Close Range Interactions between Nucleotide Bases and Tryptophan Residues in an *Escherichia coli* Single-stranded DNA Binding Protein-mercurated Poly(uridylic acid) Complex

A STUDY BY OPTICALLY DETECTED MAGNETIC RESONANCE SPECTROSCOPY*

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Optically detected triplet state magnetic resonance spectra are reported for the complex formed between mercurated poly(Urd) and *Escherichia coli* single-stranded DNA binding protein. Upon forming a complex, the triplet state properties of Trp residue(s) in the protein are perturbed by the heavy mercury atom and are characterized by a shortened triplet state lifetime and the appearance of a strong D + E slow passage optically detected magnetic resonance signal. These features, which signal an external heavy atom effect, provide direct evidence for a close range interaction between mercurated nucleotide bases and Trp residues owing to the requirement of a van der Waals contact between the perturbed molecule and the heavy atom perturber. The amplitude-modulated phosphorescence microwave double resonance technique selectively displays the phosphorescence spectrum of the heavy atom-perturbed Trp triplet states. A van der Waals contact manifested through a stacked structure of the mercurated uridine base and the indole moiety of Trp is strongly suggested as the most plausible mode of interaction from steric considerations, since other approaches of the mercury atom are blocked by the covalent attachment of 2-mercaptoethanol to mercury. The magnitude of the heavy atom perturbation also is consistent with Hg approach to the π-system from above or below the indole aromatic plane, and is at least an order of magnitude larger than effects expected from an edge-on approach.

Stacking interactions between aromatic amino acid residues and nucleotide bases have been suggested as one of the recognition processes involved in forming specific protein-nucleic acid complexes (1, 2). A large volume of work has been reported in support of this idea through studies of model complexes formed between polynucleotides and aromatic amino acid-containing peptides (3). Specifically, monitoring the intrinsic Trp and Tyr fluorescence changes upon complex formation often has provided evidence for the involvement of these residues in complex formation (3). Detailed structural information about the complexes cannot be extracted from this method alone, however, since many different mechanisms may account for fluorescence quenching or enhancement in the complex environment (4). In particular, no direct evidence has been provided for stacking interactions in naturally occurring protein-nucleic acid complexes.

For this reason, we have been interested in establishing methods that will provide direct information about specific structural details, especially aromatic stacking interactions should these occur in biologically important protein-nucleic acid complexes. Previously, we have introduced a powerful spectroscopic technique, ODMR (5), in conjunction with an external heavy atom effect (6) to study the close range interaction between Trp residues and nucleotide bases in a model system in which complexes were formed between the mercurated polynucleotide poly(5-HgU), and the tripeptide Lys-Trp-Lys (7). ODMR spectroscopy (5) is a method that provides detailed information on the triplet state properties, especially in zero-applied magnetic field. Magnetic resonance within a triplet manifold is detected through optical quanta (phosphorescence) which gives the advantage of optical sensitivity over conventional microwave detection techniques. No external magnetic field is needed and the information about triplet state dynamics can be readily extracted from ODMR measurements. The external heavy atom effect (6) has the added advantage for detection sensitivity for reducing the triplet state lifetime and frequently enhancing its radiative strength. As a result, time-resolved ODMR experiments (8) can be performed from which the perturbed state responses can be selected from the background of unperturbed molecules. Significant heavy atom effects occur only if the perturber and perturbed molecules are within the van der Waals distance (6). Thus, the perturbation acts as a probe of extremely close range interactions.

As an extension of our previous work (7), we report in this paper our ODMR investigations of the complex formed between poly(5-HgU) and a native protein, *Escherichia coli* SSBP. SSBP is a protein with a high binding affinity toward single-stranded DNA in *vitro* (9). Its biological role in *vitro* is believed to involve the stabilization of single-stranded regions of DNA that are structurally essential in DNA replication, repair, and recombination activities through the formation of specific complexes with DNA (10). This protein contains 3 Trp residues (11, 12) whose fluorescence is found to be quenched by about 70% upon forming complexes with polynucleotides (13, 14). In this work, we present direct evidence that at least 1 of the Trp residues of SSBP is in the close vicinity of nucleotide bases in these complexes. Furthermore,

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1 The abbreviations used are: ODMR, optically detected magnetic resonance; SSBP, single-stranded binding protein; D and E, zero-field splitting parameters; AM-PMDR, amplitude-modulated phosphorescence-microwave double resonance; poly(5-HgU), mercurated poly(uridylic acid).
Aromatic stacking is the most plausible type of interaction for steric reasons, as well as from a consideration of the magnitude of the external heavy atom perturbation.

