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THE REACTION OF AROMATIC PEPTIDES WITH DOUBLE HELICAL DNA. QUANTITATIVE CHARACTERISATION OF A TWO STEP REACTION SCHEME

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The binding of LysTrpLys and LysTyrLys to calf thymus DNA has been investigated by the field jump method using fluorescence detection. Two separate relaxation processes, clearly distinguished on the time scale and by opposite amplitudes, are observed for the binding of LysTrpLys to DNA with ~30000 base pairs. The concentration dependence of the relaxation time constants demonstrates a mechanism with a bimolecular step followed by a slow intramolecular transition with a forward rate of 6.4×10^3 s^{-1} and an equilibrium constant of 11. Measurements at various degrees of peptide binding demonstrate that the binding mechanism associated with low binding rates is restricted to a rather low number of binding sites (roughly one site in 15 base pairs). The binding of LysTyrLys to the same DNA is not associated with relaxation processes of opposite amplitudes; nevertheless two processes could be identified and assigned to a two step mechanism corresponding to that observed in the case of LysTrpLys. In the presence of sonicated DNA both peptides show a single relaxation process with characteristics similar to those observed for the slow process in the binding to high molecular DNA. The data indicate that the intramolecular step is faster for low than for high molecular DNA. These results suggest an assignment of the intramolecular step to an insertion of the aromatic residues into the DNA associated with bending of the helix. The increase in the rate of the intramolecular step with decreasing chain length of the DNA may then be explained by a higher flexibility of the double helix at lower chain lengths.

1. Introduction

Amino acids with aromatic groups are generally expected to make relatively strong contacts with nucleic acids because of their ability to stack upon the aromatic base residues of the nucleic acids. In the case of nucleic acids with a double helical structure, this type of interaction requires some rearrangement of the helix. One of the possible rearrangements is unstacking of two adjacent base pairs and subsequent insertion of the aromatic residue between these base pairs. Such a process is usually called intercalation [1]. In the case of aromatic amino acids, the possibility of an insertion into a nucleic acid double helix has been analysed in various model systems [2–8]. Unfortunately, different authors arrived at different conclusions concerning the mode of interaction or the extent of insertion of aromatic residues. In the present investigation, a kinetic approach is used to obtain information about this reaction. Although this method does not provide direct information about the structure, it allows the characterisation of the number of species involved in the reaction, their distribution at equilibrium and the rates of their reactions.

2. Materials and methods

DNA from calf thymus was obtained from Boehringer GmbH, Mannheim. One sample of this DNA was sheared strongly by sonification for 30 minutes in an ice bath using a Branson sonifier B12. Both the original and the sonified DNA were dialysed extensively against 1 mM NaCl, 1 mM Na-cacodylate pH 7.0, 0.2 mM EDTA (standard buffer). The average length of the DNA samples was determined by analytical zone sedimentation in 1 M NaCl using a Beckman model E centrifuge equipped with a photoelectric scanner. The evaluation was based upon the data given by Studier [9]. According to this procedure,

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the original "long" DNA had 30000 base pairs on the average, whereas the sonified "short" DNA had 500 base pairs on the average. The melting curves of the two samples were quite similar with respect to their shape. However, the T_m-value and the hypochromicity h were slightly lower for the sonicated sample ("long" DNA $T_m = 54.2°$, $h = 27.6\%$; "short" DNA $T_m = 52.0°$, $h = 26.7\%$). These differences may be attributed to end effects. In order to remove any single stranded parts from the DNA a control sample of the long DNA was incubated with S_1-nuclease from Boehringer GmbH (Mannheim) as described by Wiegand et al. [10]. After the incubation the sample was dialysed against the standard buffer. The S_1-nuclease was checked with a sample of the long DNA denatured by heat and was found to be active.

LysTrpLys and LysTyrLys were obtained from Bachem Feinchemikalien AG (Switzerland). The peptides were chromatographed on DEAE cellulose to remove excess salt. The pH was adjusted by addition of HCl and the concentration determined after acidic hydrolysis by ninhydrin according to the method described by Moore and Stein [11]. The concentration of the peptides is given in mol oligomer units/l, whereas the concentration of DNA is given in mol phosphates/l.

