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The Binding of Protamines to DNA; Role of Protamine Phosphorylation

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Abstract. The thermodynamics of protamine-DNA interaction was investigated with clupeine Z from herring labeled at its amino terminus with fluorescein. The ionic strength dependence, the influence of protamine phosphorylation, of the native DNA conformation, using native and heat-denatured DNA, and of the protamine primary structure, using two oligoarginine peptides of similar length as the clupeine, was thoroughly studied. The unusually high cooperativity of interaction found is strictly correlated to the native DNA conformation and the protamine primary structure. Cooperativity is explained by cross-linking of DNA segments resulting in an increase of the negative charge density. The importance of protamine phosphorylation lies in the fact that thermodynamically governed interaction with DNA and favorable cross-linking of DNA are shifted to physiologically reasonable ionic strengths.

Key words: Peptide-DNA interaction – Protamine-DNA condensation – Phosphorylation of chromosomal proteins.

Introduction

The nucleosome, the basic structural element of the somatic chromosomes of all eucaryotes, is a general device which allows both activation of chromosomal domains for gene expression and the formation of higherorder structures to condense great amounts of DNA (for review cf. Felsenfeld, 1978). In the strongly condensed nonsomatic chromatin of the male gametes packing of DNA is, however, basically different. As the physiological role of these specialized nuclei do not imply transition to active chromatin, nucleosomal organization is not required, and as DNA compaction is emphasized obviously continuous structures are favored (Johnson et al., 1970; Duckett and Racey, 1975). Spermatogenesis replaces somatic histones for a variety of basic sperm proteins; the arginine-rich protamines common with several fishes represent a well-studied subclass (Ando et al., 1973). The question of how protamines dis-

Abbreviations: FITC – fluorescein isothiocyanate; FTC-clupeine – clupeine labeled at its amino terminus with fluorescein via a thiocarbamate bond

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place the somatic histones and build up a different and rather compact DNA-protein structure should be seen in connection with the fact that protamines after synthesis are extensively phosphorylated (Louie et al., 1973; Marushige and Marushige, 1978). Obviously phosphorylated protamines displace the histones and the ultimate compaction of the nucleoprotamine appears to be correlated with the removal of the protamine phosphoryl groups. Phosphorylation-dephosphorylation is obviously in general related to processes which change DNA compaction. This holds for H5 in erythropoesis (Sung, 1977) and also for H1 and H3 in mitosis (Bradbury et al., 1974; Gurley et al., 1978).

The present work was aimed to elaborate the thermodynamics of protamine-DNA interaction in vitro. With the use of phosphorylated protamines bearing one, two or three serine phosphoryl groups per molecule the consequences of this modification upon the binding parameters could be evaluated. Moreover preparation of $(Arg)_n$ with n values of 16 and 24, respectively, allowed a comparison with arginine oligopeptides of similar size as a protamine molecule. By this means the role of protamine primary structure and by using native and denatured DNA the role of DNA secondary structure could be elucidated.

Materials and Methods

Clupeine Z was isolated from herring sperm according to Ando et al. (1973) using 0.2 mM PMSF throughout. Phosphorylation of the clupeine Z was performed as described previously (Willmitzer and Wagner, 1975). Calf thymus DNA, purchased from Boehringer, was sonicated and fractionated on Sepharose 4B (Wehling et al., 1975). The sedimentation coefficient was determined to be 6.8 s (in 50 mM Tricine, 1 mM EDTA, 10 mM NaCl, pH 7.3). Denaturation of DNA was performed by incubation in boiling water for 10 min and rapidly cooling down to 4° C; the residual hyperchromicity was 2%. Comparative series of experiments were performed with the same preparation of DNA.

Preparation of the arginine-peptides: Poly-L-arginine sulfate (MW 20,000), purchased from Miles Yeda, was submitted to partial hydrolysis (6 N HCl, 80 min, 60° C) and subsequently to gel filtration on Bio-gel P2 and P6 (0.5 M acetic acid), to remove low molecular and high molecular weight peptides. Further fractionation was performed on Bio-gel P4 and after lyophilization on Sephadex CM 25 using exponential NaCl gradients ranging from 1.0–2.0 M NaCl for the $(Arg)_{16}$ peptide and 2.0–2.8 M NaCl for the $(Arg)_{24}$ peptide (20 mM sodium acetate buffer of pH 5.8). From the series of peaks obtained, one eluted at 1.7 M NaCl and one at 2.3 M NaCl, respectively, were chosen, desalted on Bio-gel P2 (0.5 M acetic acid) and lyophilized. The length of the peptides was determined from the amount of N-terminal NH_2 groups (by quantitative FITC-labeling) and quantitative amino acid analysis thus obtaining the amount of arginines. No other amino acid than arginine could be detected.

Preparation of FITC-cellulose: 500 mg cellulose (avicel from Merck, Darmstadt) was activated for 20 min at 150° C. After cooling the cellulose was added to a solution of 100 mg FITC (Sigma Chemical Co.) in 40 ml dry acetone. After evaporation of the acetone at 30° C and subsequent lyophilization for 2–3 h, the FITC-cellulose was ready for use. The preparation proved to be stable for several months at 4° C.

