Involvement of Tryptophyl Residues in the Binding of Model Peptides and Gene 32 Protein from Phage T4 to Single-Stranded Polynucleotides. A Spectroscopic Method for Detection of Tryptophan in the Vicinity of Nucleic Acid Bases†

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ABSTRACT: A spectroscopic method for the direct detection of aromatic residues interacting with nucleic acid bases has been developed. It rests upon the perturbation of luminescence properties of aromatic molecules by neighboring heavy atoms. Mercury derivatives of pyrimidine bases can be used as probes for the presence of tryptophyl residues in their vicinity. Low-temperature luminescence measurements have been carried out to provide evidence for the mercury heavy atom effect in complexes of oligopeptides and gene 32 protein from phage T4 (gp 32) with poly(5-mercuriuracil). Cysteinyl residues of the protein have been protected by reaction with *N*-ethylmaleimide before binding to the mercurated polymer in order to prevent covalent attachment of the protein to the polynucleotide. An enhancement of the tryptophan triplet-state population (and consequently of phosphorescence intensity) as well as a drastic shortening of the triplet-state lifetime has been observed for both tryptophan-containing peptides and gp 32. These effects require a van der Waals contact between the heavy atom and the perturbed tryptophyl residues, suggesting direct interactions between tryptophyl residue(s) and nucleic acid bases.

There is a class of proteins termed single-strand binding (SSB)[1] proteins that bind in a specific manner to single-stranded DNA or polynucleotides as compared to double-stranded ones. These proteins are known to be involved in basic processes of viral DNA metabolism [Hélène et al., 1982; see Toulmé et al. (1984) and references cited therein]. The origin of the specificity for this class of proteins is so far not elucidated. In previous works (see below) and in the accompanying papers (Toulmé et al., 1984; Casas-Finet et al., 1984), we have tried to shed some light on the possible role of aromatic residues in the binding specificity of SSB proteins. Aromatic residues (tyrosine and tryptophan) were shown to be involved in the binding of oligopeptides to single-stranded nucleic acids through stacking interactions with nucleic acid bases [for a review, see Hélène & Maurizot (1981)]. Tryptophyl residues were also found to be involved in the binding of the gene 32 protein (gp 32), a SSB protein from phage T4, onto single-stranded polynucleotides (Hélène et al., 1976; Toulmé et al., 1984). The methods used in these works provided only indirect evidence for the presence of tryptophyl residues in the binding site of the SSB protein–DNA complexes. In a previous study (Hélène, 1979; Hélène et al., 1979), we have proposed a spectroscopic method for direct detection of aromatic residues that are in close contact with nucleic acid bases. This method is based upon the external heavy atom effect on the spin–orbit coupling in aromatic molecules (McGlynn et al., 1969). The close contact of a heavy atom with an aromatic molecule results in an enhancement of the intersystem crossing rate in the excited state of the perturbed molecule, leading to (i) a quenching of fluorescence, (ii) an increase of the triplet-state population (although this is not always experimentally observed), and (iii) a drastic reduction of the triplet lifetime. This effect is an extremely localized one as the two interacting molecules have to be in van der Waals contact for the heavy atom effect to be observed. Moreover, this method is very sensitive as demonstrated in the case of the Lys-Trp-Lys–poly(HgU) complexes (Hélène et al., 1979; Cha & Maki, 1982). At 77 K, the fluorescence of the peptide was almost completely quenched, its phosphorescence was strongly enhanced, and the tryptophyl triplet lifetime was reduced by about 3 orders of magnitude. Similar results have also been obtained in complexes of CH_3Hg^+ and tryptophan derivatives at low temperature (77 K) by Svejda et al. (1978).

As will be shown in this paper, the heavy atom effects described above are readily observed in several model peptide–poly(HgU) systems, and the method has been used to provide evidence for the presence of at least one tryptophyl residue in the binding site of gp 32 and poly(HgU).

Materials and Methods

Materials. L-Histidyl-L-tryptophan (His-Trp), L-lysyl-L-tryptophan (Lys-Trp), lysyltryptophanyl-α-lysine (Lys-Trp-Lys), lysylglycyltryptophanyllysine *O-tert*-butyl ester (Lys-Gly-Trp-Lys-O*t*Bu), lysyltryptophanylglycyllysine *O-tert*-butyl ester (Lys-Trp-Gly-Lys-O*t*Bu), tyrosyllysyllysylvalyltryptophan (Tyr-Lys-Lys-Val-Trp), and tyrosylhistidyllysylglutaminyltryptophan (Tyr-His-Lys-Gln-Trp) were purchased from Bachem, L-arginyl-L-tryptophan (Arg-Trp) was from Cyclo Chemical, and L-tryptophyl-L-lysine (Trp-Lys) was from Mann Research. Peptide solutions were prepared in standard buffer I containing 1 mM sodium cacodylate, pH 6.0, 1 mM NaCl, and 0.2 mM EDTA.