The fluorescence titrations and the field jump experiments were performed as described previously [12]. The field jump data were stored in a datalab DL920 transient recorder and transferred to a digital computer via a cassette tape. As usual, relaxation times were obtained from the "off-field" relaxation curve, i.e. at zero field strength. In order to improve the signal to noise ratio, the relaxation curves were measured at response time constants of the detector that were only slightly lower than the relaxation time constants to be characterised. The folding of the relaxation signal with the response curve of the detector was then accounted for by a simple "unfolding" routine according to the following procedure: the response time of the detector was measured independently using a fast light diode Folding of the response exponential with chemical relaxation exponentials was calculated according to the standard equations (cf. e.g. ref. [13]). Theoretical folded relaxation curves obtained in this way were then fitted to the experimental curve using an automatic error minimisation routine.

Table 1
Equilibrium parameters obtained from fluorescence titrations according to two different methods A and B (cf. text). K: stability constant, α: quantum yield of bound peptide relative to free peptide, n: number of nucleotide residues per binding site. Estimated accuracy ±10%

DNA	Peptide	Method	K (M^{-1})	α	n
DNA-500	LysTrpLys	A	6.1×10^4	0.66	–
DNA-500	LysTrpLys	B	8.0×10^4	0.68	2.7
DNA-30000	LysTrpLys	A	8.6×10^4	0.65	–
DNA-30000	LysTrpLys	B	7.6×10^4	0.66	2.8
DNA-500	LysTyrLys	A	5.3×10^4	0.28	–
DNA-30000	LysTyrLys	A	5.9×10^4	0.31	–

3. Results

3.1. Equilibrium parameters

The equilibrium constants were determined by fluorescence titrations using two different procedures. Since the experimental procedures as well as the methods of evaluation were described previously [6,12], it will not be necessary to describe the details again. The main difference between the two titration procedures A and B is as follows: A) The titration is restricted to low degrees of binding Θ and thus does not provide information about the number of nucleotide residues per binding place n. B) Titrations up to high Θ-values allow the determination of n-values, but require more extensive corrections for inner filter effects. The results obtained by these procedures are compiled in table 1.

3.2. Relaxation experiments

3.2.1. The reaction of peptides with short DNA

It is well established that electric fields may produce strong perturbations of chemical equilibria involving charged species [14]. The structure of DNA may also be perturbed by strong electric field pulses [15,16]. For the present investigation, it was essential to avoid any field-induced conformation change of the DNA double helix. This was accomplished by selecting a relatively high ionic strength and avoiding extreme field strengths [17]. In the standard buffer (1 mM NaCl, 1 mM Na-cacodylat, 0.2 mM EDTA pH 7) and at the maximal field strength (23.1 kV/cm) used in the present experiments, there was no indication for any field-induced

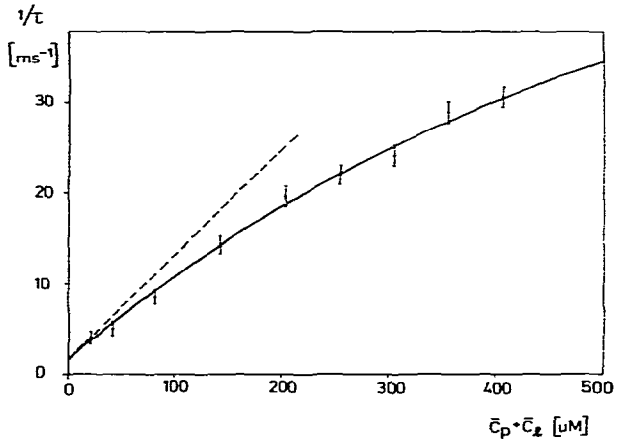

Fig. 1. Reciprocal relaxation time $1/\tau$ as a function of the free reactant concentration $\bar{c}_p + \bar{c}_l$ for short DNA + LysTrpLys (standard buffer, 20°C). The dashed line gives the "initial" slope $k_{12} \cdot k_{01}/k_{10}$ according to eq. (2). Degree of binding $\Theta = 0.08$ = constant (±0.02).

conformation change of the DNA. The peptides LysTrpLys and LysTyrLys also did not show any field-induced reaction under the conditions of the present experiments.

