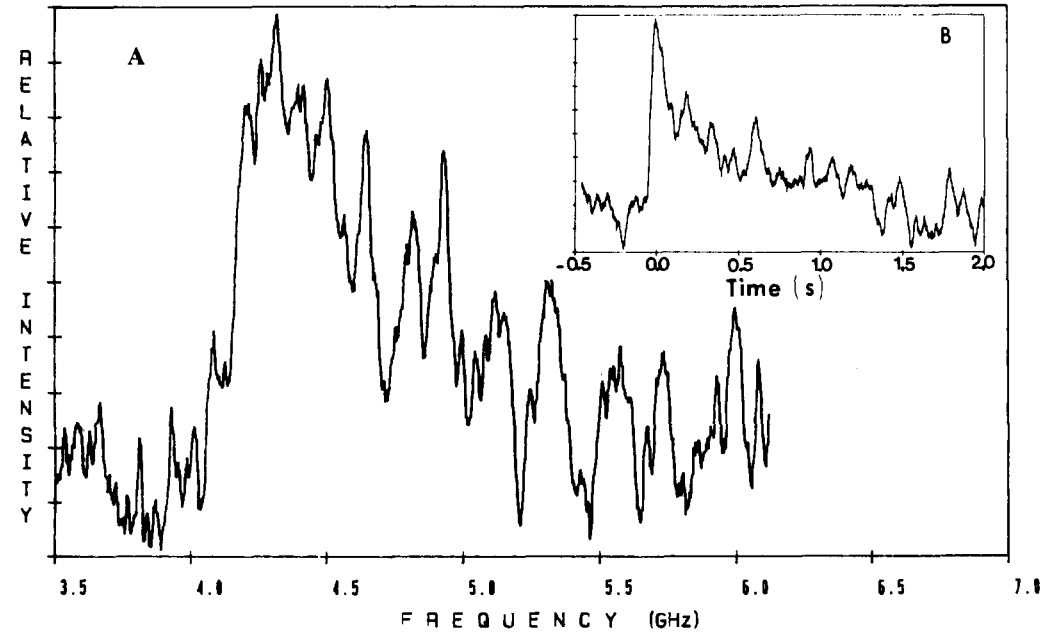

FIGURE 5: (a) Slow-passage $|D| + |E|$ ODMR signal of perturbed Trp in the GP32–poly(5-BrU) complex. The sweep rate was 0.46 GHz s^{-1}, and 1525 scans were signal averaged. (b) Fast-passage response of the perturbed Trp in the GP32–poly(5-BrU) complex in the $|D| + |E|$ region. The microwaves were swept from 3.7 to 8.3 GHz at ca. 37 GHz s^{-1}. For both parts, the phosphorescence at 1.2 K was monitored at 415 nm with 3-nm slits.