The gene 32 protein (gp 32) from phage T4 was prepared according to the procedure of Alberts & Frey (1970). The protein migrates as a single band corresponding to a molecular weight of 35 000 (Toulmé et al., 1984). All experiments with gp 32 were carried out at 5 °C in a pH 7.6 buffer (buffer II) containing 10 mM sodium cacodylate, pH 7.6, 10 mM NaCl,

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[1] Abbreviations: poly(HgU), poly(5-mercuriuridylic acid); d-DNA-(Pt), heat-denatured DNA modified by *cis*-$[Cl_2(NH_3)_2Pt]$; SSB protein, single-stranded DNA binding protein; gp 32, gene 32 protein from phage T4; gp 32-*S*-NEM, gp 32 having two cysteinyl residues blocked with NEM; NEM, *N*-ethylmaleimide; ME, β-mercaptoethanol; Lys-Trp-Lys, lysyltryptophyl-α-lysine; EDTA, ethylenediaminetetraacetic acid.

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and 2 mM EDTA. Dissociation of the protein–polynucleotide complex was achieved by increasing the ionic strength of the solution (addition of NaCl or $MgCl_2$).

Poly(U) was obtained from Miles, poly(HgU) from P-L Biochemicals (70% of the uracil bases were mercurated), and DNA (calf thymus) from Sigma. Heat-denatured DNA was prepared by heating the DNA solution at 100 °C for 15 min and then chilling in an ice bath. Modification of DNA by platinum derivatives, mostly with the *cis*-dichlorodiammineplatinum, was performed as previously described (Macquet & Butour, 1978; Toulmé et al., 1983).

Absorption, Circular Dichroism, and Fluorescence Measurements. Absorption spectra were recorded with a Cary 15 spectrophotometer and circular dichroism spectra on a Roussel Jouan III dichrograph. The concentrations of peptide or protein solutions and those of polynucleotides were determined by using the following molar extinction coefficients (in M^{-1} cm^{-1}): $\epsilon_M^{280} = 5600$ for peptides containing one Trp residue; $\epsilon_M^{280} = 6800$ for peptides containing one Trp and one Tyr residue; $\epsilon_M^{280} = 37\,000$ for gp 32; $\epsilon_M^{260} = 10\,000$ for poly(U); $\epsilon_M^{267} = 9120$ for poly(HgU); $\epsilon_M^{260} = 6500$ for native DNA. Fluorescence measurements were performed with an Aminco 500 spectrofluorometer. The protein fluorescence was measured at 335 nm and that of peptides at 355 nm ($\lambda_{exc} = 290$ nm).

Phosphorescence and Triplet Lifetime Measurements. Phosphorescence measurements were carried out with an Aminco spectrofluorometer equipped with a laboratory-made phosphoroscope. The rotation rate of the phosphoroscope could be varied from 0 to 9000 rpm by monitoring the direct-current voltage of the driving motor. A Suprasil quartz tube (2-mm internal diameter) containing about 100 μL of sample was dipped slowly in a Dewar filled with liquid nitrogen. Phosphorescence decays were displayed on the screen of a Tektronics oscilloscope (Model R5 103 N) and triplet lifetimes determined graphically from photographs of phosphorescence decay profiles.

Protection of Protein SH Groups and Poly(HgU) Mercury Atoms. As mercuriuracil in poly(HgU) might react with cysteinyl residues of proteins, it was necessary to block either the SH groups of the protein or the mercury atoms of poly(HgU).

