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Influence of the Mercury Blocking Reagent 2-Mercaptoethanol on the Spectroscopic Properties of Complexes Formed between Lysyltryptophyllysine and Mercurated Poly(uridylic acid)†

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ABSTRACT: Optical detection of magnetic resonance (ODMR) studies are reported for complexes formed between the tripeptide Lys-Trp-Lys and poly(5-HgU), both in the absence and in the presence of the blocking reagent 2-mercaptoethanol (ME). Complexes formed both with and without ME show characteristics of a heavy atom effect: quenching of Trp fluorescence, enhancement of the Trp phosphorescence quantum yield, a drastic reduction of the Trp phosphorescence lifetime, and the appearance of a Trp $|D| + |E|$ ODMR signal at ca. 4.2 GHz. Significant differences are found, however, in the photophysical properties of the complexes formed with and without ME. In the absence of ME, the Trp phosphorescence bands are broad, the 0,0 band is shifted to 415.3 nm, and the $|D| - |E|$ and $2|E|$ ODMR transitions are broad and poorly resolved. These features are characteristic of an inhomogeneous Trp environment. In the presence of ME, the phosphorescence peaks are narrower, with the 0,0 band shifted to 411.6 nm. The $|D| - |E|$ and $2|E|$ ODMR transitions are well resolved and shifted in frequency relative to the unblocked complex. These features point to a more homogeneous Trp environment in the presence of ME. UV difference spectra show hypochromicity in the poly(5-HgU) absorption band (indicating induced stacking) which occurs on binding of Lys-Trp-Lys with ME present, while in the absence of ME, hypochromicity occurs primarily in the Trp absorption bands. Reversal of these effects with added $NaClO_4$ occurs in both cases, but higher ionic strength is required with ME present. These results are consistent with the formation of stacked complexes in the presence of ME, but with additional types of complexes in its absence. The additional complexes formed in the absence of ME do not contribute to stacking of poly(5-HgU) and may involve direct binding of mercury to amines of Lys-Trp-Lys; binding occurs between Lys-Trp-Lys and the monomer 5-HgUTP in the absence of ME, but not when the Hg is blocked with ME.

Protein–nucleic acid interactions are by any measure among the most important processes operating in life. Numerous model systems have been subjected to extensive study in order to arrive at an understanding of the nature and the specificity of the interactions involved in protein–nucleic acid associations. In particular, studies of the complexes formed between oligopeptides and polynucleotides have provided some valuable information about these interactions (Hélène, 1981). Among the various types of interactions which may be operating in protein–nucleic acid associations, stacking interactions between aromatic amino acid residues and the nucleotide bases have been proposed as important in the specific recognition of nucleic acids by proteins (Gabbay et al., 1976; Toulmé & Hélène, 1977).

In this paper, we report on our investigations of complexes formed between the tripeptide Lys-Trp-Lys and the heavy atom derivatized polynucleotide poly(5-HgU). We make particular use of optical detection of triplet state magnetic resonance (ODMR)[1] in this work. ODMR spectroscopy has been developed in several laboratories over the last decade and has proven to be a useful method for studying the excited triplet state properties of proteins and nucleic acids (Maki & Zuclich, 1975; Kwiram, 1982; Maki, 1982). The external

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[1] Abbreviations: ODMR, optical detection of (triplet state) magnetic resonance; ME, 2-mercaptoethanol; ZFS, zero-field splitting; D and E, ZFS parameters; EDTA, ethylenediaminetetraacetic acid; EG, ethylene glycol; EGB, 50% v/v mixture of ethylene glycol and aqueous buffer; poly(U), poly(uridylic acid).