Reaction of the peptides with FITC-cellulose: The reaction can be performed in Eppendorf vials. 5 mg of clupeine acetate (1 μmol) is dissolved in 0.5 ml of a 0.05 M $NaHCO_3$-Na_2CO_3 buffer of pH 8.6 and added to 20 mg FITC-cellulose (10 μmol FITC) which is suspended in 0.5 ml of the same buffer. If necessary the pH of the mixture is adjusted to pH 8.6. After 15 min shaking at room temperature, 1 ml of 5 M guanidinium hydrochloride is added and after mixing for 2–3 min the vials are centrifuged and the supernatant is given onto a Sephadex G 25 column. Upon elution with distilled water, FTC-labeled clupeine Z is well separated from unreacted FITC and salt. Quantitative labeling was proved by gel electrophoresis on 20% polyacrylamide gels containing 0.6% bisacrylamide, using 50 mM Tris-acetate of pH 7.0 as buffer (Wehling et al., 1976).

Performance of the binding measurements: All measurements were performed in 50 mM Tricine, 1 mM EDTA, pH 7.4 and at the NaCl or urea concentrations indicated. Binding of the fluorescent clupeine by DNA was measured by adding increasing and equal amounts of fluorescein-labeled clupeine to a constant amount of DNA (sample cuvet) and to the reference cuvet containing only the buffer. Fluorescence intensity was measured 4 min after the additions. Fluorescence measurements were performed on a Schoeffel RRS 1000 fluorometer by excitation at 490 nm and recording at 513 nm. Intensity changes of the 1000 W Xenon lamp are corrected for by a Rhodamin B reference. A small fraction of the FTC-clupeine is adsorbed to the cuvet walls; however, with reproducible handling of the cuvets the relative amount of this fraction is constant in the concentration range applied, thus giving rise to a linear concentration dependency of the fluorescence intensity in the reference cuvet. It was further shown using radioactive labelled protamine that the relative amount of the adsorbed fraction is the same in the presence and absence of DNA. Fluorescence intensity was correlated with FTC-clupeine concentration by a simultaneous determination of the absorption at 500 nm (pH 7.3) using a molar extinction coefficient of $6.08 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$. This correlation was performed at a 20–50 fold higher concentration of FTC-clupeine than used in the binding experiments; at this higher range the adsorbed fraction could be neglected, however absorption and fluorescence intensity were still linearly dependent upon the concentration (Willmitzer, 1977; Wehling et al., 1976).

Results

a) Evaluation of the Method Applied

Preparation of the Fluorescein-Labeled Clupeine: As fluorescein exhibits a high quantum yield in aqueous environment as well as a dramatic change of its fluorescent properties upon contact with DNA, fluorescein isothiocyanate was used for labeling the N-terminus of clupeine. Wehling et al. (1976) described the labeling at pH 9. As the labeling efficiency was about 50%, unlabeled clupeine had to be separated from labeled clupeine by a combination of ion exchange and gel filtration chromatography. This procedure was not amenable to us because of the long reaction time and the rather high pH which might result in a loss of phosphates from the phosphorylated protamine species because of β-elimination (Willmitzer and Wagner, 1975).

As the main difficulty of the above procedure is due to the different solubilities of the polycationic protamine and the hydrophobic fluorescein derivative, in the present procedure a heterogenous reaction was applied. FITC was adsorbed onto activated cellulose as described in Materials and Methods and added to an aqueous solution of the clupeine. Due to the high concentration of both the clupeine and the FITC, labeling was complete within 15 min at pH 8.6, as proved by gel electrophoreses (Wehling et al., 1976). Addition of guanidinium hydrochloride was found to be necessary for the desorption of the clupeine from the cellulose as well as desorption of unspecifically adsorbed FITC from the clupeine; FITC-labeled clupeine was separated from unreacted FITC by gel filtration on Sephadex G25. This labeling procedure proved to be very effective as it allowed the quantitative labeling of small amounts of protein within a very short time and at only slightly basic pH.

Characterization of the Labeled Clupeine Z: Extinction coefficient of the fluorescein labeled clupeine as well as the fluorescence spectra and their pH-dependence did not differ from the data described by Wehling et al. (1976) and thus will not be shown in this paper. In order to prove whether FITC had only reacted with the N-terminal amino acid alanine, FTC-clupeine Z was treated analogous to an Edman degradation (Guidotti et al., 1962) and the amino acid composition of residual protein determined. Whereas the arginine-alanine ratio of untreated clupeine Z was found to be 6.5, that of the degraded FTC-clupeine was 9.8. As clupeine Z contains 21 arginines and 3 alanines (Ando et al., 1973), these data reveal that the Edman degradation of FTC-clupeine Z resulted in the loss of one, obviously the N-terminal, alanine. Furthermore it could be shown that more than 98% of the total fluorescence was split off by this degradation thus again proving exclusive labeling at the N-terminal amino acid.

