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Time resolved fluorescence of bacteriophage Pfl DNA binding protein and its complex with DNA

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Abstract. The DNA binding protein of the filamentous bacteriophage Pfl exhibits fluorescence from a single tryptophan residue. The location of the emission maximum at 340 nm ist quite common for proteins, but the single lifetime of 7.8 ns is one of the longest yet reported. Protein fluorescence is quenched more efficiently by Cs^+ than by I^-; the Trp is located in a partially exposed pocket, in the vicinity of a negative charge.

In the native complex of the binding protein with Pfl DNA the fluorescence emission maximum is at 330 nm, indicating a more apolar environment for Trp 14. The native nucleoprotein complex exhibits a similar fluorescence lifetime (6.5 ns) and an approximately equal fluorescence yield, indicating the absence of Trp-DNA stacking. The tryptophan in the complex is virtually inaccessible to ionic quenchers, and thus appears to be buried.

Fluorescence depolarisation measurements have been used to examine the rotational mobility of the tryptophan in the protein and in the nucleoprotein complex. In the protein alone a single rotational correlation time (Φ) of ~ 19 ns is observed, corresponding to rotation of the entire dimeric molecule; in the native nucleoprotein complex with Pfl DNA, a Φ of ~ 500 ns is observed, corresponding to a rigid unit of at least 50 subunits. In neither case does the tryptophan exhibit any detectable flexibility on the subnanosecond time scale.

Key words: Time resolved fluorescence, Pfl-phage, DNA binding protein, fluorescence depolarisation

Introduction

The DNA binding protein of Pfl filamentous bacteriophage is one of the major proteins produced during infection of Pseudomonas aeruginosa (Kneale and Marvin 1982). The protein is present in the cell predominantly as a stoichiometric complex with single-stranded viral DNA (Gray et al. 1982; Kneale and Marvin 1982) and, like the gene 5 protein of fd filamentous bacteriophage, is probably responsible for inhibition of double-stranded viral DNA synthesis, and for packaging the viral DNA strands in a conformation suitable for incorporation into virions (Henry and Pratt 1969; Salstrom and Pratt 1971; Mazur and Model 1973).

Measurements of nucleoprotein mass by scanning transmission electron microscopy (Kneale et al. 1982) indicate that there are approximately four nucleotides per protein subunit. X-ray fibre diffraction studies show that the complex is a helical assembly with pitch 45 Å; the asymmetric unit is a protein dimer linking the two antiparallel DNA strands, and there are six such dimers per helical turn (Kneale et al. 1982). However, the helix parameters can vary considerably as a function of ionic strength and humidity.

The amino acid sequence of the Pfl DNA binding protein has been determined (Maeda et al. 1982); the aromatic residues of the protein (M_r = 15,400) are one tryptophan, three tyrosines, and five phenylalanines. The fd gene 5 protein (M_r = 9,690) contains no tryptophan and shows a typical tyrosine fluorescence spectrum that is quenched on DNA binding (Pretorius et al. 1975). In view of the unusually high resistance of the Pfl nucleoprotein complex to dissociation by high salt concentrations (Kneale and Marvin 1982; Kneale 1983) and the generally accepted role of aromatic amino acids in binding to single-stranded DNA (Helene and Maurizot 1981; Helene and Lancelot 1982) it is of interest to study the

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interaction of DNA with aromatic residues in the Pfl DNA binding protein.

For a proper investigation of the interaction of the DNA binding protein with nucleic acids by fluorescence, it is necessary to characterize the fluorescence by time-resolved spectroscopy. As a first step in this investigation we have studied the Pfl DNA binding protein and its native complex with DNA, using fluorescence emission spectra, fluorescence lifetime and depolarisation measurements. Quenching studies have also been performed to give additional information on the local environment around the tryptophan. The results reveal a significant change in this environment upon binding to DNA, and a long fluorescence lifetime of the tryptophan in the free protein.

Materials and methods

The Pfl nucleoprotein complex was prepared from Pfl infected Pseudomonas aeruginosa according to the method of Kneale and Marvin (1982). The Pfl DNA binding protein was isolated from crude extracts of the nucleoprotein complex, by dissociation of the complex in 1 M $MgCl_2$, followed by chromatofocusing on Pharmacia PBE 74 (Kneale 1983). The purity of the samples was checked by SDS gel electrophoresis and UV spectroscopy. Concentrations were estimated from the extinction coefficients of the protein $A_{278}^{1\%} = 7.7$, and nucleoprotein, $A_{260}^{1\%} = 23$ (Kneale 1983).

