Chromatin compaction under mixed salt conditions: Opposite effects of sodium and potassium ions on nucleosome array folding

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It is well known that chromatin structure is highly sensitive to the ionic environment. However, the combined effects of a physiologically relevant mixed ionic environment of K\(^+\), Mg\(^{2+}\) and Na\(^+\), which are the main cations of the cell cytoplasm, has not been systematically investigated. We studied folding and self-association (aggregation) of recombinant 12-mer nucleosome arrays with 177 bp DNA repeat length in solutions of mixtures of K\(^+\) and Mg\(^{2+}\) or Na\(^+\) and Mg\(^{2+}\). In the presence of Mg\(^{2+}\), the addition of sodium ions promotes folding of array into 30-nm fibres, whereas in mixtures of K\(^+\) and Mg\(^{2+}\), potassium ions abrogate folding. We found that self-association of nucleosome arrays in mixed salt solutions is synergistically promoted by Mg\(^{2+}\) and monovalent ions, with sodium being slightly more efficient than potassium in amplifying the self-association. The results highlight the importance of a mixed ionic environment for the compaction of chromatin under physiological conditions and demonstrate the complicated nature of the various factors that determine and regulate chromatin compaction in vivo.
The vast majority of studies investigating the ionic dependencies of folding and self-association of nucleosome arrays were performed by varying the concentration of a single ionic species, either Na⁺ or Mg²⁺. Investigations of the influence of cations other than Na⁺ or Mg²⁺ (e.g. K⁺, Ca²⁺, or cations with charge higher than +2 like ubiquitous polyamines spermidine or spermine) are rare. There exist a few exceptions (e.g. Ref. 23) when in vivo relevant conditions of mixtures of cations of different charge (like Na⁺ and Mg²⁺) was studied. Widom how studied folding and self-association of chromatin fibres, which included a careful analysis (by electron microscopy, small angle X-ray scattering and AUC sedimentation velocity measurements) of fibre properties in mixed NaCl + MgCl₂ solutions. At concentration above 45 mM it was found that Na⁺ caused fibre folding but at lower concentrations Na⁺ competed with multivalent species (Mg²⁺ and trivalent Co(NH₃)₆³⁺) for binding to the fibre, thus reducing the folding. Oligomerization of the arrays was observed at submillimolar and millimolar concentrations of Mg²⁺ both in the absence and in the presence of NaCl and it was also noted that the arrays have a tendency to self-associate when folding to the 30-nm fibre is still incomplete. Similar conclusions about competition between Na⁺ and Mg²⁺ for binding to the array was made by Borochov et al observing self-association of chromatin fibres. These results were interpreted considering chromatin as a cation-exchange polyelectrolyte, using the Manning counterion condensation model to predict that more Mg²⁺ is required to fold or to aggregate chromatin fibre with increase of monovalent salt. However, another study using similar chromatin fibres and ionic concentrations came to the opposite conclusion, namely that the simultaneous presence of Na⁺ and Mg²⁺ (or Co(NH₃)₆³⁺) makes a synergistic influence on the fibre self-association; more added Na⁺ requires less Mg²⁺ (or Co(NH₃)₆³⁺) to precipitate the fibre. To the best of our knowledge these discrepancies were not later addressed, scrutinised and resolved. The cited works were performed using fibres extracted by limited micrococcal nuclease digestion of chromatin from chicken erythrocytes that were inhomogeneous with respect to number of nucleosomes, nucleosome repeat length, amount of linker histone H5 and with unknown number and nature of histone posttranslational modifications.