However, when field pulses were subjected to solutions containing both DNA and LysTrpLys or LysTyrLys, clear relaxation effects could be easily detected by following the fluorescence intensity. The electric field pulses induced an increase of the fluorescence intensity corresponding to a decrease in the amount of bound peptide. When the field was turned off, the fluorescence intensity decreased to the level before application of the field pulse. In order to avoid difficulties due to excluded binding effects [18,19], the measurements were restricted to low degrees of binding Θ (number of nucleotide residues covered by ligands divided by total number of nucleotides) <0.1. For this purpose the DNA concentration was usually much higher than the peptide concentration. The relaxation curves obtained in this manner could be represented with high accuracy by single exponentials. Variation of the concentrations revealed a clear concentration dependence. When the reciprocal relaxation time constant $1/\tau$ is plotted against the free reactant concentration $\bar{c}_p + \bar{c}_l$ (calculated with the equilibrium constant obtained from fluorescence titrations), it becomes apparent that $1/\tau$ is not a linear function of $\bar{c}_p + \bar{c}_l$. The decrease in the concentration dependence of $1/\tau$ observed at high $(\bar{c}_p + \bar{c}_l)$-values suggests the validity of the following reaction mechanism:

$$P + L \underset{k_{10}}{\overset{k_{01}}{\rightleftharpoons}} C_I \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} C_{II} \,. \qquad (1)$$

Since the first step is a fast preequilibrium [13], the slow relaxation process may be described by

$$1/\tau = k_{21} + \frac{k_{12}(\bar{c}_l + \bar{c}_p)}{(\bar{c}_l + \bar{c}_p) + k_{10}/k_{01}} \,. \qquad (2)$$

The constants were fitted by a least squares procedure using the stability constant $K = (k_{01}/k_{10})(1 + k_{12}/k_{21})$ obtained from the equilibrium titrations. The experimental data and the fitted curve is shown in fig. 1. Similar data were obtained for the reaction of LysTyrLys with DNA. The resulting parameters are compiled in table 2.

3.2.2. The reaction of peptides with long DNA

A DNA with 500 base pairs is usually considered to be sufficiently long for the reaction with a peptide that covers approximately three nucleotide residues. In such a case it will usually be argued that the contribution of end effects is relatively small and the reaction is equivalent to that of the peptide with a DNA of "infinite" chain length. From this point of view, it would be expected that the relaxation data for peptide binding to a DNA with 500 base pairs are rather similar to those obtained with a much longer DNA. This expectation is verified to a relatively large degree in the case of LysTyrLys binding to a DNA with 30000 base pairs. The field jump relaxation is similar to that observed with the 500 base pair DNA. Electric fields induce an increase in the fluorescence intensity, which is reverted upon turning off the field in a monotonous function. Differences are not detected, unless the relaxation curves are analysed more closely (see below).

A very different type of relaxation curve is found, however, for the binding of LysTrpLys to the DNA with 30000 base pairs. The reaction is characterised by two separate phases, shown in fig. 2 for the relaxation induced by turning off the electric field. In the first reaction phase the fluorescence intensity increases with a time constant of about 10 μs; in the second phase the fluorescence decreases with a time constant in the range around 400 μs. The time constants of both relaxation processes are dependent upon the reactant concentrations (cf. fig. 3). The time constant τ_2 associated with the slow relaxation process approaches a limit value at high concentrations and thus exhibits a concentration dependence simi-

Table 2
Thermodynamic and kinetic parameters of peptide binding to DNA. K: equilibrium constant (from fluorescence titrations) k_{01}, k_{12} and k_{21}: rate constants according to eq. (1). k_{01} is given in terms of molar concentrations of nucleotide residues. $K_2 = k_{12}/k_{21}$. Estimated accuracy ±20% for LysTrpLys and ±30% for LysTyrLys; the numbers in brackets are preliminary