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heavy atom effect (Kasha, 1952) has been utilized in connection with ODMR spectroscopy to probe the interactions of heavy atom probes with biological chromophores such as tryptophan (Hershberger & Maki, 1980a) and the nucleotide bases (Luk et al., 1975; Anderson et al., 1980; Maki & Ott, 1981). In the present work, we use ODMR to investigate the heavy atom effects induced by the close approach (within the van der Waals radii) of the Hg atom of the polynucleotide to the Trp of the associated peptide. If the peptide–polynucleotide association involves stacking between Trp and the mercurated nucleotide bases, the Hg atom may be brought in direct contact with the aromatic π electrons and thereby alter the Trp excited-state properties. Heavy atom effects would be observed. Nonspecific complexes, which may not involve the stacking of Trp with the perturber nucleotide, will also exhibit a heavy atom effect provided the Hg atom is located in the vicinity of the Trp π electrons. The association of Lys-Trp-Lys with poly(5-HgU) has been studied previously by Hélène and coworkers (Hélène, 1979; Hélène et al., 1979), who reported the presence of typical external heavy atom effects: Trp fluorescence quenching, reduction of the Trp phosphorescence lifetime, and enhancement of the Trp phosphorescence quantum yield. The heavy atom effects were assigned to stacking of Trp with the heavy atom perturber 5-HgU. The complexes dissociate as the salt concentration is increased, suggesting that Coulombic forces between the charged amines and phosphates are largely responsible for the association.

The main object of our work is to compare the spectroscopic properties of the complexes formed in the presence of and in the absence of sulfhydryl blocking reagent, mercaptoethanol (ME). Mercury is known to bind to the α-amino group of amino acids, and the stability of CH_3Hg^{II} complexes of aromatic amino acids is particularly high (Rabenstein et al., 1974; Svejda et al., 1978; Hershberger & Maki, 1980b). Thus, the binding of unblocked Hg to the amines of Lys-Trp-Lys in the association with poly(5-HgU) is a distinct possibility. Dale & Ward (1975) have shown that mercurated DNA acts as a template for in vitro synthesis of RNA only if the Hg atom is blocked with a mercaptide. In the absence of blocking reagent, the mercurated nucleotides are thought to complex directly with accessible cysteines and possibly other residues of RNA polymerase.

Materials and Methods

Mercurated nucleotides and polynucleotides were obtained from P-L Biochemicals. The poly(5-HgU) contains about 70% mercurated uridine residues according to the manufacturer. L-Tryptophan was purchased from Calbiochem-Behring while Lys-Trp-Lys was a product of Research Plus. All other commercial reagents were of the highest available purity.

Poly(5-HgU), 5-HgUTP, Lys-Trp-Lys, and Trp were dissolved in 1 mM pH 7 cacodylate buffer containing 1 mM NaCl and 0.1 mM EDTA. A slight excess of 2-mercaptoethanol (Sigma) was added to the nucleotide solutions in order to block the Hg. Complexes were prepared by mixing appropriate volumes of stock solutions in an ice bath. All samples subjected to luminescence and ODMR measurements were mixed with an equal volume of ethylene glycol, EG, (Matheson Coleman & Bell, chromatography grade) before being transferred to 1-mm i.d. quartz sample tubes for measurement.

The apparatus for measurement of 77 K phosphorescence spectra and slow passage ODMR spectra at ca. 1.1 K has been described previously (Maki & Co, 1976). The luminescence was passed through a McPherson, Inc., Model 2051 monochromator with 3-nm slits and was detected with a cooled EMI Model 9789QA photomultiplier. The excitation light was passed through an Instruments SA H-10 grating monochromator with a 16-nm band-pass. A Corning CS 7-54 filter was used in the excitation path, and Schott WG-345-2 cutoff filter was used in the emission path. Phosphorescence decays were analyzed and deconvoluted by computer using the procedure described earlier (Maki & Co, 1976). The nonexponential decays were arbitrarily fit to a maximum of three exponential components. The analysis was considered satisfactory when differences between the theoretical decay curves and the experimental data were less than 5%. Slow passage ODMR frequencies were obtained by averaging the peak frequencies observed with microwaves swept in both directions at the same scan rate. Signals from perturbed, short-lived triplet states were sorted out by altering the microwave sweep rate as described earlier (Hershberger & Maki, 1980a).

