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Evidence for stacking interactions between 5-mercurated polyuridylic acid and HIV-1 p7 nucleocapsid protein obtained by phosphorescence and optically detected magnetic resonance (ODMR)

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The photoexcited triplet state of Trp-37 in the C-terminal zinc finger of the HIV-1 p7 nucleocapsid protein was used as a probe of p7 interactions with the heavy atom-derivatized RNA homopolymer, poly-5-mercuriuridylic acid (5-HgU). Binding of p7 to 5-HgU (Hg blocked with 2-mercaptoethanol) produces an external heavy atom effect (HAE) on Trp-37 characterized by fluorescence quenching, reduction of the phosphorescence lifetime by three orders of magnitude, and the appearance of the D + E phosphorescence-detected ODMR signal, absent in unperturbed Trp, but induced by a HAE. The details of the HAE are consistent with out-of-plane van der Waals contact of Hg with the indole chromophore of Trp-37 Steric requirements suggest further that the Trp–RNA contact occurs via an aromatic stacking interaction.

External heavy atom effect; Tryptophan phosphorescence; ODMR spectroscopy; Zinc finger–RNA interaction

1. INTRODUCTION

The human immunodeficiency virus type-1 (HIV-1) is a retrovirus whose RNA genome contains the *gag*, *pol*, and *env* genes that encode proteins in the mature virus. The gag proteins have a structural role. During virus assembly and budding, thousands of copies of the gag protein participate in the recognition and packaging of the RNA genome [1–3]. After budding, the gag precursor polyprotein is cleaved by viral proteases into six smaller products, one of which is the p7 nucleocapsid (NC) protein [4]. Mature viruses contain 2,500–3,000 copies of the HIV-1 p7 NC protein (p7) that apparently stabilize the RNA through formation of a ribonucleoprotein complex [5–9]. Retroviral NC proteins and their gag precursors contain, without exception, one or two copies of an invariant CCHC array, Cys-X_2-Cys- X_4-His-X_4-Cys (X is a variable, or conservatively substituted amino acid) [5]. p7 is a small protein (ca. 55 amino acids) that contains both of the CCHC arrays of the gag polyprotein. Site-directed mutagenesis has been employed [8–11] to establish that the CCHC array is essential for faithful genome packaging and infectivity of the virus. The CCHC array has been proposed to be a Zn binding domain in the virus [12], and is known to bind Zn tightly in vitro [13]. Recent measurements [3] have shown that the CCHC arrays of NC proteins are associated with quantitative amounts of Zn in particles of HIV-1, SIV (simian immunodeficiency virus), and many other retroviruses. A recent three-dimensional solution structure of p7 obtained by NMR [14] reveals two non-interacting structured 'zinc finger' domains linked by a short, flexible peptide chain. It was proposed that p7 interacts with RNA by hydrophobic contacts of residues on the Zn finger surfaces with RNA bases; the possibility of aromatic stacking interactions was considered but could not be confirmed [15]. p7 contains a single tryptophan, Trp-37, that is located on a 'hydrophobic patch' of the C-terminal Zn finger. The corresponding position on the N-terminal Zn finger is occupied by Phe.

Optical detection of triplet state magnetic resonance (ODMR) has become a powerful method for investigating biopolymers, and their interactions [16,17]. We report in this letter on our phosphorescence and ODMR measurements of the photoexcited triplet state of Trp-37 in free p7, and in its complex with the heavy atom-derivatized RNA, poly 5-mercuriuridylic acid (5-HgU) in which the Hg atoms have been blocked by complexing with 2-mercaptoethanol (ME). The close approach

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Abbreviations: 5-HgU, 5-mercurated polyuridylic acid; HIV-1, human immunodeficiency virus type-1; HAE, heavy atom effect; ME, 2-mercaptoethanol; MIDP, microwave-induced delayed phosphorescence; NC, nucleocapsid; ODMR, optically detected magnetic resonance of the photoexcited triplet state; SLR, spin-lattice relaxation; SSB, *E. coli* single-stranded DNA-binding protein; ZFS, zero-field splittings.