Chemical modification of poly(HgU) was carried out by adding to the polymer solution a 2-fold excess of freshly prepared cysteine or β-mercaptoethanol solutions under an inert atmosphere (N_2). Chemical modification of gp 32 was carried out by using *N*-ethylmaleimide (NEM) as the sulfhydryl reagent. Protein from stock solutions was thoroughly dialyzed against 0.1 M phosphate buffer (pH 7) to eliminate β-mercaptoethanol present in the storage buffer. The reaction was then carried out in a quartz cuvette in the above phosphate buffer at 10 °C. The course of the reaction was followed by measuring the decrease of the *N*-ethylmaleimide absorption at 320 nm ($\epsilon_{NEM}^{320} = 468$ M^{-1} cm^{-1}). At this wavelength, the absorbances of both the protein and the reacted NEM were negligible. The reaction proceeded according to two-step kinetics: one SH group was titrated after 30 min and a second one after around 3 h of reaction. A slow decrease of the NEM absorbance was still observed after 3 h of reaction, suggesting slow side reactions of NEM with other residues (histidine, amine) as already observed (Smyth et al., 1960). The reaction was stopped (after 3 h) by loading the mixture on a Sephadex G25 column (0.9 × 10 cm) to separate the protein from the sulfhydryl reagent. Circular dichroism of the modified protein with two blocked SH groups did not exhibit any detectable tertiary structural change as compared with the native protein.

Results and Discussion

Heavy Atom Effect in Peptide–Poly(HgU) Complexes. In solution, it was previously shown that peptides bearing positively charged and tryptophyl residues form different kinds of complexes with polynucleotides (Brun et al., 1975; Dimicoli & Hélène, 1974). Spectroscopic results were analyzed according to a two-step model: one type of complex involves electrostatic interactions of lysyl residues with phosphate groups of the polynucleotide whereas the other one involves both electrostatic interactions and stacking of the tryptophyl residue with nucleic acid bases (Brun et al., 1975; Behmoaras et al., 1981; Montenay-Garestier et al., 1983). In stacked complexes, the aromatic group is in close vicinity to nucleic acid bases; therefore, one may expect a heavy atom effect when a mercurated polynucleotide is used. In model systems such as Lys-Trp-Lys–poly(HgU) complexes, we observed a strong heavy atom effect resulting in an almost complete quenching of the peptide fluorescence, an enhancement of its phosphorescence quantum yield (about 3-fold), and a drastic decrease of the triplet lifetime, from 6 s to ≃5 ms. The phosphorescence spectrum of the complex at 77 K exhibits the characteristic vibronic structure of tryptophan emission with bands at 409, 435, and 460 nm. These bands are shifted by about 8 nm to longer wavelengths as compared to those of the free peptide. Dissociation of the complexes by increasing the ionic strength leads to the reversal of the effects described above (Hélène et al., 1979).

Similar results were obtained with several peptide–poly(HgU) complexes. All the peptides investigated bear a tryptophyl residue and various positively charged groups. Lifetime data are presented in Table I. The weight of each component in the two-exponential phosphorescence decay of the complexes is expressed as the percentage of the contribution of each component to the total phosphorescence. It can be seen from these results that binding of the peptides to mercurated poly(U) results in the appearance of a short-lived triplet-state component of 5–6 ms. This short component is different from that of the mercurated polymer (1 ms), and its contribution to the phosphorescence of the complex disappears when the complexes are dissociated in the presence of 1 M NaCl. This short-lifetime component (≃5 ms) can be ascribed to the phosphorescence decay of tryptophyl residues that are in contact with the heavy atom. The value of this short lifetime varied in a rather narrow range whatever the position of the tryptophyl residue in the peptide sequence or the medium used (frozen buffer or glassy mixtures of buffer and propylene glycol). The longer lifetime component (≃6 s) is always present and has a value similar to that of the free peptide. It accounts either for unbound peptide molecules or for bound peptides in such a conformation that tryptophyl residues are not in close proximity to the mercurated uracil bases, or for peptide molecules bound to nonmercurated poly(U) regions. The poly(HgU) sample used in this work was 70% mercurated. However, it should be noted that the respective contributions of each lifetime component were subject to wider variations when lysyl residues were substituted by arginyl or histidyl residues. This may be due to a lower affinity of these peptides toward the polynucleotide or a conformation of the bound peptide less favorable for a stacking interaction of tryptophyl residues with the mercurated nucleic bases as compared to lysine-containing peptides.

