Phosphorylated protamines. II. Circular dichroism of complexes with DNA, dependency on ionic strength

Lothar Willmitzer, Jürgen Bode and Karl G. Wagner

Gesellschaft für Biotechnologische Forschung, Abteilung Molekularbiologie,
Braunschweig-Stöckheim, GFR

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ABSTRACT

The influence of protamine phosphorylation upon the conformation of nucleoprotamine complexes was studied at different ionic strengths using circular dichroism. The sharp onset of CD spectral changes upon decreasing the NaCl concentration was correlated with the beginning of complex formation and can be used to determine apparent binding affinities in terms of a critical ionic strength. It is shown that phosphorylation strongly reduces the binding strength of protamines towards DNA. Directly mixed and reconstituted complexes reveal differences in their CD spectra, which decrease with increasing ionic strength. Spectra of complexes between threefold phosphorylated clupeine Z and DNA obtained by reconstitution or direct mixing at higher ionic strength resemble the ψ -type spectra of DNA and are unique for the phosphorylated species. The implications of protamine phosphorylation for chromatin or DNA condensation have been discussed.

INTRODUCTION

Phosphorylation represents one of the most important post-translational modification of chromosomal proteins during mitotic and meiotic cell proliferation: phosphorylation of histone H1, as a possible trigger for mitosis¹, and in vivo phosphorylation-dephosphorylation of protamines² connected with sperm chromatin condensation, has been thoroughly investigated.

In the preceding paper³ in vitro studies on DNA complexes with protamines of different degree of phosphorylation have been described. It was shown that phosphorylation reduces thermal stability of these complexes and that binding stoichiometry (expressed as DNA nucleotides occupied by a protamine molecule) is significantly diminished. In the present work circular

dichroism studies have been performed, in order to investigate the conformational implications of protamine phosphorylation for the formation of nucleoprotamine complexes. Introduction of three phosphates into clupeine Z from herring, possessing three serine and no threonine residues, yields a homogeneous phosphoprotein which obviously plays an important role in spermiogenesis². Complexes with this compound and DNA reveal significantly altered CD spectra relative to those with nonphosphorylated clupeine; these changes are especially obvious when the ionic strength dependence is investigated. Complete phosphorylation of this highly basic protamine reduces binding affinity as expected, it appears however to significantly enhance the DNA-condensing properties of this sperm protein.

MATERIALS AND METHODS

Calf thymus DNA and the phosphorylated clupeine species were prepared as described in the preceding paper³. Labeling of the clupeine with fluorescein isothiocyanate was performed by a modification of the procedure described previously⁴. The modification, application of cellulose adsorbed fluorescein isothiocyanate, will be described in detail elsewhere.

DNA-protamine complexes were usually formed in NaCl solutions containing 20 mM Tricine (pH 6.8) and 1 mM EDTA by either of the following methods:

Direct mixing of protamine solution (20 μ M) and the DNA solution (100 μ M) under constant stirring after both solutions had been adjusted to the desired ionic strength. The final DNA concentration was in the range of 50 - 80 μ M and the measurements were performed one hour after mixing.

Formation of reconstituted complexes by common stepwise dialysis strongly suffers from high loss of protamine not bound to DNA at high ionic strength. Therefore, before dialysis, the components were mixed at an ionic strength just high enough not to allow complex formation, i. e. 0.7 M NaCl for nonphosphorylated and 0.5 M for threefold phosphorylated clupeine (cf. Results

and Discussion) ; the resulting DNA concentration was 60 μ M. The ionic strength was then lowered by very slow continuous addition (peristaltic pump, 2 - 3 hours) of bidistilled water under constant stirring, until the ionic strength had decreased to 0.5 and 0.3 M NaCl, respectively, where binding is nearly complete. The solutions were then dialysed against the final NaCl concentration for about 8 hours. With this procedure no appreciable loss of clupeine (and DNA) was noticed; but the recovery of protamine and DNA decreased drastically when the protamine-Arg to DNA-nucleotide ratio (Arg/Nt) exceeded 0.5 and 0.75, respectively. This may be caused by precipitation and/or sticking of nucleoprotamine to the glass vessel and probably to the dialysis tubes. Circular dichroitic spectra were taken on Roussel-Jouan 185 Mod II dichrograph at room temperature using a 1 cm cell. Fluorescence measurements were performed on a Schoeffel RRS 1 000 fluorometer.