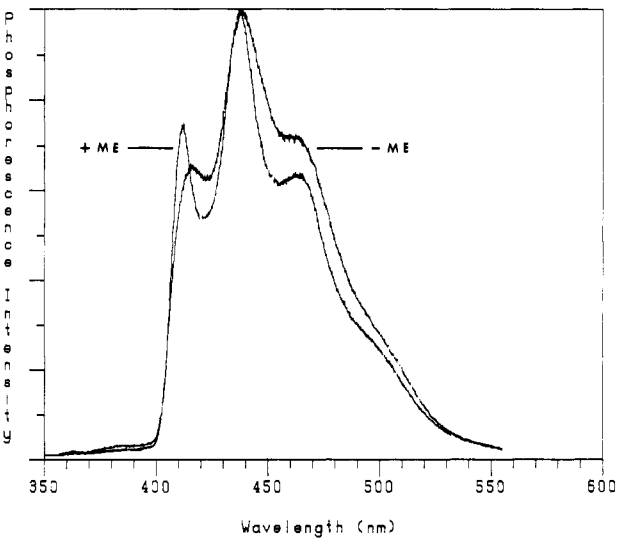

FIGURE 1: Phosphorescence spectra of complexes formed between the tripeptide Lys-Trp-Lys and poly(5-HgU), both in the presence and in the absence of the blocking reagent ME. Concentrations are 2.6×10^{-4} and 5.5×10^{-5} M for poly(5-HgU) and Lys-Trp-Lys, respectively, in EGB at $T = 77$ K.

Ultraviolet difference spectra were obtained on a Hewlett-Packard Model 8450A UV/vis spectrophotometer at ice–water temperature. Difference spectra were obtained by subtracting absorption spectra of the mixture from the sum of the absorption spectra of the separated components in the mixture. The salt concentration was altered by adding appropriate amounts of concentrated stock salt solution to the mixture. The final volumes of the mixtures were kept constant by adjusting the volume of buffer in the mixture. Sodium perchlorate was used instead of sodium chloride in order to eliminate complexing between chloride and Hg (Rabenstein, 1978).

Results

Phosphorescence Spectra and Lifetimes. The 77 K phosphorescence spectra of complexes formed between Lys-Trp-Lys and poly(5-HgU) both in the absence of ME and in the presence of ME are compared in Figure 1. The quenching of Trp fluoresence and the enhancement of the Trp phosphorescence quantum yield (relative to Lys-Trp-Lys) are observed in both samples. Significant differences, however, are present in the phosphorescence spectra of the complexes formed with and without ME. Both 0,0 bands are shifted to the red relative to that of Lys-Trp-Lys, which occurs at 406 nm (Co & Maki, 1978). In the presence of ME, the Trp 0,0 band occurs at 411.6 nm, while in the absence of ME, the 0,0 band is shifted further to 415.3 nm. The phosphorescence

Table I: Phosphorescence Decays of Lys-Trp-Lys and Its Complexes with Poly(5-HgU)[a]

sample	λ_{exc} (nm)	λ_{obsd} (nm)	α[b] (%)	τ (s)
Lys-Trp-Lys[c]	290	410	79	6.92
			21	4.38
Lys-Trp-Lys + poly(5-HgU)[d]	290	416	10	5.66
			29	0.14
			61	0.009
	280	395	43	1.98
			57	0.051
Lys-Trp-Lys + poly(5-HgU) + ME[d]	290	411	12	5.50
			32	0.15
			56	0.010
	280	395	42	2.10
			58	0.049

[a] T = 77 K. Samples are dissolved in EGB. [b] Preexponential factor of lifetime component. [c] Data are from Co & Maki (1978). [d] Concentrations are the following: Lys-Trp-Lys, 1.1 × 10^{-4} M; poly(5-HgU), 5.2 × 10^{-4} M in nucleoside phosphate.

decays predominantly with greatly reduced lifetimes (see below) and thus represents the spectrum of heavy atom perturbed Trp. The spectrum of the complexes formed in the presence of ME is better resolved, consisting of relatively narrow bands.

Phosphorescence lifetimes are reported in Table I. The lifetimes of the Trp residues were obtained by monitoring the Trp 0,0 band and exciting the sample at 290 nm. The lifetimes of poly(5-HgU) were obtained by using somewhat higher energy excitation (280 nm) and monitoring the emission at 395 nm where only the polynucleotide emits a weak phosphorescence. Nonexponential decays were obtained in all cases. The presence of long, intermediate, and short Trp lifetimes indicates the presence of unperturbed and of variably perturbed Trp residues in these samples.