Fluorescence emission spectra were recorded with a Perkin Elmer MPF 44A spectrofluorimeter operated in the ratio mode. The spectra were not corrected for spectral sensitivity changes in the detector.

Fluorescence lifetimes were measured with the EMBL double beam fluorescence lifetime spectrometer which is described in detail elsewhere (Wijnaendts van Resandt et al. 1982). In short, the system consists of a synchronously pumped, cavity dumped dye laser, the output of which is frequency doubled to 280–300 nm. The pulsewidth of the system is 10 ps with a repetition rate of 4 MHz. The tryptophan fluorescence of the sample was excited at 298 nm and observed through a 20-nm interference filter centered around 340 nm. Simultaneously, the fluorescence of a known standard (p-terphenyl) was measured. Lifetime spectra were measured using the single photon technique (Yguerabide 1972) with a very fast channelplate photomultiplier. The time response of the system is 150 ps (FWHM). The data were analysed using a nonlinear least-squares fitting procedure, where the fluorescence of the standard is used to account for the response function. The principle of the data analysis method is based on the technique reported by Gauduchon and Wahl (1978) and our implementation has been reported in detail (Wijnaendts van Resandt et al. 1982). A good fit to the data results in randomly distributed weighted residuals and a reduced chi-square of the order of unity.

Fluorescence depolarisation measurements were made using an extension of the spectrometer described above. The vertically and horizontally polarised components of the emitted light are now detected simultaneously by the same detector. This is done by using a 45-ns optical delay of the horizontally polarised component with respect to the vertically polarised emitted light. The remainder of the equipment is identical to that described for the fluorescence lifetime measurements. Since the apparatus response function is very short (150 ps) with respect to a typical re-orientation times of macromolecules, no attempt was made to convolve the model function with this response. The time dependent change of the fluorescence anisotropy upon excitation with a very short, vertically polarised pulse, is given by (see e.g., Wahl 1975)

$$A(t) = [I\|(t) - I\perp(t)]/[I\|(t) + 2\,I\perp(t)], \qquad (1)$$

where $I\|$ is light polarised parallel to the plane of polarisation of the excitation beam and $I\perp$ is perpendicularly polarised. For a spherical macromolecule Eq. (1) can be fitted with $A(t) = A_o e^{-t/\Phi}$, where Φ is the rotational correlation time. The same least-squares fitting procedure was used to describe the decay of the anisotropy as was used for the fluorescence lifetime measurements. However, the data were not convolved with the apparatus response function and the residuals were weighted in accordance with the statistical errors expected from Eq. (1) (Wahl 1979).

Quenching curves were obtained by adding up to 0.2 ml of quenching solution (1 g/ml CsCl or 0.5 g/ml KI) to 2.0 ml of solutions of N-acetyltryptophanamide, protein or complex. The concentrations of the quenched substances were chosen to give similar fluorescence intensity, corresponding to about 0.1 mg/ml protein. Since fluorescence lifetimes were used for the Stern-Volmer plots, corrections for dilution were unnecessary. However, static quenching due to the formation of nonradiative complexes will not be detected using the lifetime method. As a minor contribution from a short lifetime ($\tau = 1.8$ ns) component of variable amplitude is observed, in particular after prolonged storage at −20° C, the spectra were routinely analyzed as bi-exponential (see also result in caption of Fig. 1), and the major (long lifetime) component was used in the further analysis.