Change of the Fluorescence Properties upon Binding to DNA: Upon the addition of FTC-clupeine to a large excess of DNA (to ensure quantitative binding) the fluorescence is nearly totally quenched (Fig. 1). The almost quantitative disappearence of the fluorescence of the FTC-clupeine upon binding towards DNA was found under all conditions applied independent of ionic strength, concentration of urea and the conformation of DNA. The quenching process may be a radiationless loss of energy caused by the mere contact of the fluorophor with the DNA surface or by a change in the pK value of the fluorescein molecule upon binding (Garel, 1976). UV-spectra recorded from the DNA-bound FTC-clupeine show a decrease in the absorption at 500 nm by a factor of about 2, thus resembling the decrease found for the transition from the twofold ionized to the onefold ionized state. This transition is known to be correlated with a decrease in the fluorescence yield by a factor of 10 (Wingender and Arellano, unpublished results).

Analysis of Data: Data obtained were analysed according to the concept of the overlapping binding sites developed by McGhee and von Hippel (1974) describing the binding of a multisite ligand to a large lattice. Cooperative binding is described by three independent parameters: the affinity constants K_c for contiguous binding and K_i for binding to an isolated site. The quotient K_c/K_i is termed the cooperativity parameter ω and m is the stoichiometry parameter, which corresponds to the number of sites (nucleotides) occupied by one ligand (protamine molecule). A computer program was

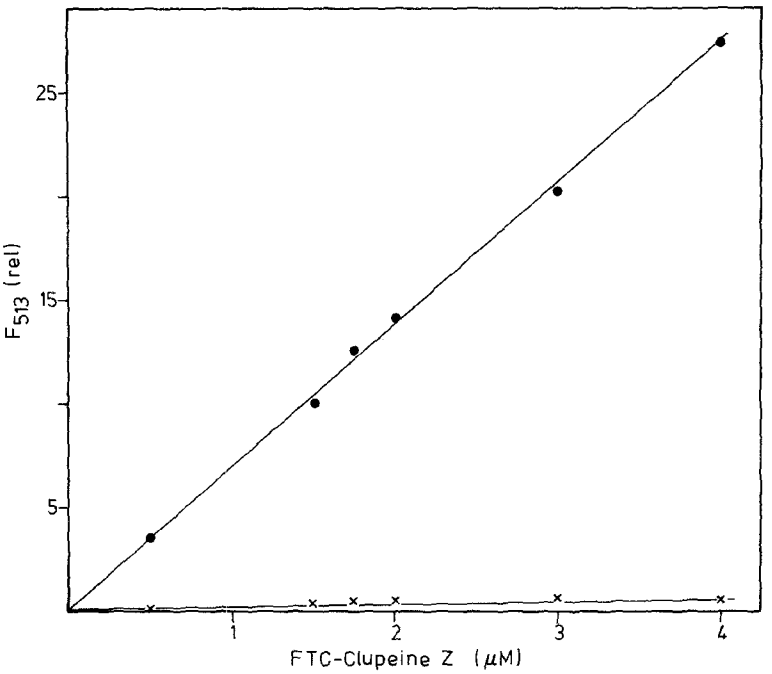


Fig. 1. Quenching of the fluorescence of FTC-clupeine Z by DNA. FTC-clupeine Z was added in equal amounts to samples devoid of (●) and containing DNA (92 μM) (×) in 2.5 ml of 50 mM Tricine, 1 mM EDTA of pH 7.3. Fluorescence was recorded at an excitation wavelength of 490 nm and emission recorded at 513 nm

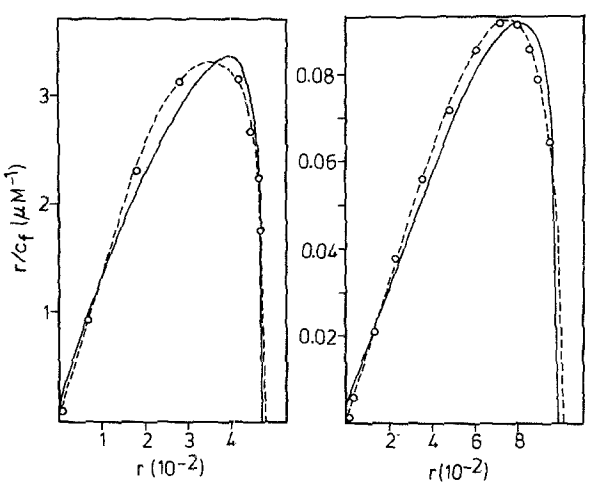


Fig. 2. Binding of nonphosphorylated clupeine Z (left part) and threefold phosphorylated clupeine Z to DNA at an ionic strength of 0.4 M NaCl, 50 mM Tricine, 1 mM EDTA pH 7.3. Binding data are represented according to the Scatchard formalism. Experimental data (–O–O–); computed data (——)

developed which included independent variations of the parameters to be derived using the least square's approach to select the optimal values. Figure 2 shows typical experimental and calculated binding curves in the Scatchard presentation, the latter is obtained from the McGhee and von Hippel equation using the computed parameters. There is obvioulsy a very good coincidence of the experimental and calculated data.

