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Specific Recognition of Single-Stranded Regions in Ultraviolet-Irradiated and Heat-Denatured DNA by Tryptophan-Containing Peptides

(protein–nucleic acid interaction/photoreactivation/fluorescence)

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ABSTRACT **Fluorescence studies of the binding of peptides containing lysyl and tryptophyl residues to nucleic acids show that two types of complexes are formed. One of them involves a direct interaction of the tryptophyl ring with nucleic acid bases, which leads to fluorescence quenching. Comparison with proton magnetic resonance and circular dichroism data indicates that this fluorescence quenching is due to a stacking of the indole ring with bases. Quantitative analysis of fluorescence data leads to the conclusion that stacking is favored in single-stranded regions of DNAs, which are produced either by heating or by UV-irradiation of the native DNA sample. The binding of the peptide Lys-Trp-Lys is about ten times tighter in these single-stranded regions as compared with double-stranded ones. A short tripeptide such as Lys-Trp-Lys is, therefore, able to discriminate between single-stranded and double-stranded regions. Moreover, bound peptide molecules photosensitize the splitting of thymine dimers in UV-irradiated DNA, thus providing a model for DNA photoreactivation.**

We are presently investigating the origin and the nature of molecular interactions responsible for the specific recognition of nucleic acid sequences by proteins (see refs. 1–4 for previous publications). Since we are interested in the role played by aromatic amino acids, we have studied the binding of oligopeptides containing lysyl and tryptophyl residues such as Lys-Trp-Lys to different nucleic acids and polynucleotides. Proton magnetic resonance (2, 4) and circular dichroism* studies have provided evidence for a stacking interaction of the tryptophyl ring with bases. A similar conclusion has also been reached by Gabbay and coworkers for different tryptophan-containing peptides (5, 6). Fluorescence and phosphorescence studies at low temperature have shown that the tryptophyl ring stacked with bases acts as a trap for the triplet excitation energy (7). At room temperature, fluorescence spectroscopy is appropriate for study of the binding of tryptophyl-containing peptides to nucleic acids since only the tryptophyl ring emits fluorescence in fluid medium. The weak fluorescence of nucleic acid bases (8, 9) at room temperature has a quantum yield that is 3 orders of magnitude smaller than that of tryptophan.

A quantitative analysis of fluorescence data is presented here which shows that two types of complexes are formed when oligopeptides such as Lys-Trp-Lys bind to nucleic acids. The role of the local structure of nucleic acids in the binding process is investigated by comparing the behavior of heat-denatured or UV-irradiated DNA with native double-stranded DNA with respect to stacking of the tryptophyl ring with bases. It is also shown that the tryptophyl ring is able to photosensitize the splitting of thymine dimers in UV-irradiated DNA.

* M. Durand, H. N. Borazan, J. C. Maurizot, and C. Hélène, to be published.

EXPERIMENTAL

Fluorescence measurements were made with a Jobin-Yvon spectrofluorimeter that was modified to correct for lamp fluctuations by deflecting part of the incident beam onto a rhodamine B quantum counter. The sample was contained in a 5-mm quartz Suprasil thermostated cell (usually 2°).

Difference absorption spectrophotometry measurements have shown that the binding of tryptophan-containing peptides to nucleic acids is accompanied by a small but reproducible absorption change. The excitation wavelength was therefore chosen at the isosbestic point of these difference spectra (292 nm).

To correct fluorescence intensities for the screening effect of the nucleic acid at the excitation wavelength, peptide–nucleic acid complexes were dissociated by increasing the ionic strength (2, 12). At high ionic strength (0.5 M NaCl), only the screening effect of the nucleic acid was assumed to lead to an apparent decrease of the fluorescence quantum yield of the peptide. All fluorescence quantum yields were measured with respect to that of the free peptide and corrected for the screening effect of the nucleic acid.