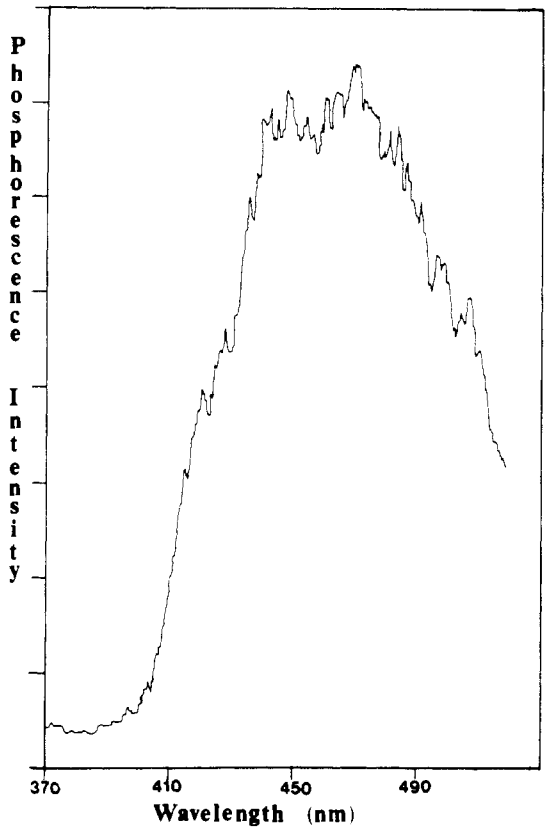

FIGURE 6: AM-PMDR spectrum of the GP32–poly(5-HgU) complex. The spectrum was obtained by amplitude modulation of microwaves at 50 Hz, which were frequency modulated at 2.0 KHz over a range of ±200 MHz about the center resonance frequency of 4.4 GHz. Phase-sensitive detection was performed at 50 Hz. Signal averaging was carried out for 4 scans. Excitation was at 295 nm with 16-nm bandpass, and the emission slit width was 1.5 nm. The temperature was 1.2 K.

the contribution of at least two distinct Trp environments to the phosphorescence of GP32. There is a blue-shifted environment, characterized by points for which $\lambda < 412.5$ nm, and a red-shifted environment, having points with $\lambda > 412.5$ nm. The data for the GP32–poly(5-HgU) complex (Figure 7, open circles) indicates the absence of the red-shifted environment as a contributor to the ODMR spectrum of the complex. It is important to note that the Hg-perturbed Trp residue(s) in the GP32–poly(5-HgU) complex do not produce ODMR signals in the $|D| - |E|$ and $2|E|$ signal regions under the slow microwave passage conditions of this experiment. Thus, the ODMR signals plotted in Figure 7 all are due to Trp sites that are not subject to an external heavy-atom perturbation. Examination of Figure 7 reveals that the red-shifted Trps, which dominate the ODMR spectrum at $\lambda > 412.5$ nm in GP32 and which have wavelength-independent zero-field splittings, are perturbed upon complex formation with the polynucleotide. This may be explained by the selective stacking of the bases with these Trp sites, leaving the blue-shifted Trp sites relatively unperturbed and dominant in the slow-scan ODMR spectrum. These Trp sites also are responsible for the residual narrow-band Trp phosphorescence emission from the complex, since the perturbed Trp phosphorescence is broad (Figure 6).

It is interesting to note that under identical conditions of optical excitation the 0,0-band peak of the GP32 is shifted slightly to the blue (from 413.4 to 413.0 nm) upon complexing with poly(5-HgU). This demonstrates that the phosphorescence peak of the Trp sites, which will be perturbed when GP32 is complexed with poly(5-HgU), lies to the red of the unperturbed Trps, probably by ca. 1 nm. From Figure 7 (open

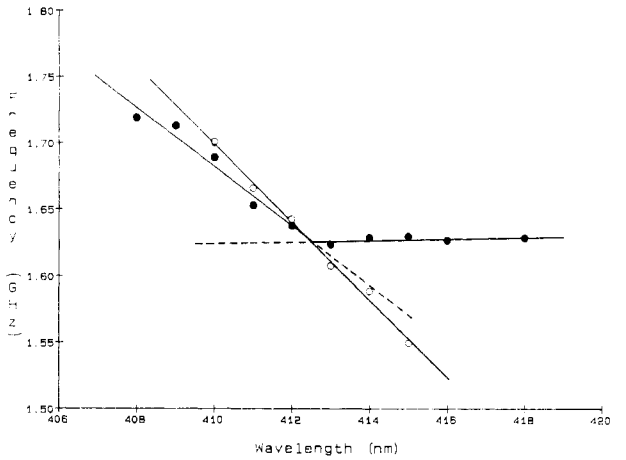

FIGURE 7: Plot of the $|D| - |E|$ ODMR frequency vs. emission wavelength through the Trp 0,0-band region in GP32 (solid circles) and in the GP32–poly(5-HgU) complex (open circles). The sweep rate for both cases was 33 MHz s^{-1}. The temperature was 1.2 K, and the emission slit width was 1 nm. The transition frequencies were corrected for rapid passage effects. The error in these frequencies was estimated to be ±10 MHz.