ODMR Spectra. The slow-passage ODMR signals observed from the samples of poly(5-HgU) complexes with Lys-Trp-Lys, with and without ME, are given in Table II. The signals are attributed to heavy atom perturbed Trp; the passage rates in these measurements were sufficiently fast that unperturbed Trp gives no ODMR responses. A strong ODMR signal is observed at 4.2 GHz, which we attribute to Trp (ν_3, $|D| + |E|$) both in the presence and in the absence of ME. These signals are compared in Figure 2. The $|D| + |E|$ signal is not observed in unperturbed Trp, since the triplet sublevels connected by this transition decay radiationlessly in the absence of a heavy atom perturbation (Svejda et al., 1978). The appearance of this ODMR signal is diagnostic for the presence of a heavy atom effect which induces the sublevels to emit phosphorescence. The lower frequency $|D| - |E|$ and $2|E|$ signal region is shown for complexes formed with and without ME in Figure 3. Two well-resolved ODMR signals are observed for the complexes formed in the presence of ME, whereas in the absence of the blocking reagent, a broad response is obtained, suggesting a large degree of heterogeneity in the ZFS of the emitting Trps.

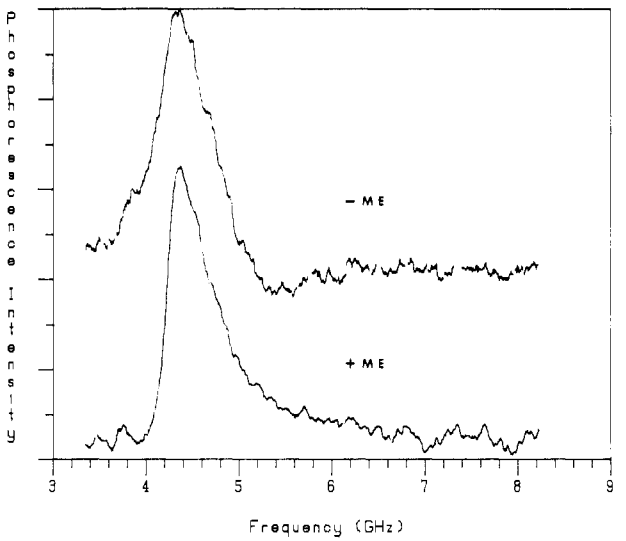

FIGURE 2: High-frequency, $|D| + |E|$, slow-passage ODMR transition of the complexes described in Figure 1. Microwave frequency is swept from 3.7 to 8.3 GHz at the rate of 15 MHz/ms. Signals are averaged for 2000 scans.

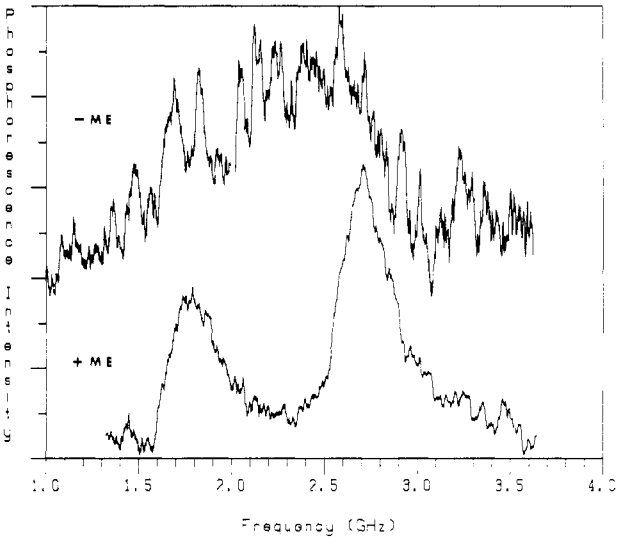

FIGURE 3: Slow-passage ODMR spectra in the ν_1 and ν_2 signal region of Lys-Trp-Lys complexed with poly(5-HgU) in the presence and in the absence of ME. In the presence of ME, the microwaves are swept from 1.4 to 3.5 GHz at a rate of 4 MHz/ms. Signal averaging is carried out over 2000 scans. In the absence of ME the microwave frequency is swept from 1.0 to 3.5 GHz at a rate of 5.8 MHz/ms with signal averaging for 3000 scans. Sample concentrations are given in Figure 1.