Polynucleotides and oligopeptides were purchased from Miles and Schwarz/Mann, respectively. *Escherichia coli* DNA was purified by the usual procedure (10). [^{14}C]Thymine-labeled *E. coli* DNA was a gift from Drs. W. L. Carrier and R. B. Setlow.

Solutions were prepared in a buffer containing 1 mM sodium cacodylate and 0.2 mM EDTA and adjusted to pH 6 (DNA) or pH 7 (polynucleotides). The ionic strength was changed by increasing the concentration of sodium chloride in the solution.

RESULTS

The binding of peptides containing lysyl and tryptophyl residues to nucleic acids is accompanied by a decrease of the fluorescence quantum yield of tryptophan (2) (Fig. 1). The fluorescence lifetime remains unaffected, as already observed for serotonin binding to DNA (11). This result indicates that only two types of peptide molecules exist in solution from the fluorescence viewpoint: those that have a fluorescence quantum yield equal to that of the free peptide and those that do not emit fluorescence at all. Quantitative analysis of

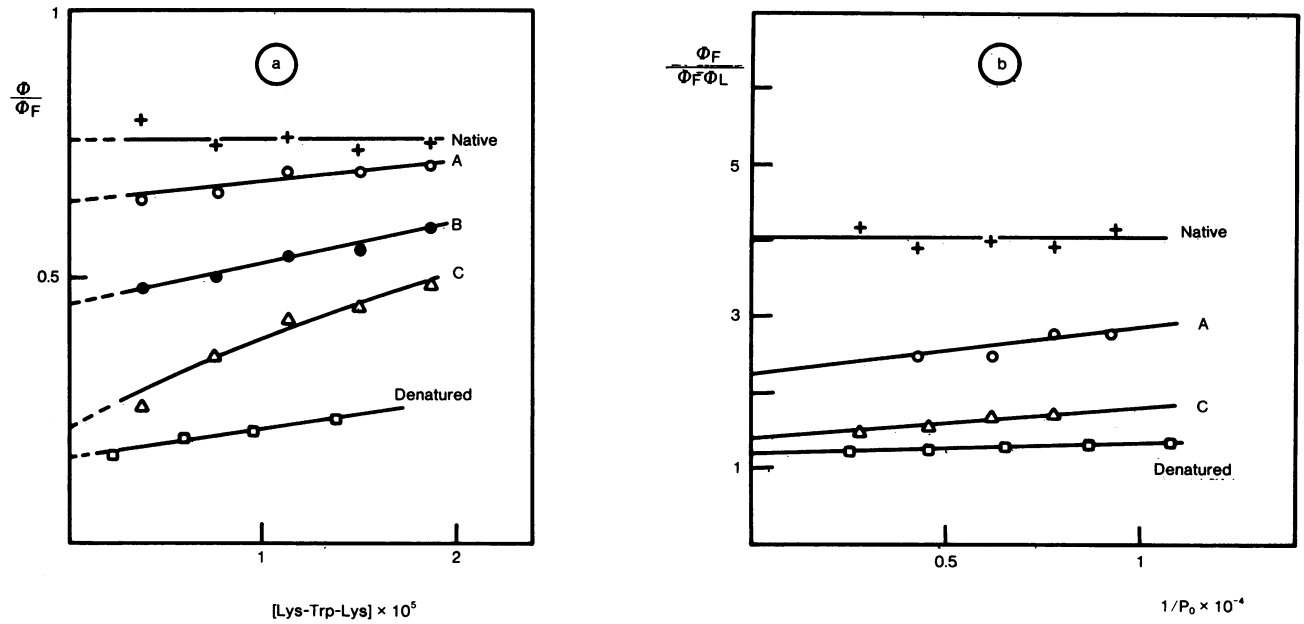

FIG. 1. (*a*) Concentration dependence of the overall fluorescence quantum yield of the peptide Lys-Trp-Lys (relative to that of the free peptide) in the presence of native, denatured, and UV-irradiated *E. coli* DNA (each 0.5 mM). Fluorescence measurements were performed at 2° in a buffer containing 1 mM NaCl, 1 mM Na cacodylate, 0.2 mM EDTA at pH 6.0. Denatured DNA was prepared by heating the native sample for 10 min at 90° and then rapidly cooling to 0°. DNA was irradiated with a HBO 200-W lamp whose radiations below 250 nm and above 310 nm were filtered out. Durations of irradiation were 2 min (*A*), 10 min (*B*), and 20 min (*C*). The percentage of thymine converted to dimers was 6, 17, and 21, respectively. Values on the *abscissa* have been multiplied by 10^5, as shown. (*b*) Analysis of fluorescence data according to Eq. **3** for native, denatured, and two samples (*A* and *C*, see *a*) of UV-irradiated DNA.

fluorescence data [whose details will be published elsewhere (12)] and comparison with proton magnetic resonance and circular dichroism data led us to the conclusion that peptides bound to the nucleic acid (*A*) may exist in two different states (called *C* and *D* hereafter) according to scheme 1†

$$A + B \underset{}{\overset{K_1,n}{\rightleftharpoons}} C \overset{K_2}{\rightleftharpoons} D \qquad [1]$$