The influence of mercury-blocking agents such as cysteine or β-mercaptoethanol (ME) on complex formation has been studied. Cha & Maki (1982) have shown that the heavy atom

Table I: Triplet Lifetime (τ_p) of Peptide–Poly(HgU) Complexes at 77 K in Frozen Buffer I (BUF) or in Glassy-State Buffer I + Propylene Glycol (1/1 v/v) (WPG)[a]

medium	peptide	Hg/Trp	τ_p [peptide + poly(HgU)]	τ_p (free peptide) (s)[b]
BUF	Lys-Trp	4	5.8 s (35%), 5.2 ms (65%)	5.3
WPG	Lys-Trp	4	6.1 s (27%), 4.6 ms (73%)	5.8
BUF	Trp-Lys	4	6.0 s (26%), 5.4 ms (74%)	5.2
BUF	Arg-Trp	4	6.0 s (55%), 7.0 ms (45%)	5.8
BUF	His-Trp	4	6.2 s (61%), 4.0 ms (39%)	5.8
BUF	Lys-Trp-Lys	5	6.0 s (20%), 5.6 ms (80%)	5.1
BUF + Cys	Lys-Trp-Lys	5	5.0 s (34%), 5.6 ms (66%)	5.5
WPG	Lys-Trp-Lys	5	5.8 s (15%), 5.2 ms (85%)	6.2
BUF	Lys-Gly-Trp-Lys-O*t*Bu	11	5.0 s (10%), 5.6 ms (90%)	5.5
BUF + ME[c]	Lys-Gly-Trp-Lys-O*t*Bu	11	5.0 s (10%), 6.7 ms (90%)	
BUF	Lys-Trp-Gly-Lys-O*t*Bu	11	7.0 s (7%), 6 ms (93%)	5.7
BUF + ME[c]	Lys-Trp-Gly-Lys-O*t*Bu	11	6.0 s (5%), 9.5 ms (95%)	
BUF	Tyr-Lys-Lys-Val-Trp	4.5	5.9 s (27%), 4.0 ms (73%)	5.9
WPG	Tyr-Lys-Lys-Val-Trp	4.5	6.0 s (27%), 4.6 ms (73%)	6.3
WPG	Tyr-His-Lys-Gln-Trp	4.5	6.6 s (40%), 6.0 ms (60%)	6.2

[a] Peptide concentration = 2.5×10^{-5} M; λ_{exc} = 290 nm; λ_{em} = 435 nm. ME is β-mercaptoethanol. [b] The phosphorescence lifetime of the free peptide under the same solvent conditions. [c] ME/Hg = 2.

effect observed in the complex formed by Lys-Trp-Lys and poly(HgU) in the presence of ME corresponds to a more homogeneous environment of the interacting tryptophan as compared to the case where ME is absent. In such a complex, tryptophyl residues are believed to be involved in stacking interactions with the mercurated uracil bases. Our results with tetrapeptide–poly(HgU) complexes in the presence of ME confirmed the observations of Cha and Maki; the vibronic structure of the phosphorescence spectra was more resolved in the presence of ME as compared to that in the absence of ME even though the phosphorescence intensities were not markedly affected (Figure 1). The phosphorescence enhancement factor (f), measured by the ratio of the phosphorescence maximum intensity of the complexed peptide to that of the free peptide, is particularly high in the case of these tetrapeptides. f was found to be equal to $\simeq$100 for both tetrapeptides, due likely to higher complex concentration (association constant $\simeq 5 \times 10^5$ M^{-1}; Toulmé, 1982) as compared to Lys-Trp-Lys (f = 3) whose association constant with poly(U) was found to be $\simeq 5 \times 10^4$ M^{-1}. A more favorable contact of tryptophyl residues and the heavy atoms in tetrapeptide–poly(HgU) complexes could also account for both the high phosphorescence enhancement factor and the enhanced contribution of the short-lifetime component in the phosphorescence emission. When cysteine is used instead of β-mercaptoethanol to block the mercury atoms in Lys-Trp-Lys–poly(HgU) complexes, the f value is smaller (f = 2) than in the absence of cysteine (f = 3). The contribution of the short-lifetime component to the total phosphorescence is also smaller (66% with cysteine and 80% without cysteine).

Detection of a Heavy Atom Effect in the Emission Properties of Tryptophyl Residues in Complexes of gp 32 and Poly(HgU). (*A*) *Chemical Modification of gp 32 Cysteinyl Residues.* Study of the reaction of *N*-ethylmaleimide with SH groups of gp 32 have shown that two out of four SH groups are titratable by this method. The reaction kinetics have shown moreover that the two reactive SH groups have quite different accessibility to the reagent (see Materials and Methods). This difference in exposure of the two SH groups was also observed when an ionic reagent such as Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid)] was used instead of NEM (Casas-Finet et al., 1984). Although circular dichroism measurements did not allow us to detect any significant conformational change in the tertiary structure of the modified protein, local conformational changes were revealed by a quenching of the