DNA length in base pairs	Peptide	K (M^{-1})	k_{01} ($M^{-1}\,s^{-1}$)	k_{12} (s^{-1})	k_{21} (s^{-1})	K_2
500	LysTrpLys	7.0×10^{4}	–	8.3×10^{4}	1.7×10^{3}	49
30000	LysTrpLys	8.1×10^{4}	3.2×10^{5}	6.4×10^{3}	4.4×10^{2}	15
500	LysTyrLys	5.3×10^{4}	–	(2.8×10^{5})	1.9×10^{3}	(140)
30000	LysTyrLys	5.9×10^{4}	–	(2.5×10^{4})	7.6×10^{2}	(33)

lar to that observed for the single relaxation process in the case of LysTrpLys binding to the short DNA. Apparently the slow process again reflects the second step of the reaction scheme (1) and the fast process is mainly associated with the first, bimolecular step. Using this model, the experimental data can be represented with good accuracy (cf. fig. 3). The parameters resulting from a least squares fit are compiled in table 2.

When the relaxation curves obtained for the long DNA + LysTyrLys are analysed more closely, it becomes apparent that they cannot be described by a single exponential. Analysis by least squares minimisation routines demonstrates the existence of two relaxation processes. The slower process represents the main part of the relaxation curves – usually more than 80% of the total amplitude. Under these conditions it was not possible to evaluate the time constant associated with the fast process with sufficient accuracy. According to the approximate figures available, these time constants are in the range around 10 μs, similar to the corresponding ones observed for LysTrpLys. Least squares fitting of the relaxation time constants associated with the slow process to the equation (2) yielded the parameters included in table 2.

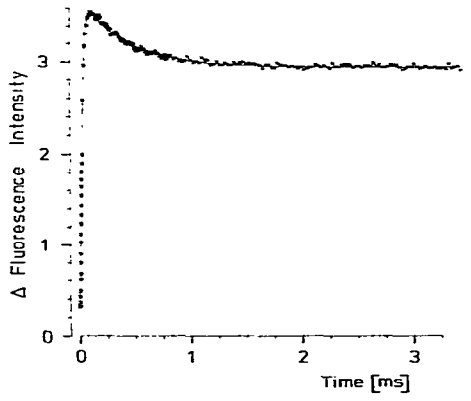

Fig. 2. Relaxation curve for long DNA + LysTrpLys upon turning off the electric field (standard buffer; $c_P^0 = 86.7\ \mu M$; $c_I^0 = 3.0\ \mu M$).

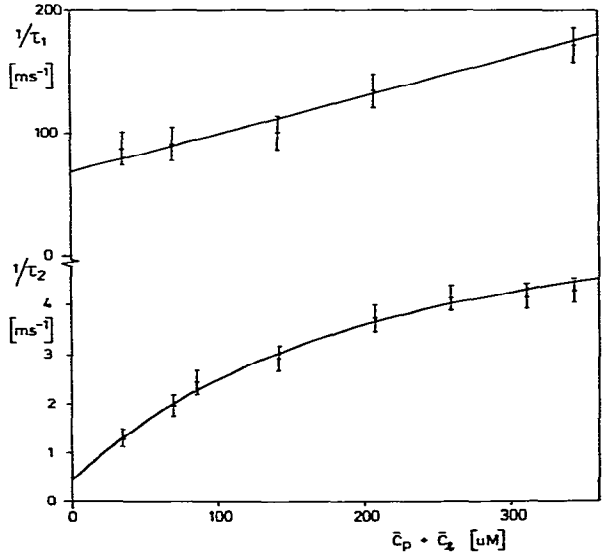

Fig. 3. Reciprocal relaxation times $1/\tau_1$ and $1/\tau_2$ as a function of the free reactant concentration $\bar{c}_P + \bar{c}_I$ for long DNA + LysTrpLys (standard buffer, 20°C). Degree of binding Θ = 0.08 = constant (±0.02).

3.2.3. Amplitudes

The amplitudes observed in the relaxation experiments could be very useful for the characterisation of the individual steps of the present system. Unfortunately, however, in the present case it is not possible to analyse the amplitudes in a quantitative manner, since the field pulses could not be maintained sufficiently long to attain true stationary equilibria. Thus the amplitudes can only be analysed qualitatively.