RESULTS AND DISCUSSION

Influence of the degree of protamine phosphorylation

Fig. 1 demonstrates the influence of phosphorylation of clupeine Z indicating that the differences in the CD spectra are highest with the completely phosphorylated species. The spectra are blue-shifted, which is opposite to the small change occurring with nonphosphorylated protamine⁵. Ellipticity at 275 nm increases and that at 245 decreases; a new band arises at about 305 nm. The complexes were formed by direct mixing at low ionic strength, in order to allow a direct comparison with the thermal denaturation studies of the preceding paper.

Ionic strength dependence of complexes obtained by direct mixing.

As DNA and protamines are highly ionized polyelectrolytes, their mutual interaction is governed by strong electrostatic

Figure 1: Dependence of CD spectra of DNA-clupeine Z complexes upon the degree of protamine phosphorylation: The spectrum of DNA alone (---) is shown throughout; the labels 0P to 3P indicate the number of the serine phosphates present in clupeine Z. The spectra were recorded in 1.6 mM NaCl and 0.3 mM EDTA of pH 6.8; the average DNA concentration was 50 μ M. The input ratios (Arg/Nt) applied, 0.9 for 0P and 1P and 1.2 for 2P and 3P, were chosen as to obtain about the same fraction of the DNA phosphates saturated with protamines (90 %).

affinities the magnitude of which depends upon ionic strength. Complexes of DNA with synthetic basic polypeptides^{6,7,8} and

also with histone H1⁹ have been investigated hitherto for the ionic strength dependence of the circular dichroism and significant changes have been detected. To elaborate the effect of the serine phosphates upon complex formation with DNA at different ionic strengths, only nonphosphorylated and the fully phosphorylated (threefold) clupeine Z was used. The CD spectra of these complexes are shown in Fig. 2; Fig. 3 indicates the molar ellipticity at 275 nm plotted versus the NaCl concentration.

The complexes formed with nonphosphorylated protamine reveal a continuous reduction of the band about 275 nm up to an ionic strength of 0.5 M; above 0.6 M NaCl the CD spectra appear rather similar to those of free DNA (cf. Fig. 3). The behavior of the complexes with threefold phosphorylated clupeine Z is obviously rather complicated. With increasing ionic strength ellipticity increases tremendously (note the expanded scale relative to that of nonphosphorylated protamine); from 0 to 0.2 M NaCl the positive long-wave band is blue-shifted, at 0.3 M/0.4 M NaCl a very strong negative band appears. At ionic strength higher than 0.4 M NaCl the CD spectra again become like those of pure DNA (cf. also Fig. 3), which apparently indicates the dissociation of the complexes (see further below).

The spectra displayed by DNA complexes with threefold phosphorylated clupeine at ionic strengths up to 0.2 M are similar to those reported for pure DNA in 70 % methanol solutions^{10,23}. Those spectra have formerly been correlated with transitions of the DNA secondary structure i. e. a B→A transition³⁰. However, recent findings, especially from X-ray experiments, rather suggest that the B conformation of DNA is maintained¹¹; furthermore hydrodynamic investigations indicate that A-type spectra result from a condensation of DNA¹⁰. This supposition is strengthened by the curious inversion of CD spectra of threefold phosphorylated protamine-DNA complexes displayed at 0.3 and 0.4 M NaCl (Fig. 2), where large negative ellipticities are obtained. These so-called ψ -type spectra have been shown not to result from changes in the DNA secondary structure^{12,13}, but to indicate highly condensed DNA¹⁴.

Figure 2: Comparison of CD spectra of DNA complexed with nonphosphorylated (top) and threefold phosphorylated (bottom) clupeine Z at different ionic strength: The complexes were formed by direct mixing of the components in 20 mM Tricine and 1 mM EDTA of pH 6.8; the molar NaCl concentration is indicated at the respective spectra. The Arg/Nt input ratio was 0.5 for nonphosphorylated and 0.7 for threefold phosphorylated clupeine Z. These different ratios were chosen as to compensate the differences in the binding stoichiometries of these clupeine species.

Inversion of CD spectra from positive to large negative bands in the 260 - 280 nm region has also been reported to occur with

Figure 3: Molar ellipticity of directly mixed complexes as a function of ionic strength. The upper part contains the data for DNA ($\square$) and nonphosphorylated clupeine Z (Δ) the lower part those for the threefold phosphorylated species ($\circ$).

histone H1-DNA complexes by increasing the protein-DNA ratio¹⁵. Studies on threefold and nonphosphorylated protamine were performed at constant ionic strengths changing the Arg/Nt ratio. The relatively small spectral changes with nonphosphorylated clupeine Z were similar to those already described by Carroll⁶. Threefold phosphorylated clupeine Z-DNA complexes revealed a continuous increase in the molar ellipticity at 275 nm with increasing Arg/Nt ratio, which was positive at 0.2 M and negative at 0.4 M NaCl and reached values at an Arg/Nt ratio of 1.5 of +260 000 and -283 000, respectively.