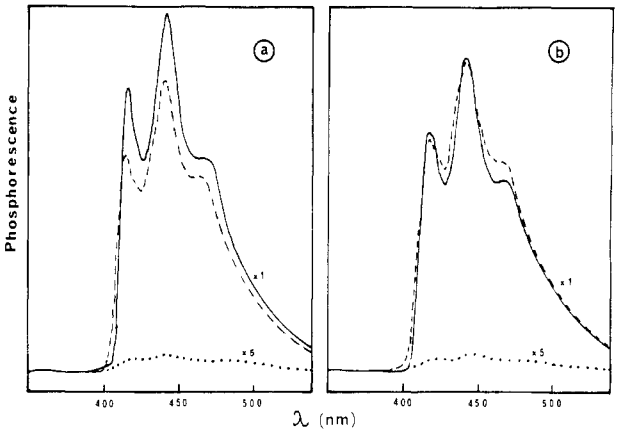

FIGURE 1: Phosphorescence spectra of frozen aqueous solutions at 77 K of complexes of Lys-Gly-Trp-Lys-O*t*Bu (a) and Lys-Trp-Gly-Lys-O*t*Bu (b) with poly(HgU) in the presence (—) and in the absence (– – –) of β-mercaptoethanol (ME). [peptide] = 2.5×10^{-5} M; [poly(HgU)] = 2.9×10^{-4} M; [ME] = 5.8×10^{-4} M. The dotted curves represent the phosphorescence spectra of a peptide solution at the same concentration as in the mixture and are recorded at ×5 sensitivity. In these experiments, ME was added to poly(HgU) 5 min before low-temperature measurements were made to avoid possible demercuration reactions (see text).

protein fluorescence after chemical modification. Although no shift was observed in the fluorescence spectrum, the fluorescence intensity of the modified protein was reduced by 17% and 60% as compared with the native protein, when one and two SH groups were titrated, respectively. We checked that there was no dynamic quenching of tryptophan by this reagent, and to our knowledge, no reaction of NEM with tryptophan has been previously reported. The fluorescence quenching observed following SH modification of gp 32 is likely due to a local conformational change of protein regions containing both cysteinyl and tryptophyl residues. In the protein sequence determined by Williams et al. (1981), there are some regions where proximity of the two residues does exist, e.g., Trp-72 and Cys-77 or Trp-168 and Cys-166. Assuming that one or both of these cysteinyl residues are accessible to the reagent, perturbation of the emission properties of one or both of the above tryptophyl residues could be expected following chemical modification of their neighboring cysteinyl residues. When CH_3Hg^+, a highly reactive SH reagent, was used instead of NEM, the fluorescence emission of the protein was also strongly quenched, and a short com-

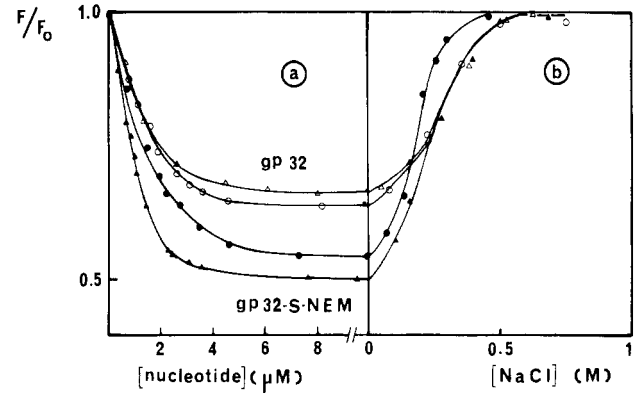

FIGURE 2: (a) Fluorometric titration of native gp 32 and modified (gp 32-*S*-NEM) protein by heat-denatured DNA (△, ▲) and poly(U) (○, ●). A typical experiment consists of sequential additions of polynucleotides to 1.5 mL of 3×10^{-7} M protein solution in buffer II (pH 7.6). The solution was stirred and allowed to equilibrate at the cell holder temperature (4 °C) before the fluorescence signal was recorded. Fluorescence quenching is expressed by the ratio of F/F_0, F and F_0 being the fluorescence intensities in the presence and in the absence of polynucleotides, respectively, measured at the maximum of the protein emission (λ_{max} = 340 nm). (b) Dissociation of protein–polynucleotide complexes by addition of NaCl.