In complex (*C*) the tryptophyl ring does not interact with bases and has the same proton chemical shifts and the same fluorescence quantum yield as the free peptide (*B*). In complex (*D*), the tryptophyl ring is stacked with bases (2, 4); it has upfield shifted proton resonances; and its fluorescence quantum yield is reduced to zero. K_1 and K_2 are equilibrium constants and n is the number of binding sites per phosphate.

† This scheme is formally identical to and cannot be distinguished on the basis of equilibrium data from a superposition of two equilibria involving the same number of binding sites

$$A + B \overset{K_I,n}{\rightleftharpoons} C$$

$$A + B \overset{K_{II},n}{\rightleftharpoons} D$$

with $K_I = K_1$ and $K_{II} = K_1K_2$. In both cases the value of K_2 represents the ratio of the concentrations of the two types of complexes *C* and *D* ($K_2 = [D]/[C]$). In a previous paper (2), fluorescence data were analyzed according to a model involving only one complex whose fluorescence quantum yield was assumed to be zero. This was not correct since further experiments demonstrated that the association constant determined under these conditions changed with the nucleic acid concentration.

The overall fluorescence quantum yield (ϕ) of a solution containing the peptide and the nucleic acid is given by

$$\phi = \phi_F \left(\frac{[B] + [C]}{[B]_0} \right) \qquad [2]$$

where ϕ_F is the fluorescence quantum yield of the free peptide. At very low peptide concentration, the number of occupied sites

$$r = \frac{[C] + [D]}{[P]_0}$$

(where $[P]_0$ is the phosphate concentration) is low as compared to the total number of sites (n). The overall fluorescence quantum yield tends toward a limit (ϕ_L) such that Eq. **3** is obeyed:

$$\frac{\phi_F}{\phi_F - \phi_L} = \frac{1 + K_2}{K_2} + \left(\frac{1}{K_1K_2n} \right) \left(\frac{1}{[P]_0} \right) \qquad [3]$$

Extrapolation of plots of ϕ against $[B]_0$ at constant $[P]_0$ gives the value of ϕ_L (Fig. 1). Then, a plot of $\phi_F/(\phi_F - \phi_L)$ against $1/[P]_0$ gives straight lines, and from x and y axis intercepts the value of K_2 and K_1n can be obtained. The values of K_1 and n can then be obtained independently by a Scatchard representation. It must be noticed that the overall binding constant is $K_1(1 + K_2)$.