The most interesting amplitude effects are observed in the system LysTrpLys + long DNA. The relatively strong increase of the fluorescence intensity in the first relaxation process indicates that the fluorescence intensity of complex C_1 is higher than that of the free peptide. It remains to be explained, however, why this separate process is observed in the long DNA but not in the short DNA. This difference may be correlated to a different fraction of bound peptides existing in the state C_1: according to the K_2-values obtained by fitting of the relaxation time constants this fraction appears to be higher for the long than for the short DNA. It seems to be more likely, however, that the amplitude dependence is due to varying influences of the electric field on the DNA peptide complexes depending upon whether short or long DNA is used. It is known that equilibrium shifts by electric fields are usually more pronounced in long than in short polymers ([17]; Diekmann and Pörschke, in preparation).

In this context it was important to test for any field induced conformation change in the long DNA. Under the conditions of the field jump experiments with the peptides there was no field induced absorbance change of the long DNA (measured at the magic angle). The field strength was below the threshold (cf. refs. [15–17]) observed for the field induced helix coil transition of the long DNA. Furthermore the relaxation curve observed for LysTrpLys + long DNA remained essentially unchanged on the relative scale, when the electric field strength was reduced to much lower values. For these reasons it is unlikely that the fast amplitude is associated with a field induced helix-coil transition in the long DNA.

Another explanation for the unusual relaxation effects observed for the long DNA might be an exchange reaction of peptides between double and single stranded regions of the DNA molecule. In order to exclude this possibility a sample of DNA was incubated with a nuclease, which digests only single stranded regions (cf. section 2). Field jump experiments using this sample with LysTrpLys showed the same relaxation effects as described above for samples, that had not been treated with the single strand nuclease. Thus it is not likely that any single stranded part of the DNA produces any major contribution to the observed effects.

3.2.4. Test for orientation effects in fluorescence detection

It is generally accepted that orientation effects in the absorbance detection of chemical reactions can be avoided by using polarised light with its polarisation plane oriented at an angle of 54.7° (magic angle) with respect to the axis of orientation [20]. Polarised light oriented at the magic angle was also used in the present investigation for excitation, in order to prevent any influence of orientation processes on the excitation. However, this alone does not prevent perturbations of fluorescence signals by orientation of the fluorescing molecules, since the emission may also be influenced by orientation [21,22].

To the knowledge of the authors the problem of fluorescence detected chemical relaxation in the presence of orientation effects has not yet been treated explicitly in the literature. It is not intended to give a detailed theoretical derivation here. Instead an attempt will be made to solve the problem by simple analogy arguments: absorbance and emission can be considered as closely corresponding events. Thus the general laws governing the orientation dependence of absorbance should be identical to those of emission. By this argument the independence of absorbance upon orientation at the magic angle should be valid for emission too.

This expectation may be tested by experiments. A suitable system for this purpose is that of DNA + acridine orange (AO) at low degrees of binding. For this system it is known that the fluorescence intensity of AO is increased by a factor of roughly 3, when AO intercalates between the base pairs of DNA [23]. The binding of AO is not mainly driven by electrostatic contacts, such that electric field pulses of low amplitudes will not perturb the extent of binding very much. Application of electric field pulses to this system led to a strong decrease of the fluorescence intensity, when the emission polariser was oriented parallel to the electric field vector. Conversely an increase

of the fluorescence was observed when the emission polariser was oriented perpendicular to the field vector. When the emission polariser was adjusted at the magic angle with respect to the field vector, no change of emission intensity was observed upon application of field pulses.

Another procedure to avoid orientation effects in the fluorescence signal is the measurement of $2 \times I_{\perp} + I_{\parallel}$ where $I_{\perp}$ and $I_{\parallel}$ are the fluorescence intensities at perpendicular and parallel orientation of the polariser with respect to the field vector. The signal $2 \times I_{\perp} + I_{\parallel}$ is proportional to the total fluorescence emission independent of orientation [24]. This was also verified by experiments with the system DNA + acridine orange.