Ionic strength dependence of the interaction affinity

The ionic strength dependence of the CD spectra, illustrated in Fig. 2 and especially in Fig. 3, makes obvious that differences observed between the complexes and pure DNA are registered only below a critical ionic strength. This sharp ionic strength dependence has also been observed with complexes of DNA and other basic polypeptides^{6,7,8}. Apparently this behavior reflects the dissociation of the DNA-protein complexes at higher NaCl concentrations,

which in the present case occurs at about 0.4 M and 0.6 M for the threefold and nonphosphorylated clupeine Z, respectively. This supposition was directly proven using clupeine which had been labeled with fluorescein isothiocyanate at the α -amino group (Fig. 4).

Figure 4: Influence of the NaCl concentration on the binding of fluorescein-labeled clupeine Z to DNA: The fraction of clupeine Z bound to calf thymus DNA, determined according to Ref. 4 is plotted versus the NaCl concentration. DNA concentration was 60 μ M (the same concentration was used in the CD experiments) the fluorescein-labeled clupeines were added to cover 75 % of the DNA phosphates, starting at 0.2 M and 0.4 M NaCl with threefold ($\circ$) and nonphosphorylated (Δ) protamine, respectively. The ionic strength was stepwise enhanced by addition of small volumes of 5 M NaCl solution. All solutions were buffered to pH 6.8 with 20 mM Tricine and 1.0 mM EDTA.

The fluorescence of the fluorescein-labeled protamines is quenched to a large extent upon binding to DNA⁴. Using this fact the amount of bound protamine was determined. The point, where 50 % of the protamine is bound, corresponds to 0.65 M NaCl (non-phosphorylated) and 0.42 M NaCl (threefold phosphorylated protamine). Comparing Fig. 4 with Fig. 3 clearly demonstrated, that with decreasing ionic strength the onset of changes in the CD spectra coincides with the beginning of binding. This correlation opens a simple way for determination of relative binding affinities

by recording CD spectral changes as a function of the ionic strength.

Reconstituted complexes; ionic strength dependence of the CD spectra

From several investigations on the interaction of different basic polypeptides with DNA there is evidence that complexes formed by direct mixing adopt conformations which may deviate from those of lowest free energy^{6,21,35,36}. In order to approach equilibrium conditions complexes are usually formed at high salt and dialysed slowly to low salt concentration (reconstitution). Protamines, having molecular weights of about 5 000 are hardly suitable to dialysis at high salt; thus a modified reconstitution method, described in Materials and Methods, was adapted to this problem. The CD spectra of reconstituted complexes, obtained at different ionic strengths, are shown in Fig. 5.

Spectra of reconstituted complexes formed with nonphosphorylated clupeine Z exhibit nearly no ionic strength dependence between 0.1 and 0.5 M NaCl, which is different from the behavior of the directly mixed complexes (cf. Fig. 3 and the inset of Fig. 5). As already obvious from the complexes formed by direct mixing (Fig. 2) threefold phosphorylated protamine induces spectral changes which are of significantly higher magnitude than those induced by nonphosphorylated protamine. Interestingly reconstitution always leads to the strong negative band at about 275 nm with molar ellipticities up to $150 \cdot 10^3$. These ψ -type spectra are obtained by direct mixing only at rather high ionic strength (Fig. 2), indicating that the molecular complex generating these spectra has a stable equilibrium conformation.

The differences in the CD spectra for the complexes formed either by direct mixing or by reconstitution are very large at low ionic strength (Figs. 2 and 5); at 0.1 and 0.2 M NaCl direct mixing leads to positive bands (Fig. 2, bottom) whereas reconstitution leads to the very strong negative ellipticities mentioned. Reconstitution has often been applied to DNA-basic polypeptide interactions and from DNA-polylysine complexes

Figure 5: CD spectra of complexes with threefold phosphorylated clupeine Z formed by reconstitution to different ionic strengths: The spectra were recorded in 20 mM Tricine and 1 mM EDTA of pH 6.8; the molar NaCl concentration is indicated at the respective spectra. The DNA concentration was 60 μ M and the Arg/Nt ratio was 0.7 (0.5, nonphosphorylated clupeine Z). In the lower part the molar ellipticity at 275 nm is plotted versus the NaCl concentration, for the threefold phosphorylated species ($\circ$, right hand scale) as well as the unphosphorylated species (Δ , left hand scale).

similar differences in the CD spectra have been reported^{6,21}. An increase in the cooperativity of protamine binding to DNA, assayed by sedimentation³⁵, and in the melting cooperativity of different DNA-basic polyamino acid complexes due to reconstitution have been described³⁶.