Control Experiments: An estimation whether the binding of the protamines to DNA is influenced by the fluorescein label was made using a 1 : 1 mixture of labeled and unlabeled clupeine. Titration of DNA was performed as with fully labeled clupeine taking into account the unlabeled fraction. The parameters extracted were identical with those of a normal titration within the error of estimation. Furthermore, the binding data obtained from phosphorylated clupeine which had been dephosphorylated by alkaline phosphatase (Willmitzer, 1977) revealed that the effect of protamine phosphorylation upon the interaction with DNA (to be presented below) is reversible.

Comparison with Data Presented Earlier: Comparison of the binding data presented in this contribution with binding data published earlier (Wehling et al., 1976) shows a very good coincidence concerning the qualitative binding behaviour such as the high cooperativity of the binding process. They differ however in the absolute values. This was shown to be due to the different labeling procedure used. Thus the degree of fluorescence quenching upon binding to DNA was found to depend upon the age of the preparation indicating a cleavage of the FTC-molecule upon storing at 4° C (Willmitzer, 1977). Using the new labeling procedure described in this contribution and storing the FTC-clupeine at −20° C, we did not find any change over 2 years. Thus although the earlier paper (Wehling et al., 1976) described the qualitative binding behaviour in a very good manner, the quantitative data cannot be relied on.

b) Interaction of Nonphosphorylated Clupeine Z with DNA

The McGhee and von Hippel (1974) concept describes a vagrant ligand interaction, i.e., a ligand covering more than one lattice residue has the choice to select any segment of lattice fitting to its size. The interaction of a protamine molecule with its 20 positively charged arginine residues with DNA molecules of about 500 nucleotides may be adequately described by this concept. Table 1 indicates the equilibrium parameters for the binding of nonphosphorylated clupeine Z with DNA at different ionic strengths. The affinity constants are strongly dependent upon the NaCl concentration, revealing a strong electrostatic contribution as one would have expected from a 20 arginine containing peptide and DNA. There is a large binding cooperativity which significantly increases with ionic strength. The binding site covered by a protamine molecule is 22 nucleotides at 0.3 M NaCl roughly corresponding to the 21 arginine in clupeine Z, with increasing ionic strength this stoichiometry decreases.

The ionic strength dependence of affinity constants can be exploited to estimate the number of ionic bonds participating in the respective interaction. According to a proposal of Record et al. (1976) a plot of the logarithm of the affinity constants versus the negative logarithm of the NaCl concentration, shown in Fig. 3, should be linear and

Table 1. Binding of non-phosphorylated clupeine Z to DNA at different ionic strength. The experimental conditions are described in the legend of Fig. 1. The binding data, exemplarily presented in Fig. 2, were analysed according to the vagrant multi-site model of McGhee and von Hippel (1974). K_i and K_c are the affinity constant for isolated and contigous binding (chain propagation), respectively. ω is the cooperativity factor equal to K_c/K_i, m the binding stoichiometry corresponding to the number of DNA phosphates occupied by a clupeine molecule

NaCl concentration	K_c (μM^{-1})	K_i (μM^{-1})	ω	m
0.3	990	5.50	180	21.9
0.4	102	0.071	1430	20.3
0.5	11.5	0.0061	1900	15.0

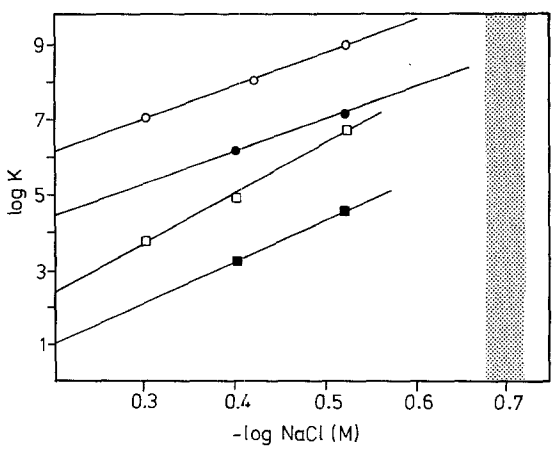


Fig. 3. Plot of the binding affinity constants K_c (○, ●) and K_i (□, ■) according to Record et al. (1976). Data are shown for the nonphosphorylated (○, □) and threefold phosphorylated (●, ■) clupeine Z. The shaded area represents an estimate of the physiological ionic strength within a nuclei

the slope should be equal to $m'\phi$, where m' is the number of the ionic bonds per ligand and ϕ the fraction of counterion bound per phosphate; for native DNA ϕ is 0.88. From the slopes of Fig. 3 the number of the ionic bonds were calculated to be 10 for the K_c and 15 for the K_i values. The number of 15 ionic bonds extracted from the ionic strength dependence of the K_i values correlates with the stoichiometrie value of 15 DNA-nucleotides or phosphates occupied by one protamine molecule determined at 0.5 M NaCl (Table 1).