Table II: Zero-Field ODMR Frequencies and Zero-Field Splittings[a]

sample	λ_{exc} (nm)	λ_{obsd} (nm)	ν_1 (GHz)	$\Delta\nu_1$ (MHz)	ν_2 (GHz)	$\Delta\nu_2$ (MHz)	ν_3 (GHz)	$\Delta\nu_3$ (MHz)	$\lvert D\rvert$ (cm^{-1})	$\lvert E\rvert$ (cm^{-1})
tryptophan[b]	295	406	1.74	165	2.45	345		[c]	0.0988	0.0408
CH_3Hg^{II}-Trp[d]	290	409	1.97	200	2.17	340	4.23	270	0.1048	0.0362
5-HgUTP + Trp[e]	290	408		[f]		[f]	4.22	290		
5-HgUTP + Lys-Trp-Lys[g]	280	408	1.74	240	2.47	310	4.18	495	0.0992	0.0412
poly(5-HgU) + Lys-Trp-Lys	290	416		[f]		[f]	4.25	635		
poly(5-HgU) + Lys-Trp-Lys + ME	290	411	1.69	215	2.61	310	4.17	485	0.0998	0.0435

[a] Phosphorescence is monitored at the peak 0,0-band wavelength. T = 1.1 K. Width of ODMR transitions is given as the full width at half-maximum intensity. Assignments of $|D|$ and $|E|$ are made assuming that $\nu_2 = 2|E|$. [b] Data are from Zuclich et al. (1973). [c] No ODMR signal is observed. [d] Data are from Svejda et al. (1978). [e] 5-HgUTP is 8.4 × 10^{-4} M, and Trp is 8.4 × 10^{-5} M in EGB. [f] Signals are intense, but broad and unresolved. The ODMR maximum is found near 2 GHz. [g] 5-HgUTP and Lys-Trp-Lys are each 2.8 × 10^{-4} M in EGB.

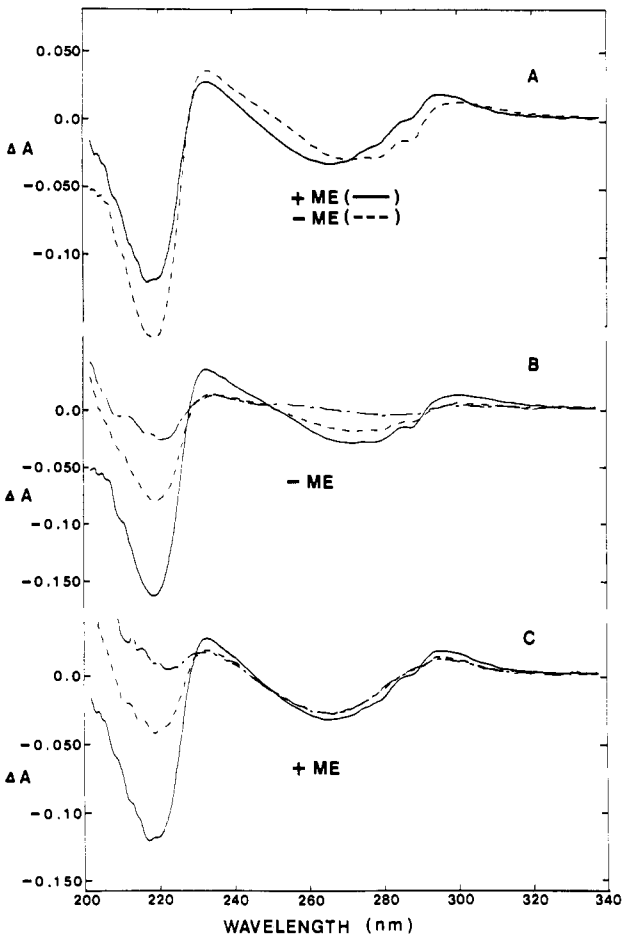

FIGURE 4: Ultraviolet difference spectra of poly(5-HgU) complexed with Lys-Trp-Lys. In (A), the solid line represents the complex formed in the presence of ME, and the dashed line represents the complex formed in the absence of ME. In (B) and (C), $NaClO_4$ concentrations are varied from 0 to 100 mM and are as follows: 0 (—), 25 (- - -), and 100 mM (– – –). Concentrations of poly(5-HgU) and Lys-Trp-Lys are 1.3×10^{-4} and 2.7×10^{-5} M, respectively.

For determination of whether complexes exhibiting a Trp heavy atom effect are formed when either Trp or Lys-Trp-Lys interact with a mercurated mononucleotide, Trp and Lys-Trp-Lys were mixed with a solution of 5-HgUTP both in the absence and in the presence of ME. In the presence of ME, the phosphorescence 0,0 bands of Trp and of Lys-Trp-Lys are found at unperturbed wavelengths, and the ODMR signal in the $|D| + |E|$ signal region is extremely weak, indicative of very little complexing with blocked 5-HgUTP. With unblocked 5-HgUTP, on the other hand, the 0,0 bands of Trp and Lys-Trp-Lys are noticeably red shifted, and intense ODMR signals are found, indicating the formation of complexes which induce a heavy atom effect. The ODMR data are given in Table II.