Corresponding experiments were performed with DNA in the presence of peptides. In this case changes in the orientation of the emission polariser only led to minor changes in the relaxation curves. As described above, the electric field causes strong perturbations in the degree of peptide binding leading to large changes of the fluorescence intensity. In the case of the peptide DNA complexes these changes due to binding reactions are much larger than any changes due to orientation. Thus it was possible to measure the fluorescence relaxation of peptide binding without emission polarisers. In order to keep the signal to noise ratio as high as possible, most of the measurements reported in the previous section were done without emission polarisers.

3.2.5. Relaxation measurements at high degrees of binding

All the relaxation measurements reported above were performed at low degrees of binding, in order to avoid artifacts due to excluded binding phenomena [18,19]. In the case of LysTrpLys + long DNA the measurements were extended to high degrees of binding. The dependence of the reciprocal relaxation time constant observed for the slow process (cf. previous section) upon the LysTrpLys concentration at a fixed polymer concentration is shown in fig. 4. For comparison fig. 4 also contains the concentration dependence expected on the basis of the kinetic parameters obtained at low degrees of binding. The free reactant concentrations required for this theoretical curve were calculated from the equilibrium parameters obtained from fluorescence titrations using the excluded binding model. It is obvious that the experimental data

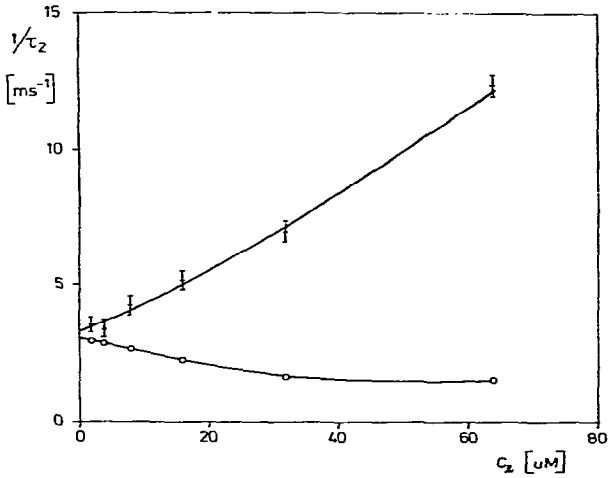

Fig. 4. Reciprocal relaxation time $1/\tau_2$ [+] as a function of the total peptide concentration c_l^0 for long DNA + LysTrpLys at fixed DNA concentration $c_p^0 = 141\ \mu M$ (standard buffer, 20°C). The circles represent calculated $1/\tau_2$-values using the equilibrium parameters from fluorescence titrations and rate constants from the measurements at low degree of binding (cf. text).

and the calculated curve are not at all consistent: the calculated curve has a minimum around $c_l = 50\,\mu M$, which is not observed in the experimental data. Furthermore, the experimental $1/\tau$ values observed at high peptide concentrations are much higher than the limit $1/\tau$ value observed at low Θ.

It may be suspected that the deviations are due to excluded binding phenomena. This possibility was tested by Monte Carlo calculations using a procedure developed by Epstein [19]. Simulations of the binding kinetics demonstrated that the excluded binding mechanism produces some deviations from the usual kinetics. The simulations also demonstrated, however, that the deviations expected for the present case are not nearly as extensive as observed (Jung and Pörschke, in preparation). Thus, the main part of the deviations cannot be explained on basis of excluded binding phenomena.

The experimental data shown in fig. 4 can be fitted to a simple one step mechanism or to a two step mechanism if it is assumed that the number of nucleotide residues per peptide binding site is around 30. An analysis of the amplitude associated with the fast relaxation process suggests a similar number of nucleotide

residues per peptide binding site. Unfortunately, it is not possible to analyse both relaxation amplitudes quantitatively, since the true stationary relaxation amplitudes cannot be measured with sufficient accuracy due to the limited length of the field pulses (cf. previous section).

Although it is not possible to give a complete quantitative description of the relaxation data observed at low and high Θ, it is obvious that the parameters of peptide binding strongly depend upon Θ. The present data indicate that the two step mechanism observed at low Θ is restricted to a relatively small number of binding places along the DNA chain.