Experiments with DNA were performed at different ionic strengths (1–10 mM NaCl). The association constant K_1 decreased when the ionic strength increased, as expected for electrostatic interactions between lysine and phosphate groups (2). The equilibrium constant K_2 did not change appreciably with ionic strength (Table 1). Since we were mostly

TABLE 1. *Ionic strength dependence of the binding of Lys-Trp-Lys to E. coli native DNA*

NaCl mM	$K_1n(\times 10^{-4})$ (M^{-1})	K_2
1	7.3	0.36
5	3.2	0.31
9	1.2	0.36

The buffer (pH 6) contained 1 mM sodium cacodylate and 0.2 mM EDTA. Temperature: 2°. Analysis of fluorescence data is made according to scheme 1 and Eq. 3. Values for K_1n have been multiplied by 10^{-4}, as shown.

interested in the influence of the structure of the nucleic acid on the value of K_2, all experiments described below were performed at low ionic strength (1 mM NaCl). Under these conditions, the changes in fluorescence quantum yield due to peptide binding are high enough to obtain an accurate value of K_2 although the small slope of the straight lines obtained when plotting $\phi_F/(\phi_F - \phi_L)$ against $1/[P]_0$ does not allow us to obtain accurate values for K_1 (because K_1 is too high at low ionic strength). It should be noted that circular dichroism data obtained for single-stranded polynucleotides led to values of K_1 $(1 + K_2)$ in quite good agreement with those deduced from fluorescence measurements*.

There is a large difference in K_2 values between native DNA and denatured DNA (Fig. 1; Table 2). The behavior of the latter is similar to that of single-stranded polynucleotides such as poly(A) at pH 7. The value of K_2 represents the ratio of stacked and unstacked tryptophyl rings. It can be concluded (Table 2) that stacking is favored in single-stranded molecules. Although the K_2 value obtained with native DNA is small, it is not zero. Whether tryptophan stacks with bases in "opened" regions of native DNA (13) or whether it intercalates between base pairs in double-stranded regions remains to be determined (structural defects in our DNA samples may also contribute to the value of K_2).

In order to determine the behavior of the peptide Lys-Trp-Lys with respect to a DNA containing single-stranded regions, we introduced defects in the DNA double helix by submitting our DNA sample to UV-irradiation. Such an irradiation produces pyrimidine dimers, and the two strands separate in these dimer-containing regions (14). These structural defects rather than their chemical nature are recognized by the photoreactivating enzyme (15) or by the UV-endonuclease whose action is the first specific step in dark repair of UV-irradiated DNA (16). By the use of [^{14}C]thymine-labeled DNA, the number of thymine-containing dimers (which represent most of the dimers) can be determined from a chromatographic analysis of hydrolyzed DNA (17).

As shown in Fig. 1, the overall fluorescence quantum yield of Lys-Trp-Lys decreases much more in the presence of UV-irradiated DNA than in the presence of native DNA. Fluorescence data were then submitted to a quantitative analysis in terms of two types of complexes, as described above (Eq. 1). The K_2 value determined for a particular sample of UV-irradiated DNA represents an average value over native and denatured parts of DNA. When the percentage of thymine dimers in DNA is high, plots of ϕ against B_0 are not straight lines (see Fig. 1*a*), which makes it difficult to obtain accurate values of ϕ_L. However, this does not introduce a large error in the determination of K_2. The main conclusion that can be

TABLE 2. *Equilibrium constant K_2 (see scheme 1) determined from fluorescence data analyzed according to Eq. 3 for the binding of Lys-Trp-Lys to native, denatured, and UV-irradiated E. coli DNA*

	K_2
Native DNA	0.36
Denatured DNA	4.9
UV-irradiated DNA (2 min)	0.87
UV-irradiated DNA (20 min)	2.5
Poly(A)	2.18
Poly(U)	3.84
Poly(A)·poly(U)	0.56

Polynucleotides are included for comparison. Measurements were performed at 2° and pH 6.0 for DNA or pH 7.0 for polynucleotides. Solutions were made in a sodium cacodylate buffer (1 mM), 0.2 mM EDTA, in the presence of 1 mM sodium chloride. The DNA samples, irradiated for 2 and 20 min, contained 6% and 21% thymine as dimers, respectively.

reached (Table 2) is that K_2 is increased by a factor of about 10 in denatured regions of DNA. The K_2 value is a measure of the relative proportion of bound peptide molecules whose tryptophyl ring is stacked with bases. Since electrostatic interactions between Lys-Trp-Lys and phosphate groups are not expected to be markedly affected in locally denatured regions of double-stranded DNA, the above result means that a simple oligopeptide such as Lys-Trp-Lys is able to "recognize" single-stranded regions in a nucleic acid structure.