(to van der Waals' contact) of the Hg atom and the indole chromophore is expected to produce a large external heavy atom effect (HAE), characterized by fluorescence quenching, an enhanced triplet state quantum yield, reduction of the phosphorescence lifetime, and the appearance of the normally absent D + E signal in the phosphorescence-detected ODMR spectrum of Trp. Previous studies of the 5-HgU-binding of *Escherichia coli* single-stranded DNA-binding protein (SSB) [18] and of the p10 NC protein from murine leukemia virus [19] have revealed HAEs on intrinsic Trp residues, demonstrating the occurrence of Trp–Hg van der Waals contacts in these complexes. Blocking of the remaining coordination site of Hg in 5-HgU with ME introduces steric constraints that allow the close approach of Hg along the out-of-plane direction of indole only via an aromatic stacking interaction. It is known from previous model studies [20,21] that the HAE produced by the out-of-plane van der Waals contact of a Hg atom with an aromatic residue such as indole is at least an order of magnitude larger than that produced by in-plane coordination or contact.

2. MATERIALS AND METHODS

The nucleotide sequence encoding the NC protein (gag p7) of HIV-1 MN has been cloned into an inducible *E. coli* expression vector (p-Mal-c, New England Biolabs, Beverly, MA) (P Powell and L.E. Henderson, unpublished) and used to express recombinant NC (rNC) protein. The protein is expressed as a fusion protein where the NC protein is fused to the C-terminal end of a maltose binding protein through a tetrapeptide linker that confers a cleavage site for Factor Xa. A method for purification of the maltose binding-NC (MB-NC) fusion protein and cleavage by factor Xa and subsequent purification of the recombinant NC (rNC) protein has been developed The final purification step was accomplished by HPLC The protein was fully characterized, including complete amino acid sequence analysis and exact molecular weight determinations by mass spectrometry as described [4] The protein was reconstituted at pH 7 with two equivalents of $ZnCl_2$ [14]. Before the measurements, it was dissolved and subjected to exchange with a 5 mM, pH 6.5, phosphate buffer. 5-HgU, 70% mercurated, purchased from PL Biochemicals, was dissolved in the same buffer and used without purification. Excess ME was added to the solution to block the Hg atom. The concentration of p7 in the uncomplexed sample was ca. 0.25 mM, and ca. 0 03 mM in the p7/5-HgU complex. The concentration of 5-HgU was ca 20-fold larger in mononucleotide than that of p7. Concentrations were determined by UV absorption spectroscopy using extinction coefficients of 5.7×10^3 M^{-1} cm^{-1} at 280 nm for p7 and 1.1×10^4 $M^{-1} \cdot cm^{-1}$ at 268 nm for 5-HgU. The complex was formed at ambient temperature and allowed to stand for 10 min prior to phosphorescence measurement at 77K. All samples contained 20% v/v ethylene glycol as cryosolvent. Slow passage ODMR spectra were obtained and triplet sublevel kinetics measurements [22,23] were carried out at 1.2K in order to suppress spin-lattice relaxation (SLR). Excitation was at 295 nm with 16 nm bandpass and the emission bandpass was 3 nm. ODMR spectroscopy has been described in two recent reviews [16,17]. The phosphorescence and ODMR instrumentation and methods currently used by us have been described previously [18].

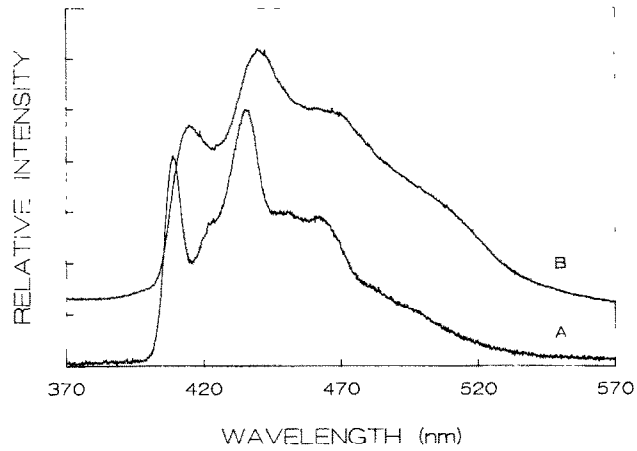

Fig. 1. (A) Phosphorescence spectrum of p7 using a rotating sector to eliminate fluorescence. (B) Total luminescence spectrum of p7/5 HgU in the absence of a rotating sector.