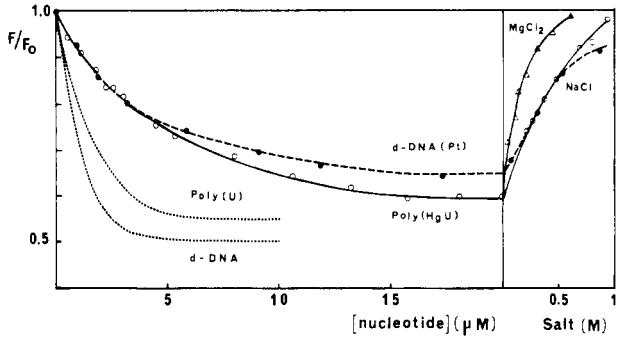

FIGURE 3: Fluorometric titration of gp 32-*S*-NEM with poly(HgU) (—) and of gp 32 with DNA modified by *cis*-$[Cl_2(NH_3)_2Pt]$ [d-DNA(Pt)] (– – –). Binding curves of gp 32-*S*-NEM to unmodified polynucleotides, poly(U), and denatured DNA (···) are redrawn on the figure for comparison. Dissociation of the gp 32-*S*-NEM–poly(HgU) complex was obtained by adding either $MgCl_2$ (△) or NaCl (○). Dissociation of gp 32–d-DNA(Pt) was achieved by adding NaCl (●). Other experimental conditions are identical with those described in Figure 2.

ponent (≃0.1 s) was observed at 77 K in addition to the normal phosphorescence lifetime of tryptophyl residues (6 s). These results suggest that in the tertiary conformation of the protein some cysteinyl residues are in close proximity to tryptophyl residues. A similar conclusion was reached by Hersberger & Maki (1980) after CH_3Hg^+ modification of glyceraldehyde-3-phosphate dehydrogenase.

(B) Binding Properties of Modified gp 32 onto Single-Stranded Polynucleotides. Titration of gp 32 having two cysteinyl blocked with *N*-ethylmaleimide (gp 32-*S*-NEM) by heat-denatured DNA (d-DNA) or poly(U) showed that binding properties of the modified protein were not altered by the chemical modification (Figure 2). Both native and modified proteins were characterized by a binding site size of six to seven nucleotides. The fluorescence quenching observed when all protein molecules were bound to nucleic acids was found to be higher for the modified protein [45% and 50% of the original fluorescence quenched by poly(U) and d-DNA, respectively] than for the native protein (35% of fluorescence quenching). Apparent association constants derived from the binding curves have shown that the chemical reaction does not change significantly the protein affinity toward single-stranded polynucleotides. The complexes were dissociated upon increasing the ionic strength as shown in Figure 2. At ≃0.5 M NaCl, all complexes were fully dissociated.

When mercurated poly(U) or DNA modified by Pt complexes was used instead of poly(U) or heat-denatured DNA, the binding curves of gp 32-*S*-NEM to these modified polynucleotides were different, even though the fluorescence quenching reached when all proteins were bound to the modified polynucleotides was similar to that observed with unmodified polynucleotides (Figure 3).

The difference in the binding curves obtained when gp 32-*S*-NEM interacts with poly(U) and d-DNA, on the one hand, and poly(HgU) and d-DNA(Pt), on the other hand, suggests that the presence of Hg or Pt atoms prevents cooperative binding. Assuming such a noncooperative binding process, the association constant was calculated from the nucleic acid concentration required to reach 50% of the maximum quenching. Values of 5×10^5 and 7×10^5 M^{-1} were obtained for gp 32-*S*-NEM binding to poly(HgU) and d-DNA(Pt), respectively. The complexes could be dissociated by adding either NaCl up to 1.0 M or $MgCl_2$ up to 0.5 M. This dissociation was not observed when the native protein was titrated by poly(HgU) due to covalent bond formation between Hg and Cys residues. This result shows that protection of protein SH groups is necessary to avoid their reaction with mercury. This also indicates that no exposure of unprotected SH groups occurred during the binding process, at least in the binding site.

Binding of native gp 32 to mercurated poly(U) whose mercury groups were blocked by either cysteine of β-mercaptoethanol has been assayed. The binding curves lie in between those obtained for the binding of gp 32-*S*-NEM to poly(HgU) and poly(U) with comparable maximum fluorescence quenching (results not shown). The complexes were also readily dissociated by addition of salt. However, the reproducibility of these experiments was rather poor especially in the presence of β-mercaptoethanol, due likely to demercuration reactions as shown by Van Broeckhoven & De Wachter (1978).

The binding affinity of gp 32 (native) toward single-stranded DNA bearing 0.2 Pt per phosphate group was found to be very similar to that with poly(HgU) (dotted curve in Figure 3). The complex was dissociated by adding NaCl, suggesting that no SH reaction with the Pt atom was observed with this polynucleotide.