Ultraviolet Difference Absorption Spectra. The UV difference spectra obtained from poly(5-HgU) complexed with Lys-Trp-Lys in the presence and in the absence of ME are compared in Figure 4A. The spectra clearly differ, indicating differences in the interactions. At wavelengths above 280 nm and near 220 nm, where the Trp absorption dominates, more hypochromicity is observed in the complexes formed without ME present. At wavelengths near 265 nm, where poly(5-HgU) absorption dominates, more hypochromicity is observed with ME present. The effect of added $NaClO_4$ on the difference spectra is shown in Figure 4B, which is in the absence of ME, and in Figure 4C, in the presence of ME. The complexes formed in the absence of ME are seen to dissociate completely in the presence of 100 mM $NaClO_4$, but dissociation is incomplete in the presence of ME. Dissociation of the complexes formed in the presence of ME does occur, but at considerably higher ionic strength.

Discussion

The association constant of Lys-Trp-Lys with poly (5-HgU) is not known, although this quantity has been measured for poly(U) (Brun et al., 1975). Assuming similar affinities of Lys-Trp-Lys with poly(U) and poly(5-HgU), we expect that approximately 70% of Lys-Trp-Lys is complexed with poly-(5-HgU) under the conditions we used for obtaining UV difference spectra with no added salt. We have no quantitative estimate of the extent of complexing in EGB at low temperatures, although the observed Trp heavy atom effects (see below) indicate that complexes are present.

The phosphorescence spectra, UV difference absorption spectra, and ODMR spectra presented earlier indicate that structurally distinct complexes are formed between poly(5-HgU) and Lys-Trp-Lys with and without ME. The UV difference spectra, Figure 4A, provide direct evidence for the different nature of the complexes formed. In the absence of ME, the difference spectrum in the 240–300-nm region resembles the absorption of Trp very closely (Wetlaufer, 1962), while in the presence of ME the difference spectrum is shifted to the blue and appears to contain a significant hypochromic contribution from the polynucleotide absorption bands. The hypochromic effects disappear completely in the absence of ME when the ionic strength is increased to 100 mM $NaClO_4$, whereas with ME present the hypochromism persists at this salt concentration (Figure 4B,C). The broadened phosphorescence spectrum of Lys-Trp-Lys complexed with poly-(5-HgU) in the absence of ME, along with the broadened ODMR spectra, suggests a more heterogeneous Trp environment in these complexes, in comparison with complexes formed with ME which yield narrower Trp phosphorescence and ODMR spectra. This heterogeneity could well be related to direct binding of amino groups of the peptide to unblocked Hg atoms. The Trp heavy atom perturbed ODMR spectrum observed in the interaction of Trp or Lys-Trp-Lys with the unblocked monomer, 5-HgUTP (but not with the blocked monomer), provides direct evidence that binding of Hg to the amines results in stable complexes exhibiting a Trp heavy atom effect. Since this mode of binding occurs with monomers, it will probably occur also in coiled, nonordered regions of polynucleotide. Polyamines, including Lys_3, which complex with the phosphates of polynucleotides, lead to enhanced stacking of the bases and the formation of helical structures (Porschke, 1979). We find from the UV difference spectra that hypochromicity in the polynucleotide absorption band is induced by binding Lys-Trp-Lys when the Hg is blocked with ME, but to a lesser extent when it is unblocked. A hypochromic contribution from Trp is observed in both cases. It appears, then, that with blocked Hg, Lys-Trp-Lys behaves as a typical polyamine, stabilizing a base-stacked polynucleotide conformation. On the other hand, when Hg is available to bind directly with the amines, the interactions which stabilize a base-stacked structure are inhibited.