3. RESULTS

3.1. *Phosphorescence spectra and overall decay kinetics*

The emission spectra of p7 and its complex with 5-HgU are compared in Fig. 1. The total luminescence spectrum of p7 is dominated by Trp fluorescence; the

Table I

Triplet state properties of p7 and its 5-HgU complex

Sample	0,0-band (nm)	Decay kinetics (s)	ODMR frequencies[a] (GHz)			ZFS values (GHz)	
			D − E	2E	D + E	D	E
p7	409.2	6 6 (93%)	1.75 (150)	2.53 (240)	–	3.02	1.27
p7 + 5HgU	414.6	0.008 (64%) 0.061 (26%)	1.74 (260)	2 48 (200)	4.26 (270)	3.00	1.26

[a]Linewidths, fwhm in MHz, are given in parentheses

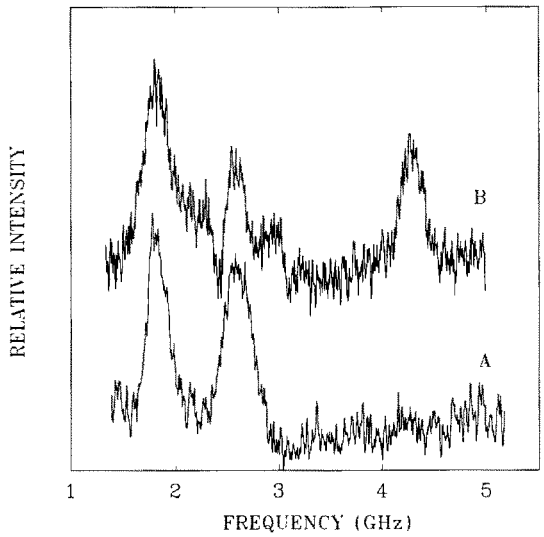

Fig. 2. ODMR slow passage spectrum of (A) uncomplexed p7, and (B) p7/5–HgU. The signals are, from lowest to highest frequency, D − E, 2E, and D + E. The microwave frequency was swept between 1.4 and 5 GHz, and sweep rates were 68 MHz/s in (A) and 10 GHz/s in (B). In (B), the baseline dip at ca. 2.4 GHz results from automatic switching of frequency bands in the microwave sweeper. The discontinuity at ca. 3 GHz probably is due to microwave reflections in the transmission line and helix that holds the sample.

spectrum shown in Fig. 1 was obtained through a rotating sector that eliminated the fluorescence. The spectrum of the p7/5–HgU complex, on the other hand, exhibits little, if any fluorescence; the phosphorescence of the complex shown in Fig. 1 was obtained without the sector. The well-resolved phosphorescence 0,0-band of free p7 peaks at 409.2 nm indicating, along with its fairly large bandwidth, that Trp-37 lies in an essentially solvent-exposed environment. In the 5-HgU complex, the Trp 0,0-band is shifted by 5.4 nm to 414.6 nm. The red shift is consistent [24] with the relocation of Trp-37 to a more polarizable local environment as a result of complex formation. The results of phosphorescence decay analysis of p7 and its complex with 5-HgU are given in Table I. The decay of the p7 phosphorescence is largely (93%) a single exponential at 77K with a lifetime of 6.6 s, which is in the normal range for Trp in the absence of an external HAE, or interactions with intrinsic triplet quenchers such as cystine [25,26]. When complexed with 5-HgU, the phosphorescence lifetime of Trp-37 is dramatically reduced. As presented in Table I, the decay consists principally of two components, 8 ms (64%) and 61 ms (26%). The major lifetime component is about three orders of magnitude shorter than the lifetime of free p7.

3.2. *ODMR results*

The slow passage phosphorescence-detected ODMR spectrum of p7 is shown in Fig. 2. Only two signals are observed, corresponding to the D−E and 2E zero field transitions, as is customary for unperturbed Trp. The frequencies and linewidths, corrected for rapid passage effects, are listed in Table I. In the p7/5–HgU complex, all three of the ODMR signals are observed. They are displayed in Fig. 2. In addition to the D−E and 2E signals, a strong ODMR signal is observed for the D + E transition, which occurs at the sum of the frequencies of the two other signals. An intense D + E signal is diagnostic for the presence of an external HAE. The corrected frequencies and bandwidths of these ODMR signals are given in Table I.