circles) we can determine that at the 0,0-band peak of the unperturbed Trps (413.0 nm), the $|D| - |E|$ transition occurs at 1.608 GHz, while at the 0,0-band peak of the Trps that become perturbed (ca. 414 nm, which is in the region of wavelength-independent zero-field splittings), the $|D| - |E|$ transition is found at 1.629 GHz. These ODMR frequencies and phosphorescence 0,0-band peak wavelengths are both characteristic of Trp sites that are buried in a nonpolar but polarizable region of the protein (Hershberger, et al., 1980). The principal distinguishing feature of the Trp residues that undergo close stacking interactions with the DNA bases is that they are located at sites with wavelength-independent zero-field splittings. The Trp residues that remain unperturbed, however, are located at sites which produce characteristic wavelength-dependent zero-field splittings (Figure 7, open circles).

Both types of behavior of Trp zero-field splittings with monitored wavelength through the 0,0-band region have been observed in previous work. For instance, both the exposed and buried Trp sites of horse liver alcohol dehydrogenase show a linear decrease of $|D| - |E|$ with increasing wavelength, as does Trp in aqueous glassy solvents (von Schütz et al., 1974). The buried Trp residues of glyceraldehyde-3-phosphate dehydrogenase from pig and rabbit, however, show no measurable dependence of D and E on the detection wavelength (Davis and Maki, 1984). This lack of wavelength sensitivity also is characteristic of the single buried Trp residue of ribonuclease T1 from *Aspergillus oryzae* (Hershberger et al., 1980). A theory for the wavelength dependence of D and E based on spin–orbit coupling (Zewail, 1979) should not be applicable to Trp since the model is valid only for molecules in which spin–orbit coupling makes a significant contribution to the zero-field splittings. Spin–orbit effects are not of great importance in Trp on the basis of its long triplet-state lifetime. It is more likely that shifts in the zero-field splittings of Trp are caused by solvent electric fields, which induce the mixing of higher excited triplet states with the phosphorescent state. As shown by Van Egmond et al. (1975) [see also Gradl et al. (1986)], this model can lead to a linear dependence of the zero-field splitting parameters on phosphorescence wavelength. It is possible also, and this appears to be the case for many *buried* Trp sites in proteins, that there is very little correlation between the environment-induced Stark shift of the triplet energy and the zero-field splitting parameters. Thus far,

wavelength independence of the zero-field splittings has been observed only for buried Trp in proteins. Consequently, we can take this characteristic of the Trp residues that undergo heavy-atom interactions in GP32 as additional evidence that they are buried in the protein interior.

Conclusions

The red-shifted origin of the 0,0-band of Trp in GP32 and the low frequencies of the $|D| - |E|$ ODMR signals indicate that the residues are buried in polarizable, hydrophobic regions of the protein structure. The heavy-atom effect produced by poly(5-HgU) and poly(5-BrU) binding leads to the appearance of short-lived Trp phosphorescence lifetime components and to the occurrence of the $|D| + |E|$ slow-passage ODMR signal, which are indicative of van der Waals contact between Trp and the heavy-atom-derivatized uracil bases in the complex. The extent of the heavy-atom perturbation by Hg as well as steric considerations strongly support a stacking interaction of Trp with poly(5-HgU). Wavelength-selected ODMR spectroscopy throughout the 0,0-band of the Trp phosphorescence spectrum reveals the presence of two distinct types of Trp environments in GP32. Comparison with similar measurements made on the GP32–poly(5-HgU) complex reveals that the Trps in only one of these environments undergo a heavy-atom perturbation. This environment is characterized by wavelength-independent Trp zero-field splittings. Wavelength-independence of the zero-field splittings has thus far been found to occur only for *buried* Trp residues. The unperturbed Trp residues are located in an environment characterized by a distinct wavelength-dependence of the zero-field splittings, and a peak 0,0-band wavelength that is slightly blue-shifted relative to the Trp residues which are perturbed. The number of Trp residues present in each of these environments is not known, nor can we speculate at present on the reasons for the differences in their spectroscopic behavior. All emitting Trp residues are located in non-solvent-exposed, relatively polarizable environments, on the basis of the red-shifted phosphorescence and the ODMR frequencies observed. A consistent and reasonable model for these helix-destabilizing protein interactions with single-stranded polynucleotides is the insertion of the bases into interior hydrophobic regions of the protein. Here they are likely to encounter and undergo stacking interactions at specific sites containing Trp. Since this type of binding can occur only with single-stranded polynucleotide it serves in a direct manner to discriminate between single-stranded and duplex structures.