c) The Influence of Protamine Phosphorylation

Table 2 shows the influence of phosphorylation upon the thermodynamics of protamine-DNA interaction. Binding affinity drops dramatically for the phosphorylated

Table 2. Influence of phosphorylation upon the binding parameters of clupeine Z. The experimental conditions and the definition of the binding parameters are indicated in the legends of Figs. 1 and 2 and Table 1

Number of phosphoryl groups in clupeine Z	NaCl concentration	K_c (μM^{-1})	ω	m
0	0.4 M	102	1430	20.3
1	0.4 M	19	1270	16.0
2	0.4 M	5.2	820	15.6
3	0.4 M	1.45	920	10.4
3	0.3 M	15.6	500	15.7

protamines, obviously mainly due to the introduction of two negative charges per phosphoryl group. This results in a decrease in the binding affinity of 4.2 kJ/mol for the introduction of the first phosphate group and each 3.2 kJ/mol for the second and third phosphate group. Assuming the interaction to be completely electrostatic, the data of nonphosphorylated clupeine Z would lead to an interaction increment of 2.2 kJ/mol for each arginine residue or positive charge. Assuming that the two negative charges of a phosphoryl group compensates two arginine positive charges, a decrease in the binding energie of 4.4 kJ/mol should be expected for each phosphoryl group incorporated. The lower decrease for the two- und threefold phosphorylated clupeine Z may be explained as an apparent increase in the hydrophobic contribution.

The stoichoimetry, i.e., the DNA nucleotides covered by a protamine molecule, is also reduced by phosphorylation, further indicating that protamine-DNA interaction is strongly governed by electrostatic contributions. This result has already been found by independent methods (Bode and Willmitzer, 1975; Wagner et al., 1976; Willmitzer et al., 1977a). On the other hand binding cooperativity is only slightly affected by phosphorylation. With the threefold phosphorylated clupeine Z, binding was measured at two different ionic strengths (Table 2). The data reveal the same behaviour as already described for the nonphosphorylated species (Table 1), i.e., reduction in binding affinity with increasing ionic strength, an increase in binding cooperativity and a decrease in stoichiometry. Analysis according to Record et al. (1976) (cf. Fig. 3), reveals 10 ionic bonds when the K_c values are considered and 12 for the K_i values.

d) Role of Native DNA Structure

From the conformations of the biopolymers involved it is certainly the DNA double helix which significantly contributes to the features of the interaction. To elaborate its influence a series of binding experiments with heat denatured DNA were performed (Table 3). Denatured DNA binds protamine with higher affinity than native DNA. This is unexpected for a helix-stabilizing protein (von Hippel and McGhee, 1972). However, classification of proteins into this category is usually based on thermal melting which is

Table 3. A comparison of binding towards native and heat denatured DNA. The experimental conditions for the determination of the binding data and the definition of the binding parameters are indicated in the legends of Figs. 1 and 2 and of Table 1

DNA	K_c (μM^{-1})		ω		m	
	Native	Denatured	Native	Denatured	Native	Denatures
Nonphosphorylated clupeine Z						
0.4 M NaCl	102	283	1430	120	20.3	20.1
0.5 M NaCl	11.5	61	1900	105	15.0	18.5
Threefold phosphorylated clupeine Z						
0.4 M NaCl	1.45	7.14	920	140	10.4	12.4

Table 4. The binding parameters of $(Arg)_{16}$ and $(Arg)_{24}$ in 0.4 M NaCl. The experimental conditions and the definition of the binding parameters are indicated in the legends of Figs. 1 and 2 and of Table 1

	K_c (μM^{-1})	ω	m
Native DNA			
$(Arg)_{16}$	68	390	19.1
$(Arg)_{24}$	900	135	27.0
Clupeine Z	102	1430	20.3
Denatured DNA			
$(Arg)_{16}$	298	420	26.4
Clupeine Z	283	120	20.1

performed at rather low ionic strength in cotrast to the present binding studies. The T_m (and the enthalpy of DNA helix-coil transition) of uncomplexed DNA increases with the ionic strength (Schildkraut and Lifson, 1965; Gruenwedel, 1974), while for DNA-protein complexes these parameters decrease with increasing ionic strength (Li et al., 1974; Matsuo and Tsuboi, 1969; Klump, 1976). These opposing dependencies may at high NaCl concentration turn a helix-stabilizing protein into a destabilizing one and this should be reflected in a higher affinity of the protein towards denatured DNA (cf. also Umanskič et al., 1974; Spelsberg et al., 1970; Leng and Felsenfeld, 1966), as it is found in the present studies.