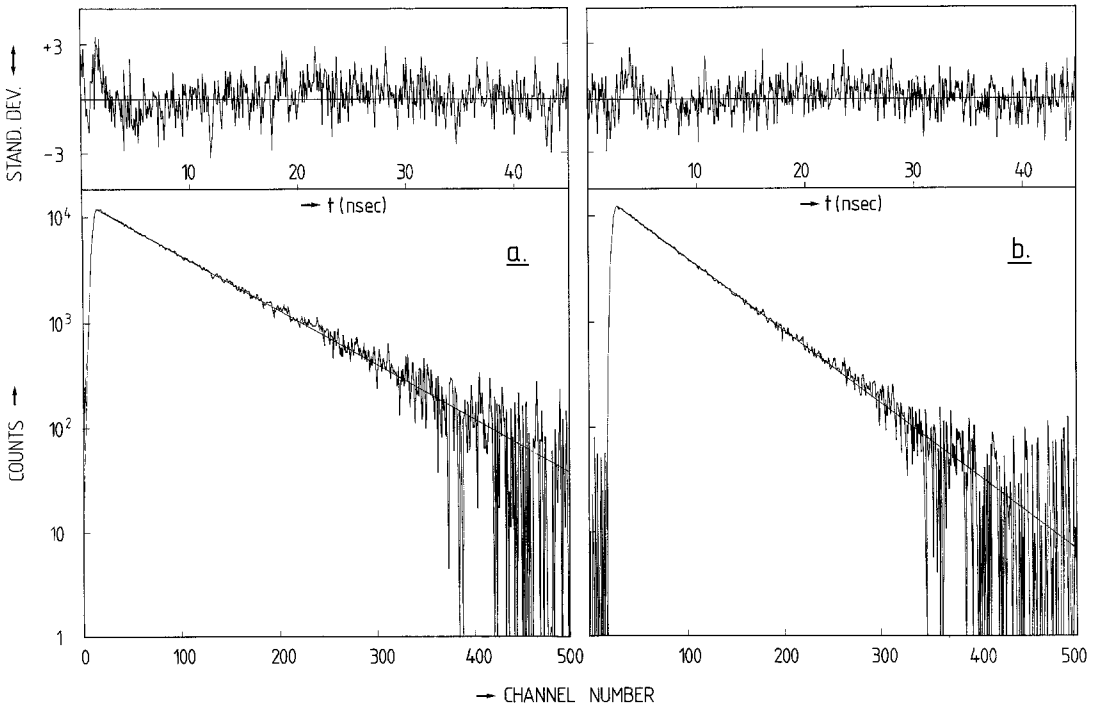

Fig. 1a and b. Fluorescence lifetime curves of the Pfl DNA binding protein (**a**) and the native Pfl nucleoprotein complex (**b**), both in 10 mM *Tris* · HCl, pH 7.5. Excitation wavelength was 285 nm; emission wavelength 340 nm. Both curves were fitted with the sum of two exponentials; (**a**) $\tau_1 = 7.75 \pm 0.06$ ns, $A_1 = 0.93 \pm 0.02$, $\tau_2 = 1.88 \pm 0.10$ ns, $A_2 = 0.07 \pm 0.02 \pm 0.02$ with a reduced chi-square of fit: $\chi^2 = 0.98$. (**b**) $\tau_1 = 6.52 \pm 0.05$ ns, $A_1 = 0.97 \pm 0.02$, $\tau_2 = 2.43 \pm 0.30$ ns, $A_2 = 0.03 \pm 0.02$ with $\chi^2 = 0.94$. In order to show the small effect of the short lifetime component a single exponential fit was made for which the resulting weighted residuals are shown above the corresponding decay curves. The results were: (**a**) $\tau = 7.29 \pm 0.02$ with $\chi^2 \pm 1.14$ and (**b**) $\tau = 6.38 \pm 0.02$ with $\chi^2 = 1.03$. In all further results presented, a two component fit was made and the irreproducible short component ignored (see text)

Results

Fluorescence emission spectra and decay curves

The fluorescence spectrum of the Pfl nucleoprotein complex has a maximum near 330 nm and a width of 50 nm (FWHM), typical of tryptophan in a hydrophobic environment (Burstein et al. 1973). The fluorescence spectrum of the isolated protein is shifted towards higher wavelength, with a peak at 340 nm and width of 55 nm (FWHM). When the complex is dissociated by $MgCl_2$ or NaSCN one obtaines the same emission spectrum as that of the free protein. Thus, fluorescence spectra can be used to follow dissociation and reconstitution of the complex. Upon denaturation by urea, the spectrum shifts to higher wavelength ($\lambda_{max} = 355$ nm) and resembles that of a free tryptophan in water.

In order to be able to compare the relative fluorescence yield of the free protein and the protein-DNA complex two spectra were recorded. First, with the native complex and then with the dissociated complex formed by addition of $MgCl_2$. After correction for dilution the spectra were integrated between 300 and 450 nm. The relative change of the fluorescence yield was found: $I_{complex}/I_{protein} = 0.82$. In all of these experiments the excitation wavelength was 298 nm.