We had previously shown that indole derivatives could photosensitize the splitting of pyrimidine dimers (18). We have shown above that the oligopeptide Lys-Trp-Lys binds preferentially to regions containing dimers. Therefore, we investigated whether the splitting of thymine dimers could be photosensitized by this oligopeptide. We first irradiated DNA with UV-light to produce dimers. Then the peptide was added and the mixture was further irradiated under the same irradiation conditions ($250 < \lambda < 310$ nm). As shown in Table 3, thymine dimers are formed by direct UV irradiation and then split after photosensitization by the peptide. Tryptophan itself, which does not bind to DNA, was ineffective as a photosensitizer of dimer splitting [this also eliminates the possibility that the tryptophyl chromophore of Lys-Trp-Lys had filtered out long-wavelength radiations and could thus have changed the photochemical equilibrium between monomers and dimers, which is sensitive to the irradiation wavelength (14)]. To show that only the peptide molecules bound to DNA were effective as sensitizers, the peptide–DNA complex was dissociated by increasing the ionic strength (see ref. 2). As shown in Table 3, the rate of photosensitized splitting is markedly decreased in the presence of 0.1 M NaCl.

CONCLUSION

The experiments described above demonstrate not only that the peptide Lys-Trp-Lys binds preferentially to regions containing pyrimidine dimers in UV-irradiated DNA, but also that bound peptide molecules are able to photosensitize the splitting of these dimers. Thus, this small oligopeptide mimics the binding and the reaction of the photoreactivating enzyme (although we do not imply that the mechanisms are identical in both cases).

TABLE 3. *Photosensitized splitting of thymine-containing dimers in DNA by Lys-Trp-Lys*

Irradiation times (Lys-Trp-Lys photosensitization)	NaCl, mM	% of dimers split
10 min	1	44
30 min	1	54
2 hr	1	59
10 min	100	10.5
30 min	100	41
2 hr	100	54

Solution of [^{14}C]thymine-labeled DNA (0.1 mg/ml) was preirradiated for 40 min with a 200-W mercury lamp, whose radiations below 250 nm and above 310 nm were filtered out. The irradiated DNA contained 25.2% of its thymines as dimers. Photosensitization by Lys-Trp-Lys (0.1 mM) was performed under the same irradiation conditions. Samples (1 ml) were then dialyzed against water and lyophilized. Hydrolysis was performed in sealed glass tubes at 170° for $^1/_2$ hr, in the presence of 0.1 ml of formic acid (according to Carrier and Setlow, ref. 17). The hydrolysate was spotted on Whatman no. 1 paper, and developed in the solvent isopropanol–concentrated HCl–water (4:1:1, v/v). The paper was cut into 1-cm strips, and the radioactivity was assayed by liquid scintillation counting.

The peptide Lys-Trp-Lys binds preferentially to single-stranded regions of a nucleic acid molecule because the stacking of the tryptophyl ring with bases is energetically favored in such regions. Such a mechanism could be operating in the recognition of altered regions of DNA (e.g., by the photoreactivating enzyme or the UV-endonuclease). It could also be involved in the interactions between aminoacyl-tRNA synthetases and tRNAs (19) or in the regulation of DNA gene expression in chromosomes (20).

The binding of Lys-Trp-Lys to native DNA induces conformational changes in the DNA molecule, as shown by circular dichroism measurements (unpublished results). The size of the indole ring is similar to that of purine bases so that the stacking of tryptophan with DNA bases may involve only one strand of the double helix. If base pairing is locally disrupted in regions where a tryptophyl ring of a protein is inserted, this could introduce new hydrogen bonding possibilities for the recognition of bases by other amino acid side chains of the protein.

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