**MATERIALS AND METHODS**

Poly(5-HgU) and *E. coli* SSBP were obtained from P-L Biochemicals and were used without further purification. About 70% of the uridine residues in the polynucleotide have a mercury atom covalently attached, according to the manufacturer. A stock solution of poly(5-HgU) was made by dissolving the polynucleotide in 1 mM cacodylate, pH 7, buffer containing 0.1 mM EDTA. *E. coli* SSBP was stored in 50 mM Tris-HCl, pH 7.5, buffer containing 1 mM EDTA, 1 mM dithiothreitol, 0.3 M NaCl, and 50% glycerol in which it was supplied. Lys-Trp-Lys was a product of Research Plus.

The complex was prepared by mixing stock solutions of mercurated polynucleotide and SSBP in the presence of a slight excess of 2-mercaptoethanol relative to Hg, and incubated at 37 °C for 10 min. A 15-pl sample was transferred to a Suprasil quartz sample tube (1-mm inner diameter) which then was immersed in liquid nitrogen or liquid helium for spectroscopic measurements. About 16% glycerol remained in the sample and served as the cryogenic solvent required for the low temperature work. The sample concentrations were calculated to be 330 μM in polynucleotide in terms of phosphate, and 33 μM for the protein, calculated from ε₉₅ = 3.3 × 10⁴ M⁻¹ cm⁻¹ (15).

The binding of SSBP to single-stranded DNA has been reported to be cooperative (18) and a ratio of 8 nucleotides to 1 protein monomer has been estimated for quantitative complexing (9, 13, 15).

The apparatus and experimental set up for measurements of phosphorescence spectra, lifetimes, and slow passage ODMR spectra have been described previously (7, 16). Briefly, slow passage ODMR experiments were performed by monitoring the phosphorescence intensity while sweeping microwaves through a pair of triplet sublevels in zero-applied field to induce magnetic resonance transitions. Signal averaging was performed. The microwave sweep rate was so adjusted that passage through a transition was slower than the longer lived triplet sublevel decay time in the resonance pair. An optical response (phosphorescence change) corresponding to microwave-induced sublevel population transfer was observed due to the differences in the steady state populations and radiative properties between the sublevels. The transition frequencies were determined by averaging the peak frequencies observed with microwaves swept in both directions at the same scan rate. Only two parameters, D and E, are sufficient to express the zero-field splittings (17). In the case of a heavy atom perturbation, the resulting short lived triplet state responds to a rapid phosphorescence spectra, lifetimes, and slow passage ODMR spectra have previously been described (14, 16).

The AM-PMDR experiment was performed by sweeping the microwaves through a transition faster than the shorter lived sublevel decay time in the transition pair. A biexponential transient response corresponding to the decays of the individual sublevels, T₁ and T₂, connected by the transition is obtained. A mathematical description of this transient response has been given previously (18).

\[ \Delta I(t) = \alpha k₁ \exp(-k₁t) - k₂ \exp(-k₂t) \]

where ΔEt is the phosphorescence transient; α is a constant which depends on the apparatus and the difference in steady state populations between T₁ and T₂; k₁ and k₂ are the total decay constants of T₁ and T₂ while k₁' and k₂' are their radiative rate constants.

A general discussion of AM-PMDR has been given (19), and the experimental details of this measurement as done in our laboratory have been reported recently (20). In this method, the microwave frequency is fixed at resonance, amplitude modulation is introduced by a pin diode to modulate the microwave power on and off. The amplitude modulation frequency is fed as a reference signal to an Omen model 9412A phase-sensitive detector. The output of the phase-sensitive detector is accumulated in a Nicolet model 102D signal averager by repetitively sweeping the emission monochromator through the sample phosphorescence region. This method is particularly useful for discriminating between chromophores that emit in the same spectral region if the microwave frequency is selected to correspond to an ODMR signal of one of them, only.