If these arguments are correct, the difference in the binding affinity towards native and denatured DNA should increase with increasing ionic strength, which is in accord with the results obtained for nonphosphorylated clupeine (Table 3). Furthermore, as the complex of DNA with phosphorylated protamine has a lower T_m than that with unphosphorylated protamine (Willmitzer et al., 1977a), at the same ionic strength the difference in the affinity towards denatured and native DNA should be larger with the

phosphorylated protamines species, which is documented with the data of Table 3. The same holds for the $(Arg)_{16}$ peptide which has a lower T_m than the clupeine species (cf. Table 4). This concept also explains why Mg^{2+} ions prefer denatured DNA at 0.2 M NaCl, but native DNA at 0.001 M NaCl (Zubay and Doty, 1958; Shack and Bynum, 1959).

It is known that denatured DNA assumes a highly compact structure at high salt concentration (Studier, 1965; Rosenberg and Studier, 1969). The length of denatured DNA decreases more than twofold by increasing the ionic strength from almost zero to 0.3 M, whereas the length of native DNA is more or less unaffected (Bujard, 1970). The higher charge density of denatured DNA relative to native DNA at the higher ionic strength thus should result in a higher affinity for the positively charged protamine.

The second unexpected result is the significant decrease in cooperativity upon binding to denatured DNA. This may be explained assuming that the cooperative binding behaviour originates in the cross-linking ability of the protamines. Cross-linking of DNA molecules would lead to an increase in charge density and this again to an increase in binding affinity (cf. Discussion). As outlined above denatured DNA has a higher charge density at higher ionic strength than native DNA. Therefore the relative increase in charge density by building up cross-links between DNA molecules should be less with denatured DNA than with native DNA. Hence, cross-linking of denatured DNA should result in a smaller binding cooperativity than in the case of native DNA. On the other hand, the smaller ω values found with denatured DNA (Table 3) support the concept that cooperativity results from the cross-linking ability of protamines.

e) Comparison with Oligoarginine Peptides

A comparison of the primary structure of protamines from several fishes reveals some conservative features concerning the position of the phosphorylation sites, the position of residues like proline, which impose some restraints on the conformation, and on the occurence of arginine cluster. Investigations with suitable oligoarginine peptides may elucidate the importance of these features. The data indicated in Table 4 show the expected dependency of the interaction affinity upon the chain length of the arginine oligopeptide, as was found for a series of oligolysine peptides (Latt and Sober, 1967). Linear interpolation leads to a K_c value of 340 μM^{-1} for an $(Arg)_{21}$ which would be equivalent to clupeine Z concerning the number of arginines residues. This value is about threefold higher than the value actually found for the nonphosphorylated clupeine species. The insertion of 10 neutral residues into a polyarginine chain (cf. Fig. 4) to form clupeine Z reduces the binding affinity towards DNA obviously due to dilution of the positive charges. The same effect has been shown with an independent method for lysine containing copolypeptides (Wehling et al., 1975). A comparison with Table 2 shows that the incorporation of the 10 neutral residues exhibits a less pronounced decrease of the binding affinity than the incorporation of only one phosphate into clupeine Z. Table 4 further shows that also $(Arg)_{16}$ interacts more strongly with denatured than with native DNA. The strongly reduced binding cooperativity relative to clupeine Z is

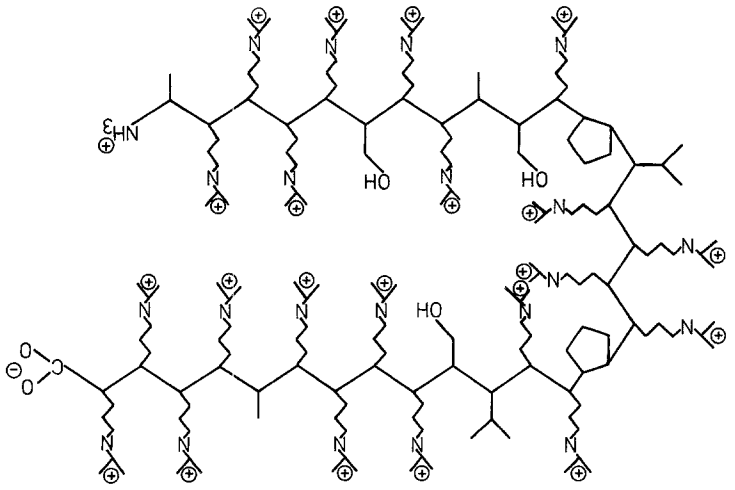


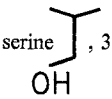
Fig. 4. Schematic drawing of the clupeine Z molecule showing the presence of kinks at the proline residues. Clupeine Z contains 21 arginine  , 3 alanine  , 3 serine  , 3 valine  , and 2 proline  residues

Table 5. The influence of 2 M urea upon the binding parameters. The experimental conditions are described in the legends of Figs. 1 and 2. The titration experiments were performed in identical solution which differed only in that one series contained in addition 2 M urea. For the definition of the binding parameters see the legend of Table 1