4. Discussion

Different binding states are often converted into each other with high rates. Under these conditions it is not possible to distinguish the binding states by conventional methods, which usually provide an average image over the various discrete states. This is also a problem for the binding of aromatic peptides to nucleic acids. The existence of two different peptide complexes has been postulated, one of them with the aromatic residue being inserted into the DNA and the other one with an almost freely mobile aromatic residue [6]. Unfortunately these complexes could not be distinguished by methods like NMR, since the exchange is too fast [8]. In such cases fast relaxation techniques can be very useful, in order to assign the different states quantitatively. In a favourable case like that of LysTrpLys + long DNA, where two relaxation processes can be readily separated and quantitatively analysed, the binding mechanism and the corresponding parameters can be evaluated with relatively high reliability. The separation of different relaxation processes is not nearly as simple and reliable in the case of LysTyrLys + long DNA; thus the parameters for this system can be given only with a relatively large error margin.

A major problem in the quantitative analysis of the present relaxation data is the assignment of the rate constant k_{12}. Unfortunately the present data do not allow the determination of k_{12} to the desired accuracy. This is essentially due to the uncertainty in the extrapolation of the $1/\tau$ values to high concentrations of free reactants. For the same reason the equilibrium constant K_2 also cannot be determined with high accuracy. The problem does not exist in the case of k_{21}, since this parameter is obtained by extrapolation to zero concentration of the reactants.

In spite of the relative uncertainty of the equilibrium constants K_2 the present data demonstrate that the K_2-values are much higher than the corresponding parameters derived previously by a simple fluorescence analysis [6,7]. This analysis was based upon the assumption, that the unstacked complex has the same quantum yield as the free peptide and the stacked complex a quantum yield zero. The amplitude associated with the fast process in the system LysTrpLys + long DNA directly indicates that one of the complex states (C_1) has a quantum yield higher than that of the free peptide. The observation of a fast decrease in the fluorescence intensity upon application of a field pulse in the system LysTrpLys + long DNA and the absence of a corresponding effect for LysTyrLys + long DNA can be correlated to the average quenching coefficients α. This coefficient is much higher for LysTrpLys than for LysTyrLys (cf. table 1). However, the molecular basis of these effects is not yet known.

The number of binding sites associated with low reaction rates (cf. section 3.2.5) is much lower than the number of sites obtained by equilibrium titrations. This demonstrates a heterogeneity of binding sites, which may be related to a heterogeneity in the sequence of base pairs. It is possible that tryptophane residues preferentially stack with a specific sequence of base pairs. However, previous experiments also demonstrated a heterogeneity of sites for the binding of LysTrpLys to a single stranded homopolymer (poly(A); cf. ref. [12]). The heterogeneity of binding sites may be a reason for the apparent discrepancy of results obtained from the present experiments and from NMR measurements. The NMR data published by Mayer et al. [8], for example, were obtained at a degree of binding $\Theta \geqslant 0.27$, whereas the present data were measured at much lower Θ values around 0.08.

The data obtained in the present investigation demonstrate a dependence of the reaction parameters upon the DNA chain length. The chain length dependence of the kinetic parameters may be explained on the basis of the DNA flexibility. If the second, intramolecular step is assigned to an insertion of aromatic residues into the DNA, the rate of this reaction will

depend upon the dynamics of the DNA base pairs, for example the unstacking rate of a pile of base pairs. The rate of this reaction may depend upon the length of the helix. It is possible that the insertion of an aromatic amino acid is associated with bending of the helix [4], since the aromatic residue is not large enough to fill up the space of a base pair in the same way as conventional intercalating dyes like ethidium [25]. The bending rate will depend upon the mobility of the residues attached to the bending site. This mobility will decrease with increasing length of the helices attached to the bending site. A bending mechanism may also explain the dependence of the equilibrium constant K_2 upon the chain length by electrostatic repulsion between the helix strands and by excluded volume phenomena. Both of these effects will act against bending and are expected to increase with increasing chain length. Thus the bending mechanism provides a reasonable explanation of the present results. Further measurements using various lengths of DNA may provide more information about this reaction, which is not only important in the context of protein nucleic acid interactions but also for an understanding of the dynamics of double helical nucleic acids.

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