The analysis of phosphorescence decays has been described previously (16). The multieponential decays were deconvoluted and analyzed by computer. The analysis was considered to be satisfactory when differences between the calculated decay curves and the experimental data were less than 5%.

**RESULTS AND DISCUSSION**

**Phosphorescence Spectra and Lifetime**—The phosphorescence spectra of *E. coli* SSBP and SSBP complexed with poly(5-HgU) are shown in Fig. 1, A and B, respectively. The phosphorescence spectrum of the protein (A) resembles Trp emission with little contribution of Tyr.² The Trp 0.0-band region is not clearly resolved and is red-shifted relative to the 0.0-band of Trp in an aqueous solvent (21). These features indicate, consistent with previous fluorescence studies (14), that the microenvironment of the Trp in SSBP is very heterogeneous and is less polar than the aqueous solvent (22). It is significant, however, that the phosphorescence spectrum of the complex (Fig. 1B) shows a sharper 0.0-band peaked at approximately 412 nm. The differences between these two spectra (Fig. 1 A and B) suggest that there exists a particular Trp residue whose 0.0-band peaks in the 415 to 420 nm region, which is selectively either quenched, shifted, or broadened upon complexing with the polynucleotide. The AM-PMDR spectrum of the perturbed Trp shown in Fig. 1C illustrates that the latter is the correct explanation. We defer this discussion until later. It is noteworthy at this point that the previously reported fluorescence studies of SSBP indicate no apparent shift in the wavelength of the fluorescence maximum, but rather a decrease in quantum yield upon binding to DNA (14). Therefore, the fluorescence spectrum does not differentiate between the different Trp sites present in the protein. The phosphorescence spectrum, on the other hand, ²Tyr residues in SSBP have been shown to be not involved in the nucleotide binding process based on chemical modification studies (14). Therefore, we focus on the study of Trp residues by exciting the sample at 295 nm (16 nm band width) where Tyr absorption is relatively insignificant.
perturbation, however, the induced spin-orbit coupling previously in a model system of Lys-Trp-Lys complexed with appearance of this ODMR signal is diagnostic for a heavy atom effect. The D+E transition obtained by sweeping microwaves through the D+E transition in about 2 ms. Signal averaging is carried out for 5000 scans. For A and B, phosphorescence is monitored at 416 nm with 3-nm slits and T = 1.1 K. A fast passage transient response characterizes the decays of the two sublevels connected by the magnetic resonance transition. The transient response observed in the complex for the D+E transition is shown in Fig. 2B. The negative decay component is not observed due to a large difference between the radiative rate constants of the coupled sublevels. Nevertheless, a decay lifetime of 3 to 5 ms can be estimated for the more radiative sublevel, and is consistent with the results of mercury-perturbed Trp sublevel lifetimes reported earlier (7, 23).

In Fig. 3, we compare the slow passage ODMR spectra of the two low frequency transitions (D-E and 2E) of the protein and the complex. It should be noted that the microwave sweep rate used to produce the spectrum of the complex (Fig. 3A) is two orders of magnitude faster than that used to produce the spectrum of the protein (Fig. 3B). Again, under the rapid sweep rate conditions, no signal is observed in the protein sample. These results indicate that the signals in Fig. 3A originate from a perturbed shorter lived triplet state than is found in the protein. Fig. 3B, obtained under slower microwave sweep conditions, represents the unperturbed Trp D-E and 2E signals. In Table I, we summarize the slow passage ODMR transition frequencies and D, E values measured in SSBP and in the complex together with those observed previously (7) in the model system for comparison.