	K_c (μM^{-1})		ω		m	
	Without urea	With urea	Without urea	With urea	Without urea	With urea
Clupeine Z						
0.3 M NaCl	990	153	180	790	21.9	21.1
0.4 M NaCl	102	8.1	1430	3300	20.3	17.7
Threefold phosphorylated clupeine Z, 0.3 M NaCl	15.6	1.3	500	730	15.7	17.7
$(Arg)_{16}$, 0.4 M NaCl	68	20	390	240	19.1	18.7

very interesting, obviously in terms of ω only clupeine Z is able to discriminate between native and denatured DNA, with $(Arg)_{16}$ there is practically no change.

f) Probing of Nonelectrostatic Contributions to Protamine-DNA Interaction

To get an idea of the importance of other than electrostatic contributions to the present interaction, a series of experiments were performed in the presence of 2 M urea. As Ta-

ble 5 shows the affinity constant K_c is significantly reduced in the presence of urea. The amount of the reduction, however depends upon the species investigated as well as the ionic strength applied. The hydrophobic contribution to binding affinity obviously increases with increasing ionic strength. This is paralleled by a decrease in the number of phosphates covered by one protamine molecule. The presence of urea reduces binding affinity of clupeine Z by about 6.3 kJ/mol whereas reduction is only 3.0 kJ/mol for $(Arg)_{16}$, clearly demonstrating the hydrophobic contribution of the neutral amino acid residues in clupeine. The influence of urea upon the binding affinity is even larger for the threefold phosphorylated clupeine Z at the same ionic strength; apparently phosphorylation converts clupeine to a more hydrophobic protein. The cooperativity parameter ω increases upon the addition of urea, thus eliminating the possibility that cooperativity my be produced by hydrophobic protein-protein interactions.

Discussion

The results of the present work demonstrate the usefulness of fluorescein as fluorescent label in peptide-nucleic acid interaction. The unique primary structure of protamines (cf. Fig. 4) allows easily to label their amino terminus; however, proteins with more regular amino acid composition would also be susceptible to this method with certainly greater effort for specific labelling. The affinity of a protamine, possessing 21 arginine residues, towards DNA is rather high. Due to the high quantum yield of the fluorescein label in water solution, affinity constants up to 10^9 M^{-1} could be determined. At the lowest ionic strength applied (0.3 M NaCl) the K_c value obtained (cf. Table 1) corresponds to a free enthalpy of 51 kJ/mol.

The magnitude of the interaction energy of protamine molecules and DNA is of interest for the physiological role of these basic peptides. In spermiogenesis DNA compaction significantly increases, accompanied by a transition of the structural order of the DNA thread from the nucleosomal pattern to a pattern unique for nucleoprotamine whose features still have to be elaborated. After fertilization this transition has to be reversed. In view of the complexity of processes concerned with folding and condensing of the tremendously long DNA thread one should assume that the interaction of a protamine molecule with DNA in vivo occurs in a thermodynamically reversible manner. To discuss this point one has to know the ionic strength in the nucleus. Figure 3 includes an estimate corresponding to 0.2 M NaCl.

Considering half-saturation of available sites at the DNA thread, reversible binding would occure at a protamine concentration roughly equal to the reciprocal K_c value. With nonphosphorylated protamine and DNA, a protamine concentration of less than 0.1 nM can be extracted from Fig. 3 (NaCl 0.2 M). The physiological concentration of protamine in the cell or cell nucleus is not known, but it should be higher than this value. Figure 3 further indicates that phosphorylation reduces the affinity by two orders of magnitude leading to a protamine concentration above the nM range for reversible binding to DNA. Thus, reduction in binding affinity is obviously one important reason for protamine phosphorylation. The same may hold for the phosphorylation of H5 in erythropoesis. Within the nucleosomal structure of somatic chromatin a considerable fraction of DNA phosphates are shielded by histone positive charges. Thus, as protamine binds to nucleosomal DNA before histone displacement, binding affinity

may be further reduced. This may shift the protamine-DNA dissociation constant into the μM range.

One of the most challenging results of this investigation is the very high cooperativity of the protamine-DNA interaction. In general cooperativity may be explained by two mechanisms: 1) protein-protein interaction; 2) DNA-mediated cooperativity. As cooperativity increases in the presence of urea, hydrophobic protein-protein interaction should be excluded. Cooperativity therefore should be due to a DNA-mediated process. We would favour cooperativity to be due to the cross-linking ability of the protamines. Using space filling models, Olins et al. (1968) showed that the proline residues in the protamine molecule are capable of kinking the extended β-sheat by almost 90°. Thus cross-linking of DNA strands would be highly favoured by the formation of kinks in the neighbourhood of the proline residues. Cooperative binding of the protamines should therefore be based on 1) an increase in negative charge density due to a general cross-linking of DNA strands or segments, 2) the primary structure of protamines apt at forming two kinks per polypeptide chain which obviously strongly favor the cross-linking of native DNA (cf. Fig. 5). Electron microscopic studies (Bode and Lesemann, 1977) and investigations on the light scattering and sedimentation of DNA-protamin complexes (Bode et al., 1979) are consistent with the supposition that larger aggregates are formed in the course of the binding process.