The preferred binding site of *cis*-$[(NH_3)_2PtCl_2]$ on DNA is the N(7) position of guanine and adenine (Mansy et al., 1978), whereas the binding site of mercury acetate used in poly(HgU) synthesis is the C(5) position in uracil bases (Dale et al., 1975). The presence of positively charged heavy atoms on base positions which are not normally involved in hydrogen bond formation in a double-stranded structure alters the binding properties of gp 32. In contrast, with poly(1,N^6-ethenoadenine), a polynucleotide having adenine bases modified at N(1) and NH_2(6), the binding of gp 32 was found to be similar to that with poly(rA) (Toulmé & Hélène, 1980; Newport et al., 1981). These results suggest that the contact region between the protein and bases of single-stranded polynucleotides might be localized on the C(4-5) side of pyrimidine bases or the imidazole side of purine bases. The probes we used in this work, d-DNA(Pt) or poly(HgU), bear heavy atoms that are located in this supposed binding site of gp 32. Any contact between aromatic residues of the protein and the heavy atoms is expected to induce a heavy atom effect. Phosphorescence measurements at 77 K were therefore carried out to provide evidence for this heavy atom effect.

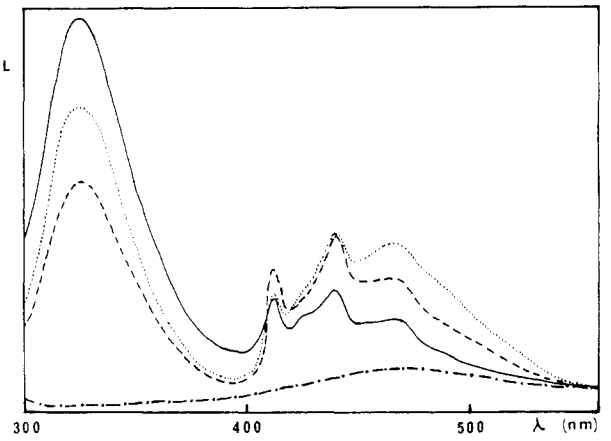

FIGURE 4: Total luminescence spectra in frozen aqueous solutions at 77 K of gp 32-*S*-NEM (—), poly(HgU) (-·-), a mixture of gp 32-*S*-NEM and poly(HgU) (– – –), and gp 32-*S*-NEM + poly(HgU) + NaCl (1 M) (···). Protein and poly(HgU) concentrations were 10^{-5} and 10^{-4} M, respectively.

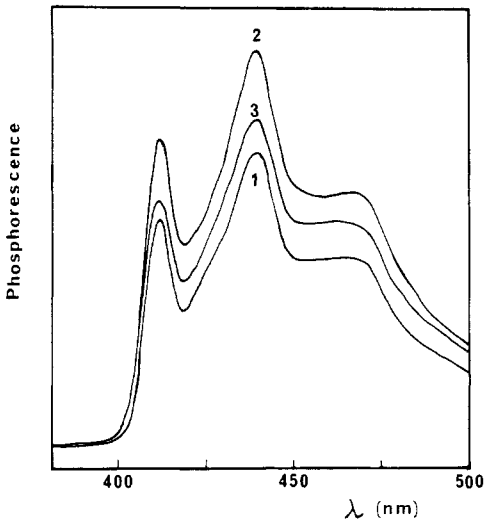

FIGURE 5: Phosphorescence spectra in frozen aqueous solutions at 77 K of (1) gp 32-*S*-NEM, (2) gp 32-*S*-NEM + poly(HgU), and (3) gp 32-*S*-NEM + poly(HgU) + 1 M NaCl. The following concentrations were used: [gp 32-*S*-NEM] = 6.7×10^{-6} M; [poly(HgU)] = 8×10^{-5} M.

(C) Study of Complexes of gp 32-S-NEM with d-DNA(Pt) or Poly(HgU) at 77 K. Complexes of gp 32-*S*-NEM with either d-DNA(Pt) or poly(HgU) were formed in solution at room temperature and then frozen down to 77 K. Fluorescence and phosphorescence spectra of the complexes were recorded and compared to those of the free components. The results obtained with poly(HgU) are shown in Figures 4 and 5.