Figure 1a gives the fluorescence decay curve of the free protein. The result of the non-linear least-squares analysis (Wijnaendts van Resandt et al. 1982) shows that it can almost be described by a monoexponential decay with a long lifetime of $\tau = 7.8$ ns. A minor, short lifetime component ($\tau \approx 1.8$ ns) of variable amplitude was observed, in particular after prolonged (> 1 month) storage of the sample at −20° C and may possibly be due to proteolysis. In fresh samples the contribution of this component was less than 2% of the total fluorescence. In Fig. 1b the lifetime curve of the native complex is given. Again, the fluorescence decay is almost monoexponential, with a lifetime of 6.5 ns. There is therefore a small but significant change in the tryptophan fluorescence properties when the protein is complexed to viral DNA. These results are also summarized in Table 2.

Accessibility of the tryptophan

In principle, the use of D_2O instead of water, as a solvent, can give information on the accessibility of a

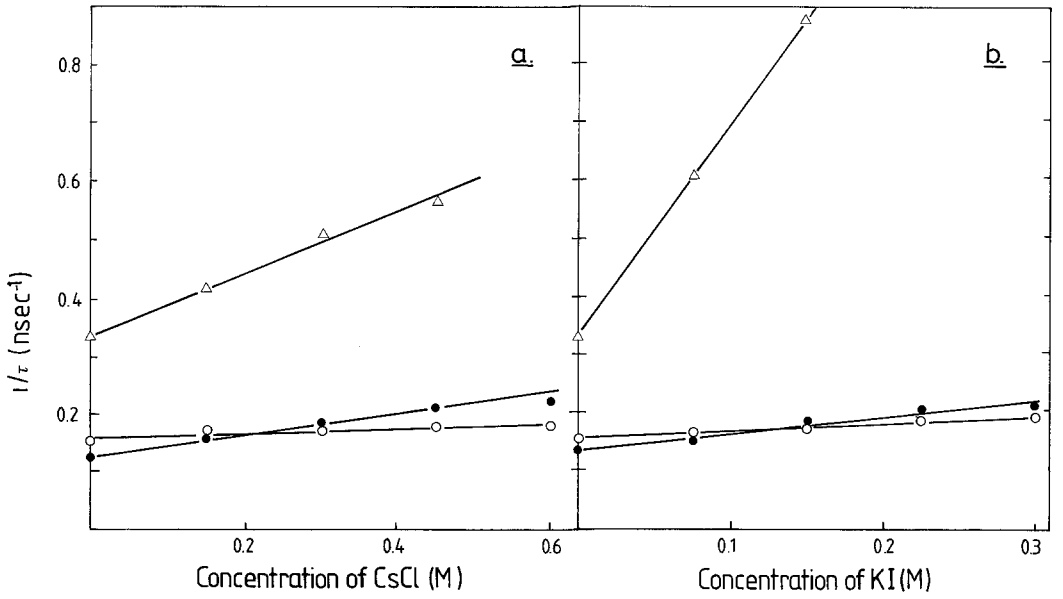

Fig. 2a and b. Stern-Volmer plots for fluorescence quenching by CsCl (**a**) and KI (**b**). Fluorescence lifetime, τ, of the free protein (●), the native nucleoprotein complex (○) and the model compound N-acetyl-tryptophanamide (△), were measured with increasing concentrations of quencher. Note that for the model compound the quenching efficiency of KI is approximately 6.5 fold that of CsCl

tryptophan group (McGuire and Feldman 1973). For example, transfer of N-acetyl-tryptophanamide or the ACTH-sequence 1–25 with its one exposed tryptophan from H_2O to D_2O causes an increase of the fluorescence yield and lifetime by approximately 20% (data not shown). However, for the Pfl DNA binding protein and the nucleoprotein complex, D_2O has no observable effect on the fluorescence lifetime.

The accessibility of a fluorescing group can also be demonstrated by the use of fluorescence quenchers. Often, the relationship between intensity, I or lifetime, τ and the quencher concentration is given by the Stern-Volmer equation.