Stacking interactions of Trp generally result in a reduction of the D value (25). The use of Hg as the perturber atom counteracts this effect (26), however. Consequently, other factors must be taken into consideration for elucidating the presence of a stacked complex. From our previous studies on the complexes formed between Lys-Trp-Lys and poly(5-HgU) (7), we found that the D-E and 2E frequency region of Trp perturbed by an unblocked Hg is characterized by broad, unresolved signals. In the present study, an excess of 2-mercaptoethanol is included in the buffer system and widely split and well resolved D-E and 2E signals are observed. These features can be associated with a Hg atom which is well protected from direct binding with the peptide. Similar well resolved D-E and 2E transitions are observed in the Lys-Trp-Lys complex with poly(5-HgU) blocked with 2-mercaptopethanol (7). From the point of view of steric constraints, the mercurated uridine and the indole moiety of Trp must be stacked in order for the Hg atom to approach van der Waals

As given in the equation under "Materials and Methods,"

![Image](https://example.com/image.png)

**Fig. 2.** D+E ODMR responses of perturbed Trp in SSBP-poly(5-HgU) complex. A, slow passage D+E ODMR signal of perturbed Trp in SSBP-poly(5-HgU) complex. Scan rate is 42 GHz s⁻¹, and signal averaging is for 5000 scans. B, rapid passage phosphorescence transient obtained by sweeping microwaves through the D+E transition in about 2 ms. Signal averaging is carried out for 5000 scans. For A and B, phosphorescence is monitored at 416 nm with 3-nm slits and T = 1.1 K. The deconvoluted lifetimes of the complex, when monitored at 416 nm, give an appreciable amount (20%) of a very short 14-ms decay component, in addition to a 5.4-s component from trace amounts of impurity present in the buffer system. The deconvoluted lifetimes of the complex, when monitored at 416 nm, give an appreciable amount (20%) of a very short 14-ms decay component, in addition to a 5.4-s component from normal Trp. This several hundred-fold reduction of the triplet state lifetime is characteristic of a heavy atom perturbation by Hg in van der Waals contact with Trp (23). In the CH₃Hg-Trp complex, a phosphorescence lifetime of approximately 10 ms is found. The Hg atom is known to lie above the indole plane in van der Waals contact with the π-system (24). The shortening of the Trp triplet state lifetime to a similar extent upon Hg perturbation has been observed previously in a model system of Lys-Trp-Lys complexed with poly(5-HgU) (7).

**Fig. 3.** D-E and 2E transitions of Trp in SSBP-poly(5-HgU) complex (A), and SSBP (B). Sweep rates are 13.6 GHz s⁻¹, 5,000 scans (A), and 32 MHz s⁻¹, 64 scans (B). T = 1.1 K, and phosphorescence is monitored at 416 nm with 3-nm slits. The phosphorescence lifetimes for both the protein and the complex were measured at 77 K and analyzed. Nonexponential decays were observed in both cases. The phosphorescence decay of the protein has a major component of 5.8 s representing the Trp lifetime. A minor lifetime component (13%) of 150 ms also is observed, however, and probably originates from trace amounts of impurity present in the buffer system. The deconvoluted lifetimes of the complex, when monitored at 416 nm, give an appreciable amount (20%) of a very short 14-ms decay component, in addition to a 5.4-s component from normal Trp. This several hundred-fold reduction of the triplet state lifetime is characteristic of a heavy atom perturbation by Hg in van der Waals contact with Trp (23). In the CH₃Hg-Trp complex, a phosphorescence lifetime of approximately 10 ms is found. The Hg atom is known to lie above the indole plane in van der Waals contact with the π-system (24). The shortening of the Trp triplet state lifetime to a similar extent upon Hg perturbation has been observed previously in a model system of Lys-Trp-Lys complexed with poly(5-HgU) (7).

**ODMR Spectra**—The D+E slow passage ODMR signal is not observed in unperturbed Trp since the triplet sublevels connected by this transition decay radiatively in the absence of a heavy atom perturbation (21). With a heavy atom perturbation, however, the induced spin-orbit coupling enhances the radiative strength of the triplet sublevels through selective intersystem crossing routes; the D+E transition becomes detectable when phosphorescence is monitored. The appearance of this ODMR signal is diagnostic for a heavy atom effect. The D+E ODMR signal observed in the complex of SSBP with poly(5-HgU) is shown in Fig. 2A. Under identical experimental conditions, no comparable signal is observed in the protein sample. These results confirm the presence of at least one heavy atom-perturbed Trp residue in the complex.