In the presence of poly(HgU), the protein fluorescence was quenched (≃40%), but no peak shift was observed. The phosphorescence intensity was enhanced [the contribution of the poly(HgU) phosphorescence was taken into account], but no detectable peak shift was observed (Figure 5), in contrast with the results obtained with model peptides (see above). Dissociation of the complexes by increasing the ionic strength restored the protein fluorescence, taking into account the screening effect of the polynucleotide. Note that addition of 1 M NaCl to the protein or poly(HgU) alone induced a slight increase (≃10%) of the phosphorescence intensity but was without any effect on the shape of the phosphorescence spectra.

Phosphorescence lifetimes were measured at two different polynucleotide to protein ratios (Table II). The phosphorescence decay of gp 32-*S*-NEM measured at 430 nm was monoexponential with a lifetime of 5.5 ± 0.3 s, whereas that of poly(HgU) was found to be biexponential [1.0 ± 0.2 ms (85%) and ≃0.5 s (15%)]. Results presented in Table II show that in the gp 32-*S*-NEM–poly(HgU) complexes, a new short-lifetime component (5.5 ± 0.3 ms) was detected, accounting for more than 50% of the observed phosphorescence. This lifetime component is remarkably close to that observed with peptide–poly(HgU) complexes where a dramatic heavy atom effect has been detected (see above). Dissociating the complexes by adding either NaCl or $MgCl_2$ decreases the contribution of the 5-ms component and concomitantly increases that of the noninteracting tryptophyl residues (τ_p = 5.5 s). The 5-ms component did not vanish in the presence of 1 M NaCl. This is very likely due to incomplete dissociation of the complexes in the frozen state.

Table II: Phosphorescence Lifetimes (τ_p) of gp 32-*S*-NEM and Poly(HgU) Mixtures at 77 K in Buffer II[a]

[nucleotide]/ [protein]	salt	τ_p
12	+NaCl (10 mM)	5.5 s (40%), 1.2 ms (8%), 5.3 ms (52%)
12	+NaCl (1 M)	5.2 s (67%), 1.0 ms (8%), 5.8 ms (25%)
21	no $MgCl_2$	5.4 s (35%), 1.2 ms (9%), 5.5 ms (56%)
21	+$MgCl_2$ (0.5 M)	5.2 s (68%), 1.0 ms (16%), ≃5 ms (16%)

[a] λ_{exc} = 290 nm. Phosphorescence decays were measured at λ_{em} = 430 nm. Protein concentration = 6.7×10^{-6} M.

These results show that a heavy atom effect can be observed in the gp 32-*S*-NEM–poly(HgU) complex. This indicates that at least one tryptophyl residue of the protein is close to mercury atoms. Measurements of phosphorescence lifetimes made at 390 nm in the tyrosine phosphorescence region have shown that the tyrosine triplet lifetime (2.3 s) was not affected when the protein was complexed to poly(HgU).

No heavy atom effect was observed in complexes of the native or modified protein with d-DNA(Pt) or poly(HgU-Cys). Steric hindrance may be invoked to explain these results since in the latter polynucleotides the heavy atoms are surrounded by more bulky groups than in mercurated poly(U).

Conclusions

We have demonstrated in this study that the heavy atom perturbation method can be used to detect the presence of tryptophyl residues of peptides or proteins interacting with nucleic acid bases. In complexes formed by tryptophan-containing peptides with poly(HgU), the tryptophyl residues which are in contact with mercury atoms are characterized by a strong quenching of their fluorescence, an enhancement of their phosphorescence intensity, and a reduction of their phosphorescence lifetime from 6 s to 5 ms. In complexes of mercurated poly(U) with the T4 gene 32 protein whose two accessible SH groups were blocked by NEM reaction, a short-lifetime component of ≃5 ms was also observed at 77 K. This result allows us to conclude that in these complexes at least one tryptophyl is in close contact with the mercury atom in the fifth position of the uracil ring. Blocking of SH groups on the protein was necessary to avoid any chemical reaction with poly(HgU). This protein modification leads to a local alteration of the protein conformation that was revealed by a change in the intrinsic fluorescence but not detected either by CD measurements or during the binding assays of the modified protein onto polynucleotides. Modification of cysteinyl residues on gp 32 by NEM does not significantly alter the protein binding affinity or the binding site size in contrast to the results obtained following protein reaction with an ionic

SH reagent such as 5,5′-dithiobis(2-nitrobenzoic acid) [see Casas-Finet et al. (1984)]. These results suggest that the conclusion reached with gp 32-*S*-NEM could be extrapolated to the unmodifed protein; i.e., at least one tryptophyl residue is in close contact with the polynucleotide bases.

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