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Registry No. L-Trp, 73-22-3; poly(U), 27416-86-0; poly(5-BrU), 27988-64-3.

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Interaction of RecA Protein with a Photoaffinity Analogue of ATP, 8-Azido-ATP: Determination of Nucleotide Cofactor Binding Parameters and of the Relationship between ATP Binding and ATP Hydrolysis†

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ABSTRACT: The binding and cross-linking of the ATP photoaffinity analogue 8-azidoadenosine 5′-triphosphate (azido-ATP) with recA protein have been investigated, and through cross-linking inhibition studies, the binding of other nucleotide cofactors to recA protein has also been studied. The azido-ATP molecule was shown to be a good ATP analogue with regard to recA protein binding and enzymatic function by three criteria: first, the cross-linking follows a simple hyperbolic binding curve with a K_d of 4 μM and a cross-linking efficiency ranging from 10% to 70% depending on conditions; second, ATP, dATP, and adenosine 5′-*O*-(3-thiotriphosphate) (ATP-γ-S) specifically inhibit the cross-linking of azido-ATP to recA protein; third, azido-ATP is a substrate for recA protein ATPase activity. Quantitative analysis of the cross-linking inhibition studies using a variety of nucleotide cofactors as competitors has shown that the binding affinity of adenine-containing nucleotides for recA protein decreases in the following order: ATP-γ-S > dATP > ATP > adenylyl β,γ-imidodiphosphate (AMP-PNP) $\gg$ adenylyl β,γ-methylenediphosphate (AMP-PCP) $\approx$ adenine. Similar competition studies also showed that nearly all of the other nucleotide triphosphates also bind to recA protein, with the affinity decreasing in the following order: UTP > GTP $\approx$ dCTP > dGTP > CTP. In addition, studies performed in the presence of single-stranded DNA demonstrated that the affinity of ATP, dATP, ATP-γ-S, and AMP-PNP for recA protein is significantly increased. These results are discussed in terms of the reciprocal effects that nucleotide cofactors have on the modulation of recA protein–single-stranded DNA binding affinity and vice versa. In addition, it is demonstrated that nucleotide and DNA binding are necessary though not sufficient conditions for ATPase activity. The significance of this result in terms of the possible requirement of critically sized clusters of 15 or more recA protein molecules contiguously bound to DNA for ATPase activity is discussed.

The recA protein of *Escherichia coli* is a DNA-dependent ATPase which has been shown to play an important role in the processes of genetic recombination and UV-inducible DNA repair [for reviews see McEntee and Winstock (1981), Radding (1982), Dressler and Potter (1982), and Little and Mount (1982)]. The enzymatic activities of this protein in vitro include the renaturation of complementary strands of single-stranded DNA (Winstock et al., 1979), the assimilation of complementary single-stranded DNA into duplex DNA molecules (Shibata et al., 1979; McEntee et al., 1979; West et al., 1981a,b), and the proteolytic cleavage of lexA and λ repressor proteins (Craig & Roberts, 1980; Little et al., 1980). A requirement of each of these reactions is that recA protein must bind ATP (or a suitable ATP analogue), and in the case of the first two reactions, recA protein must hydrolyze ATP.

Although the exact mechanistic function of ATP binding and hydrolysis in these reactions is not yet understood, ATP and other nucleoside tri- and diphosphates have been shown to have a significant effect on the single-stranded DNA binding properties of recA protein (McEntee et al., 1981; Menetski

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