$$I_o/I = \tau_0/\tau = 1 + k_q \cdot \tau_0 [Q], \quad (2)$$

where k_q is the bimolecular quenching constant. I_0 and τ_0 are values measured in the absence of quencher. Deviations from this Stern-Volmer law are more probable for fluorescence intensities than for lifetimes for reasons summarized by Eftink and Ghiron (1981). The most important effect being the static quenching, i.e., the formation of non-radiative complexes. Indeed, with the Pfl DNA binding protein, fluorescence intensities do not follow the Stern-Volmer equation. All of the fluorescence decay curves were fitted as described and the resulting fluorescence lifetimes were used in the quenching analysis. For high quencher concentrations (i.e., 0.3–0.6 M KI) an appreciable deviation from a single exponential decay curve was found (data not shown). A possible explanation is that high ionic strength was found to lead to aggregation of the protein (see next paragraph). Also, transient quenching effects (Wijnaendts van Resandt 1983) and multiple environments in the tryptophan (Eftink and Ghiron 1981) could be responsible for non-exponential decay behaviour. However, we were interested in a rough determination of the accessibility of the tryptophan to ionic quenchers and to solvent and a detailed quenching study is beyond the scope of this work. The dynamic quenching data for the protein, the nucleoprotein complex and the model substance N-acetyl-tryptophanamide are plotted in Fig. 2 according to a slightly modified Stern-Volmer equation.

$$1/\tau = 1/\tau_0 + k_q[Q], \quad (3)$$

Table 1. Fluorescence quenching data for the Pfl DNA binding protein and the native nucleoprotein complex

	k_q ($\times 10^{-8}$ $M^{-1} \cdot s^{-1}$)		k'	
	Cs^+	I^-	Cs^+	I^-
Protein	0.18	0.28	0.33	0.08
Nucleoprotein complex	0.04	0.13	0.07	0.04
N-acetyl tryptophanamide	0.54	3.70	1.0	1.0

k_q is the absolute quenching constant for CsCl and KI. The relative quenching constant k' is given by the ratio of the quenching constant of the sample to that of the model compound N-acetyl-tryptophanamide. The relative error in the determination of k_q depends on the measurement of τ (~ 1%) and the error in the concentration of the quencher (estimated ~ 4%) resulting in an error of ~ 5% in k_q and ~ 7% in k'

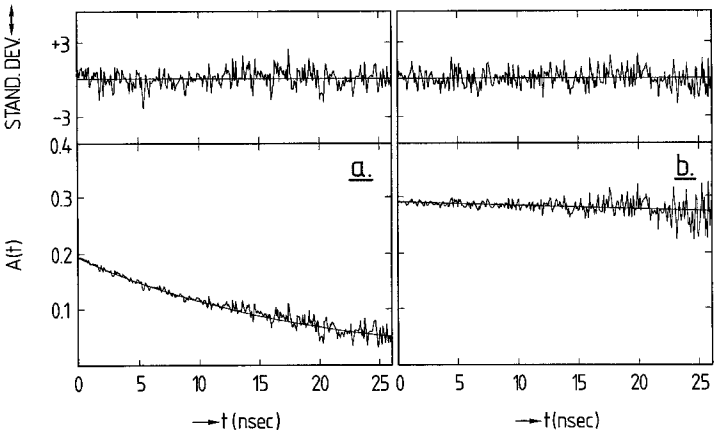

Fig. 3. Decay of fluorescence anisotropy of the Pfl DNA binding protein (**a**) and the native nucleoprotein complex (**b**). The excitation wavelength was 298 nm and the emission wavelength 340 nm. Curve **a** is fitted by a single exponential (A_0 = 0.195 ± 0.01; Φ = 19.2 ± 0.5) with a reduced chi-square of fit, 0.95. Curve **b** is fitted by a single exponential (A_0 = 0.29 ± 0.01; Φ = 493 ± 60) with a reduced chi-square of fit, 0.98. Weighted residuals are shown above the curve

where the ratio of the slopes for protein or complex to that for model compounds is a direct measure of the relative quenching efficiency of the tryptophan group. This ratio we define as k' (Table 1).

For the nucleoprotein complex the quenching efficiency of both CsCl and KI is low ($k' < 0.10$), indicating that in the complex, Trp 14 is protected against collisions with the quenching ions. For the free protein, the quenching efficiency of KI is also low ($k' = 0.07$). However, with CsCl, considerable quenching of the protein fluorescence is observed ($k' = 0.33$). The different quenching efficiencies of Cs^+ and I^- for the free protein indicate that there is a negative charge, probably Glu 12, in close proximity to Trp 14. Although quenching by Cs^+ indicates that Trp 14 is considerably exposed to solvent in the free protein, we have found no effect of D_2O on the fluorescence lifetime. Thus we conclude that Trp 14 is in a partially exposed hydrophobic pocket on the surface of the protein. The inaccessibility of Trp 14 in the native nucleoprotein complex clearly shows that the tryptophan is buried in the complex, in accord with the observed decrease in λ_{max} to 330 nm. The tryptophan site, on formation of the complex, is therefore protected either by DNA or by adjacent protein subunits.