As given in the equation under "Materials and Methods,"
Table I

| Sample            | D - E | 2E | D + E | D | E |
|-------------------|-------|----|-------|---|---|
| Lys-Trp-Lys        | 1.72  | 2.49 | -     | - | - |
| Lys-Trp-Lys + poly-(5-HgU) | 1.69  | 2.61 | 4.17  | - | - |
| Lys-Trp-Lys + poly-(5-HgU) + 2-Me | 1.65  | 2.35 | -     | - | - |
| SSBP              | 1.60  | 2.60 | 4.15  | - | - |
| SSBP + poly-(5-HgU) + 2-Me | 1.64  | 2.60 | 4.15  | - | - |

*From Ref. 25.

*Signal is not observed.

*From Ref. 7.

*Signals are broad and unresolved.

contact with the indole π-electrons and thereby produce a heavy atom perturbation of the magnitude observed. It should be pointed out that edge-on approach of a Hg atom to an aromatic molecule, such as occurs in the CH₃Hg⁺ complex of benzimidazole (24) yields sublevel lifetimes which are more than an order of magnitude longer than those observed in the SSBP-poly(5-HgU) complex.

**AM-PMDR Spectra**—As mentioned above, in a complicated system in which several sites contribute to the phosphorescence emission in the same spectral region, the AM-PMDR technique is particularly useful in discriminating between individual contributions, if the microwave frequency is chosen to correspond to an ODMR signal of only one emitting site. Since the D + E transition is observed only in the perturbed Trp, modulating this transition will produce an AM-PMDR spectrum which represents the phosphorescence spectrum of the perturbed Trp alone. Fig. 4 is introduced at this time to demonstrate the correspondence between the phosphorescence and the AM-PMDR spectrum. Fig. 4, A and B, are the phosphorescence spectra of Lys-Trp-Lys and Lys-Trp-Lys complexed with poly(5-HgU), respectively. Based on the lifetime measurements made earlier (7), >90% of the phosphorescence intensity in the complex originates from heavy atom-perturbed Trp. Fig. 4C shows the AM-PMDR spectrum obtained by modulating the D + E ODMR transition of the complex. It is seen to be nearly identical with the phosphorescence spectrum of the complex (Fig. 4B).

Fig. 1C displays the AM-PMDR spectrum of SSBP complexed with poly(5-HgU) with microwaves modulated in the D + E ODMR transition (Fig. 2A). The spectrum is broad and lacks the well resolved structure which is typical of unperturbed Trp. These features account for the changes observed in the Trp phosphorescence spectrum of SSBP upon complexing. It is very interesting to make a comparison of the AM-PMDR spectrum with a previously studied case where the enzyme glyceraldehyde 3-phosphate dehydrogenase from rabbit is perturbed by CH₃Hg⁺ binding with sulfhydryls (20). In that system, CH₃Hg⁺ complexed with a particular Cys residue perturbs a specific Trp site in the immediate vicinity. One of the optically resolved Trp 0-bands disappears from the structured phosphorescence spectrum as the result of considerable spectral broadening due to the Hg perturbation. The reported AM-PMDR spectrum (20) is very similar to that observed in the present system. Comparing Figs. 1C and 4C, the very different AM-PMDR spectra indicate that the tripeptide-polynucleotide complex differs from the protein-polynucleotide complex as far as the heavy atom-induced phosphorescence is concerned. Along similar lines, a recent report has shown that the complexes formed between gene-5 protein of bacteriophage M13 (also a single-stranded DNA binding protein) with oligonucleotides and polynucleotides are structurally distinct (27).

Comparing the spectra of Fig. 1, the interesting observation can be made that the Trp(s) which is(are) perturbed in the SSBP-polynucleotide complex has(have) a quite red-shifted (415 to 420 nm) 0,0-band. Phosphorescence 0,0-bands of solvent-exposed Trp invariably occur to the blue of 410 nm (22); it is clear, then, from the phosphorescence spectra that complex formation of SSBP with a single-stranded polynucleotide involves stacking of bases with buried Trp residues. Whether in the complex the buried Trp is pulled out of the protein interior, or whether the base is inserted into the protein interior is an interesting question which cannot be answered at present. It seems reasonable, however, that the latter mechanism would discriminate more positively between single-stranded and duplex structures.

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