Heavy atom effects are induced in Trp for complexes formed both with blocked poly(5-HgU) and with unblocked poly(5-HgU), as can be seen most directly from the phosphorescence decay analyses in Table I. The phosphorescence decay kinetics show far less distinction between the complexes formed with and without ME than do the phosphorescence, ODMR, and UV difference spectra. In each case there is a long component accounting for about 10% of the intensity which results from

Trp's which are not in close contact with Hg. The shorter, perturbed phosphorescence is resolved into a longer component of ca. 150 ms and a shorter one of ca. 10 ms. The latter lifetime is characteristic of Trp whose π system is in direct contact with a Hg atom, as in the CH_3Hg^{II}–Trp complex in which the Hg atom is situated above the aromatic plane (Svejda et al., 1978; Anderson & Maki, 1980). The 150-ms triplet lifetime is too long by about an order of magnitude to be accounted for by a direct contact. It is interesting that in light of the large spectroscopic differences between the complexes formed with and without ME, the decay kinetics are similar. The arbitrary use of three-exponential fitting of the decay kinetics does not imply that we believe only three species with unique lifetimes contribute to the emission. Unquestionably, these samples have a larger—perhaps effectively continuous—distribution of triplet state lifetimes. The significance of the three-component analysis lies in its demonstration of emission components with short, intermediate, and long lifetimes.

Our measurements do not provide any direct spectroscopic evidence for the stacking of Trp with the bases of poly(5-HgU), although much independent evidence exists for the occurrence of stacked complexes between Lys-Trp-Lys and polynucleotides (Hélène, 1981). Stacking of Trp with 5-HgU is, however, a plausible mode for bringing a Hg atom into direct contact with the π electrons, thus producing the heavy atom effects which are observed. When the Hg atom is blocked with ME, it is apparent for stereochemical reasons that stacking is required to bring the Hg atom into direct contact with the π electrons of the indole. Stacking is not required, however, if the Hg atom is not blocked. For this reason, we think that the 10-ms component of the Trp *in the presence* of ME results from stacked complexes of Trp and 5-HgU in which the Hg atom interacts directly with indole. This implies that the well-resolved ODMR signals shown in Figure 3 (+ME) are characteristic of stacked, heavy atom perturbed complexes. The relatively symmetric appearance of the ODMR signals observed in this sample (Figures 2 and 3) at the large scan rates used indicates that they must originate from Trp's having a much shorter triplet state lifetime than 150 ms. The shortest possible sublevel lifetime of a triplet state having an average decay lifetime of 150 ms is about 50 ms. A sublevel lifetime as long as 50 ms would result in noticeable transient effects in the narrow transitions of Figures 2 and 3 at the microwave sweep rates employed. A significant part of the 10-ms component of Trp phosphorescence *in the absence of ME* may originate from stacked complexes as well, although clearly the system is more heterogeneous, and other modes of indole–Hg interaction may be involved.

Finally, the 150-ms Trp component may originate from several types of Trp sites, none of which involves direct contact of the π electrons with the Hg atom. Possibilities include stacked complexes of Trp with normal U (the polymer is only 70% mercurated), which is itself stacked with Hg–U, and partially stacked complexes of Trp and 5-HgU in which the indole π electrons are not in direct contact with the Hg atom but are in indirect contact via the uracil π system.

The ODMR spectrum of the heavy atom perturbed Trp which is produced by association of Lys-Trp-Lys with poly(5-HgU) in the presence of ME is characterized by an unusually low frequency ν_1 signal, while the ν_2 signal is at an unusually high frequency for Trp. These shifts contrast sharply with those produced in the complex CH_3Hg^{II}–Trp, where the ν_1 and ν_2 signals are shifted toward one another, resulting in a broad, poorly resolved signal which peaks in the vicinity of 2 GHz. The latter type of ODMR shifts also characterizes the complexes of Trp with unblocked 5-HgUTP and of Lys-Trp-Lys with unblocked poly(5-HgU). The ODMR signals of Lys-Trp-Lys complexed with unblocked 5-HgUTP, on the other hand, are well resolved and close to those found in unperturbed Trp. The variation of the ODMR spectra in the ν_1 and ν_2 region is a clear indication of a variation in Trp environments which are present in the heavy atom perturbed sites. The ZFS of Trp can be influenced by many environmental factors, including the proximity of charged groups (Kwiram, 1982), induced spin–orbit coupling due to the interaction with the Hg atom (Davis & Maki, 1982), and the presence of stacking interactions with aromatic residues (Co & Maki, 1978). At the present time, there are insufficient model studies available to enable us to interpret the ODMR shifts in terms of specific local perturbations.

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