Decay of fluorescence anisotropy

Time resolved fluorescence depolarisation was used to examine the rotational mobility of Trp 14 for the Pfl DNA binding protein (under two solvent conditions) and for the Pfl nucleoprotein complex (Fig. 3). The spectra were analysed by a non-linear least-squares regression method. The spectra could be described by a mono exponential function, characterized by the zero time anisotropy A_0 and the rotational correlation time Φ, as shown in Table 2. For the protein Φ = 19.2 ± 0.5 ns showing that the

Table 2. Fluorescence parameters of the Pfl DNA binding protein and the nucleoprotein complex (excitation wavelength = 298 nm)

	Protein	Complex	Protein in high salt [2 M NaCl]
λ [nm]	340	330	330
I_F	1	0.82	–
τ [ns]	7.75 ± 0.06	6.52 ± 0.05	6.65 ± 0.05
ϕ [ns]	19.2 ± 0.5	493 ± 60	88 ± 3
A_o	0.195 ± 0.01	0.29 ± 0.01	0.27 ± 0.01

Relative integrated intentity (I_F) is obtained by integrating emission spectra between 300 and 450 nm

rotational motion arises from rotational diffusion of the whole macromolecule and is to be compared with the value of Φ_0 = 13 ns calculated for a hydrated sphere of M_r = 30,800 (corresponding to a protein dimer). The ratio of these two numbers (Φ/Φ_0 = 1.46) gives the rotational frictional coefficient and indicates a somewhat ellipsoidal protein dimer. A more precise calculation of the decay anisotropy for non-spherical molecules requires the fit of a sum of at least three exponentials and a knowledge of the angles between absorption dipoles and the orientation with respect to the non-spherical structure. The quality of the data does not allow for such modelling, since a very good fit with a reduced chi-square of fit of the order of unity can already be obtained by fitting just one exponential.

A much larger value of Φ, 493 ± 60 ns, is obtained for the nucleoprotein complex, and is much less accurate as this long rotation time is only observed during the relatively short fluorescence emission window. Nevertheless, it is clear that there is no rotational freedom for the individual protein dimers within the complex (based upon the observed correlation time a minimum value for the size of the rigid unit can be estimated to be around 2–3 helical

turns). For both the protein and the nucleoprotein complex it is clear than on the timescale of these experiments, the tryptophan is rigidly bound to the macromolecule and has no detectable rotational motion of its own.

Also, when the protein is allowed to aggregate in high salt concentration (2 M NaCl) the effect of aggregation shows in the increase of the observed rotational correlation time to 88 ± 3 ns.

In addition to the difference in rotational correlation time between the protein and the protein-DNA complex, a substantial difference is found for the time zero anisotropy, A_0. For the protein, $A_0 = 0.195$ and for the complex, $A_0 = 0.29$. Theoretically, in cases where the absorption and emission dipoles of the fluorophore lie in the same direction, the absolute value of A_0 should be 0.4. However, it has been shown by Lakowicz and Weber (1980) that N-acetyl-tryptophanamide displays rather complex fluorescence anisotropy behaviour, which is critically dependent on the excitation wavelength. We verified, using an excitation wavelength of 298 nm, that the A_0 for N-acetyl-tryptophanamide in propylene glycol was 0.25, in agreement with the observations of Lakowicz and Weber (1980). In order to minimize possible depolarisation effects due to energy transfer between the tryptophans in the dimer, all experiments were performed with excitation at the red edge (298 nm) of the tryptophan excitation spectrum (Munro et al. 1979). If the low A_0 of the protein is due to a rapid relaxation of the excited tryptophan with respect to the local environment this would have to occur faster than 100 ps. Apparently this environment is quite different in the protein-DNA complex, resulting in a much larger A_0.

Discussion

Environment of the tryptophan

Although it has a typical tryptophan fluorescence spectrum with an emission maximum at 340 nm, the Pfl DNA binding protein fluoresces with a lifetime of 7.8 ns. This is one of the longest lifetimes reported for a protein at room temperature in aqueous solution. The fluorescence lifetimes of the vast majority of proteins (Burstein et al. 1973), oligopeptides (Werner and Forster 1979) of tryptophan and its derivatives (Szabo and Rayner 1980b) do not exceed 5 ns. Lifetimes of the order of 8 ns are found for 3-methylindole in water (Szabo and Rayner 1980a) and tryptophan at high pH (Gudgin et al. 1981) where the NH_3^+ is deprotonated. A lifetime of 7.8 ns indicates that the indole ring system is subject to very little intramolecular quenching. In tryptophan and oligopeptides the lifetime is thought to be reduced by intramolecular quenching from the NH_3^+ group of the N-terminus (Szabo and Rayner 1980a).