3.3. *Triplet state sublevel decay kinetics*

The apparent triplet state sublevel decay constants obtained by MIDP [22] for the two slower decaying sublevels and by rapid passage phosphorescence transient methods [23] for the fastest decaying sublevel are listed in Table II. The apparent decay constants of free p7 seem to be influenced somewhat by residual SLR at 1.2K, since they lead to a predicted average lifetime that is somewhat shorter than that observed at 77K. The actual sublevel decay constants of Trp, corrected for SLR [27], are included for comparison. The apparent sublevel decay constants of free p7 are in the same range as those of Trp. The sublevel decay constants of Trp-37 in the p7/5–HgU complex (Table II) are all greatly increased relative to free p7. The HAE is selective for the T_z sublevel which dominates in the decay of the complex.

4. DISCUSSION

Our experimental results demonstrate that upon complexing of p7 with 5-HgU, the Trp-37 residue that resides in the C-terminal zinc finger is subjected to an extreme HAE. The intense fluorescence of free p7 is quenched, the phosphorescence lifetime is reduced by ca. three orders of magnitude, and the normally absent D + E signal is induced in the phosphorescence-detected ODMR spectrum (Fig. 2). A 5.4 nm red shift of the phosphorescence 0,0-band (Fig. 1) is a further indication of a change in the relatively solvent-exposed local environment of Trp-37 to one that is more polarizable in the 5-HgU complex.

The 77K phosphorescence decay analysis reveals the presence of two major lifetime components, 8 ms and 61 ms. We interpret this to be an indication of a heter-

Table II

Apparent p7 and p7/5–HgU complex triplet sublevel decay constants

Sample	k_x (s^{-1})	k_y (s^{-1})	k_z (s^{-1})	k_{ave} (s^{-1})[a]
p7	0.42	0.086	0.052	0.19
p7 + 5-HgU	13	3.7	290	100
Tryptophan[b]	0.24	0.12	0.038	0.13

[a] $k_{ave} = (k_x + k_y + k_z)/3$.
[b] Actual decay constants from [27].

ogeneous distribution of complexes. Heterogeneity is to be expected at least on the basis of the incompletely (ca. 70%) mercurated substrate. Other bases for heterogeneity of complex formation probably also are present. We believe (see below) that the data are consistent with stacking between Trp-37 and the nucleobases.

The individual sublevel decay constants of the p7/5–HgU complexes measured by transient ODMR (Table II) predict an average triplet state lifetime of ca. 10 ms, suggesting that these measurements are dominated by the Trp residues that undergo the largest HAE. The most strongly perturbed sublevel is T_z, where z is the out-of-plane component; its individual lifetime is ca. 3.5 ms. This value is similar to the shortest sublevel lifetime induced in CH_3Hg/Trp complexes in which the Hg atom is known to interact by a van der Waals contact from above the indole plane [20,21]. By contrast, the shortest sublevel lifetimes induced by CH_3Hg in-plane coordination to benzimidazole are more than an order of magnitude longer [20], demonstrating the greater effectiveness of the HAE produced by an out-of-plane location of the perturbing atom. The sublevel specificity of the external HAE has been shown [28] to be determined by the location of a perturbing atom in the magnetic axis system of the molecule. According to this theory, the perturbing atom will affect only the T_z sublevel if it is located along the out-of-plane z-axis, and will have little effect on any sublevel if it is located within the molecular plane. Location in the xz-plane affects both T_x and T_z sublevels, etc. The sublevel specificity of the HAE that is observed in the p7/5–HgU complex is consistent with the location of the Hg atom along the z-axis. The size of the HAE suggests that there is a van der Waals contact between the π-charge cloud of the indole ring and the Hg atom. Because of the steric requirements imposed by blocking of the Hg atom with ME, such a location of Hg is consistent only with an aromatic stacking interaction between indole and the modified uracil base. A stacking interaction also is consistent with the phosphorescence red shift that is observed on binding with 5-HgU.

We used 5-HgU in our initial studies of p7 complexing with RNA because this heavy atom-derivatized substrate provides the most direct evidence (via a HAE) for aromatic stacking interactions involving Trp-37. Stacking interactions with natural nucleobases produce Trp phosphorescence red-shifts and lifetime reductions as observed previously in SSB complexes with poly(dT) [18]. Recently, we have observed these effects on Trp-37 in p7 complexes with poly(U) and poly(I) (W-.C. Lam and A.H. Maki, unpublished).

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