The spectral differences (Figs. 2 and 5) decrease with increasing ionic strength and vanish at about 0.3 M NaCl (phosphorylated protamine), which is below dissociation (0.5 M) (cf. Fig. 4). The formation of an ordered, thermodynamically stable complex apparently depends upon the magnitude of the

rate constants involved. While the rate of association may not dramatically change with ionic strength it is the rate of dissociation, which significantly increases with increasing NaCl concentration. This rate becomes large enough already below thermodynamic dissociation, as to allow ordered complex formation. This justifies the method applied in the present work for reconstitution, when peptides not suitable for lengthy dialysis are involved.

CONCLUSIONS

The ψ -type spectra displayed by complexes of DNA and phosphorylated protamines strongly resemble those found for pure DNA after addition of neutral polymers¹⁴. Electron microscopy studies revealed the appearance of doughnut-like structures concomitant with ψ -type spectra^{16, 17, 18}; further evidence from hydrodynamic studies led to the suggestion that ψ -type spectra are displayed by a condensed and highly ordered DNA structure^{19, 20}.

ψ -type spectra have been further reported for DNA complexes with polylysine^{6, 8, 21} several lysine-containing copolypeptides^{7, 22}, and the lysine-rich histone H1^{9, 18, 24}. Polyarginine-DNA complexes do not exhibit this phenomenon⁸ and apparently the arginine copolypeptide clupeine reveals this effect only after phosphorylation of its serine residues. It is known that the interaction affinity of arginine residues towards DNA is stronger than that of lysine residues^{25, 29}, this is obviously a consequence of differences in the ionic bond formation between DNA phosphates and arginine or lysine side chains. For histone H1-DNA complexes it has been reported, that phosphorylation reduces the ψ -type spectral intensities²⁶. Although these studies were not extended to different ionic strengths, they may indicate different influences of phosphorylation for the different chromosomal proteins.

The physiological role of protamine phosphorylation during spermatogenesis should be considered with respect to the electro-

static conditions existing in a cell nucleus. The concentration of free ions has been estimated to correspond to about 0.2 M NaCl²⁷ and the local DNA concentration to 5 mM²⁸. Thus, it is hard to offer a way for a correct binding (binding at equilibrium conditions) of nonphosphorylated protamine to DNA. Hence, reduction of binding affinity by phosphorylation is an important step to approach equilibrium conditions, which allow an ordered condensation of the chromatin. This ordered condensation process is likely to be reflected in the in vitro formation of the ψ -type condensed DNA structure, which are only obtained with phosphorylated protamine.

However, the unique property of phosphorylated protamine may only partially be explained by the overall reduction of the electrostatic affinity. Phosphorylation may enhance the hydrophobic contribution involved in the interaction of protamines with DNA³¹. On the other hand evidence from the preceding paper³ shows that phosphorylation significantly decreases the binding area on DNA covered by a protamine molecule, suggesting that phosphorylated serine residues may give rise to loops of the protamine chain protruding beyond the DNA surface. These may facilitate crosslinking of adjacent protamine-DNA complexes through the formation of phosphate-arginine salt bridges. As threefold phosphorylated clupeine Z has its phosphoryl groups at well spaced positions along the polypeptide chain, it is tempting to speculate that these multiple salt bridges are the basis to generate ordered packing of nucleoprotamine by forming a regular matrix of phosphate-arginine ionic bonds.

On the other hand, direct binding measurements^{31,32}, light scattering, electron microscopy investigations^{33, 34} and the protamine primary structure have led to the assumption that a protamine molecule very strongly tends to crosslink adjacent DNA molecules by binding to one double-strand with about half of its chain and then jumping to the other double-strand with its second half portion. This inherent property seems to depend upon the ionic strength, obviously increasing NaCl concentrations favor the simultaneous interaction with two DNA regions. Phosphorylation has certainly also an effect, most likely it may enhance the tendency to form such crosslinks.

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