In proteins, additional intramolecular quenching can occur due to His residues and S-S bridges (Werner and Forster 1979). The Pfl DNA binding protein contains neither histidine nor S-S bridges (Maeda et al. 1982). A low content of His and Cys is also found in the coat protein of Tobacco Mosaic Virus, for which we estimate a lifetime of more than 9 ns from its high quantum yield of 0.44 (Burstein et al. 1973), assuming a lifetime of 3 ns and a quantum yield of 0.14 for tryptophan in water (Szabo and Rayner 1980a). These findings suggest that the absence of the intramolecular quenchers His and Cys is favourable for long lifetimes. The converse need not be true, as quenching is clearly dependent on the proximity of these residues to tryptophan. For instance, human serum albumin contains 8.7% His and Cys but has a long fluorescence lifetime component of 6.5 ns (Munro et al. 1979).

The tryptophan emission maximum can indicate the polarity of the local environment, and the value 340 nm could be the result of the interaction of the transition dipole of Trp 14 with the electrostatic field resulting from the nearby charged residues Glu 12 and Lys 17. This effect might also contribute to the relatively low A_0 of 0.195. The DNA binding process could then neutralise the charges of these residues and reduce the electric field near the Trp residue. This in turn could then explain the decrease in emission maximum to 330 nm and the increase in A_0 to 0.29 for the complex.

The differences in fluorescence properties of the DNA binding protein and the native nucleoprotein complex give some information on the DNA-protein and protein-protein interactions in the complex. Evidence from X-ray fibre diffraction indicates that protein dimers are the basic structural unit in the nucleoprotein helix (Kneale et al. 1982). The subunits must be related by 2-fold symmetry so that the nucleotide binding site on each of the two subunits can bind DNA strands of opposite polarity. Our fluorescence depolarisation measurements on the free protein also indicate that dimers are the predominant form in solution, in agreement with recent hydrodynamic studies (Kneale 1983). As we observe mainly a single lifetime for the free protein and an approximately linear Stern-Volmer plot, both tryptophans of the dimer are probably in identical environments, as one would expect for a symmetrical dimer. The decreased accessibility of Trp 14 when the DNA binding protein is complexed with viral DNA could result from protection by the DNA or by protein-protein interactions induced on DNA binding; these alternatives are discussed below.

Interaction with DNA

From a study of model polypeptides it is known that aromatic residues can bind to DNA through stacking interactions with the DNA bases, and show a strong preference for single-stranded DNA (Toulme and Helene 1975). In the case of tryptophan this would lead to quenching of fluorescence emission (Helene and Maurizot 1981). Many DNA binding proteins have been found to change their tryptophan fluorescence on DNA binding. For instance the fluorescence of the gene 32 protein of T4 bacteriophage is quenched by 35% on binding DNA (Kelly et al. 1976) and for the *E. coli* ssb protein 80% quenching is observed (Krauss et al. 1981). In neither case were decay curves measured and quenching of individual tryptophans could not be determined. For both the above proteins, no appreciable wavelength shift was observed on binding. In these examples it is probable that at least some of the quenching arises from direct interaction with the DNA bases. In contrast, for the Pfl DNA binding protein only a small change of the fluorescence intensity and lifetime occurs in the nucleoprotein complex, there is clearly no base-tryptophan stacking. Furthermore, we suggest, the less polar environment of Trp 14 in the nucleoprotein complex is more likely to arise from protein-protein interactions than from proximity to DNA. In fact a similar shift in the emission spectrum (λ_{max} = 330 nm), change in A_0 and change in fluorescence lifetime is observed when the protein is caused to aggregate in the absence of DNA, for example in high salt (see Table 2). The observed difference of the described fluorescence properties between the protein-dimer and the protein-DNA complex is being utilized to obtain protein-oligonucleotide equilibrium binding constants. Also, the detailed dependence of the hydrodynamic properties of the protein on the salt concentration is being investigated. The results of these studies will be reported elsewhere.

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