How does this model fit with our experimental results? Significant changes of the cooperativity parameter were observed by changing the ionic strength, the conformation of DNA and the primary structure of the polypeptide. The arginine oligopeptides also bind in a cooperative manner to DNA, cooperativity, however, is much lower than for clupeine. This should be mainly due to the reduced flexibility because there are no prolines (and other neutral amino acids) resulting in a reduced ability to build up cross-links.

The lower cooperativity observed with binding to denatured DNA relative to native DNA is explained by the higher charge density of denatured DNA (cf. Results section). The relative increase in charge density upon cross-linking DNA segments is therefore less with denatured DNA than with native DNA. Furthermore it is also feasable that the strong ability of the protamines to cross-link is highly adapted to the native DNA

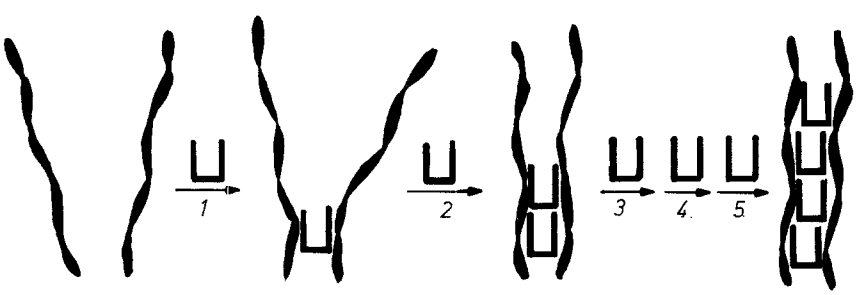


Fig. 5. Model showing the cross-linking of the two DNA strands by protamine molecules (⌊⎽⎽⌋). Binding via cross-linking creates a zone of higher charge density facilitating the binding of further protamine molecules. Increase in charge density is correlated with an increase in binding affinity. Cross-linking and hence cooperativity may be favored through the good fitting of kinked protamine molecules to the surface of adjacent parallel DNA strands

conformation. This is indicated by the fact that only clupeine and not $(Arg)_{16}$ can discriminate between native and denatured DNA with respect of binding cooperativity (cf. Table 4).

Let us consider two basically different modes of binding: 1) colinear alignment of a protamine on the DNA surface and 2) a cross-linked protamine accomodated to two (or more) adjacent DNA segments. It is conceivable that the former case is mainly governed by favorable electrostatic interactions but with some constraints in the polypeptide conformation (mostly because of the prolines). The latter case may involve conformations of less constraints; however, because of cross-linking and adaption to adjacent DNA surfaces, electrostatic neutralization may not be optimal for all arginine side chains. Hence, the colinear binding mode should be more favored at low ionic strength resulting in low cooperativity, whereas cross-linking should be favored at higher ionic strength giving rise to large cooperativity. This view is consistent with the findings (Table 1) of an increase in cooperativity with increasing ionic strength correlated with a decrease in the stoichiometry, indicating an excess of (non-neutralized) arginine residues at the higher ionic strength.

The influence of phosphorylation is also explained in this context. Phosphorylation reduces binding affinity and reduces the length of DNA covered by a protamine molecule (Table 2). These two consequences of phosphorylation should, at a given ionic strength, be correlated with an enhancement of DNA cross-linking relative to nonphosphorylated protamine. Thus, whereas both nonphosphorylated and phosphorylated protamines show an increase in cross-linking, manifested by the cooperativity and straylight measurements (Bode et al., 1979), with increasing ionic strength, it is the phosphorylation which allows efficient cross-linking at lower, physiological ionic strength.

There are consequences of phosphorylation which cannot be correlated directly to the reduction in net electric charge. In previous studies we found that complexes of phosphorylated protamine and DNA exhibit CD-spectra which are similar to those of ψ-type DNA (Willmitzer et al., 1977b). These CD-spectra could not be obtained from nonphosphorylated protamine and DNA. Thus phosphorylation provides the protamine with a new quality, i.e., the ability to build up highly ordered nucleoprotein structures. One could speculate that this property is caused by a direct influence of the phosphorylation upon the cross-linking via protamine-protamine interactions through ionic bonds between serine phosphates and arginine groups. However, it is also conceivable that the ψ-type structure is obtained by indirect effects of phosphorylation, i.e., more favorable adaption of protamines to the grooves of cross-linked DNA or higher probability of protamine kinking, as phosphorylation occurs near to the kinking points (proline residues).

The evaluation of the experimental binding data was done in the present work on the basis of the McGhee and von Hippel (1974) concept, which implies the vagrant and cooperative interaction of nearest neighbors of bound ligands on a linear lattice. The presented model of binding-induced aggregation (Fig. 5) of DNA molecules may be reconciled with nearest neighbor ligand interaction, assuming the immediate vicinity of a DNA-ligand connection provides the greatest increase in charge density. However, aggregation of lattices during the binding process is not included in the McGhee and von Hippel approach. This would further complicate the analytical display, however until now appropriate concepts for application are not available.

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