Recently, recombinant model chromatin systems have been developed and after the widespread adoption of the Widom’s 601 high-affinity nucleosome positioning sequence, large amounts of highly homogeneous, precisely positioned and fully saturated nucleosome arrays became available. Although this sequence has been generated by artificial selection and do not have natural analogues, the free energy of nucleosome formations for the 601 template is not significantly different from the free energy of nucleosome formation on generic natural DNA sequences. As shown by Widom and co-workers, the free energy of NCP formation differs by about ~4 kcal/mol for DNA sequences with the highest and lowest known histone octamer binding affinities. It is clear that the differences in free energies of nucleosome formation on different DNA sequences contribute rather marginally to the total free energy of chromatin formation, which is dominated by the huge free energy of (mostly electrostatic) non-specific DNA – histone interaction (tens if not hundreds kcal/mol) and the relatively large cost of the DNA bending (tens of kcal/mol). We took advantage of the availability of these pure and well-defined chromatin fibre systems and carried out biophysical studies to gain further understanding of the polyelectrolyte nature of chromatin. The purpose of the present work is to determine how folding and self-association of nucleosome arrays proceed when two of the three most common cations of the cell cytoplasm (K⁺ + Mg²⁺ or Na⁺ + Mg²⁺) are simultaneously present. It is generally believed that K⁺ and Na⁺ have a similar influence on DNA and chromatin and usually this view is supported by observations with a few exceptions when the small difference in binding K⁺ versus Na⁺ is multiplied due to cooperative participation of many DNA charged groups like during the co-globule transition of high molecular weight DNA. Furthermore, we recently found that for 12-mer arrays with nucleosome repeat length of 177 bp (12-177-601 array), potassium prevents complete folding of the arrays at a level almost equal to the strong effect caused by the H4 histone Lys16 acetylation (H4-K16Ac).
with 0.8 mM Mg$^{2+}$ (filled circles or filled diamonds in Fig. 2A). At 100 mM KCl, the presence of 100 mM of added KCl or NaCl and are only weakly exert different influence on the array compaction. The presence of K$^+$ inhibits the complete array folding and in 75 and 100 mM KCl the s$_{20,w}$ values are in the range 45–47 S while in the presence of 100 mM NaCl or for solutions of NaCl + MgCl$_2$, the array can reach complete compaction with s$_{20,w}$ = 52–56 S.

In the second series of experiments, the AUC-SV measurements were carried out at fixed concentration of MgCl$_2$ (1 mM) which means the arrays are fully compacted, increasing the concentration of KCl or NaCl (van Holde Weischet curves are not shown). Figure 2B summarises the results of the AUC-SV measurements. It may be noted the data in Fig. 2 from the two series of titrations (varying Mg$^{2+}$ at fixed KCl/NaCl; fixed Mg$^{2+}$, varied KCl/NaCl) obtained in the present study are combined with s$_{20,w}$ values determined earlier for the same array in solutions with only one added salt (MgCl$_2$, KCl, or NaCl). Addition of KCl or NaCl to low-salt TEK buffer array solution leads to array folding.

The “destructive” effect of K$^+$ on fibre folding is clearly illustrated by the decrease of the s$_{20,w}$ values observed upon addition of KCl to the array solution in the presence of 1 mM Mg$^{2+}$ (Fig. 2B, solid green rhombi). At 1 mM Mg$^{2+}$, the fully compacted array “opens up” when K$^+$ increases. In contrast to K$^+$, addition of Na$^+$ to array solution with 1 mM Mg$^{2+}$ leads to aggregation (Fig. 2B, solid orange triangles) and the AUC-SV data loses reproducibility which is reflected by loss of homogeneity (large error bars in Fig. 2) and by the fact that s$_{20,w}$ rises above the values possible for the maximally compacted array (Fig. 2B, data for 1 mM Mg$^{2+}$ + Na$^+$ are solid orange triangles). Solutions of the array with K$^+$ generally show higher homogeneity and AUC-SV curves are reproducible in a wider concentration range compared to the results in NaCl.

The present work confirms our earlier result$^{1,2,3}$ that K$^+$ prevents complete folding of the array and shows that this unfolding effect of K$^+$ cannot be overcome by addition of Mg$^{2+}$, which is expected to be much more powerful than monovalent cations in screening DNA-DNA repulsion and promoting chromatin compaction. In earlier work, we also found$^{4}$ that addition of KCl made a similar inhibitory effect on the array folding as H4 Lys16 acetylation, which is known as the most powerful modification that profoundly abrogates chromatin folding$^{22,34,37}$. Combining experimental data on folding of the different 12-177-601 arrays (in the wild type form, with H4 histone-tail-mediated NCP stacking even in the presence of K$^+$), see the curves with hollow symbols in Fig. 2A). It follows from our work that in the cell nucleus, where K$^+$ is the major cation, the specific tightest NCP-NCP stacking might be led to the suggestion of a novel hypothesis of the structure of the NCP-NCP stacking. In this model, NCP stacking is stabilized by hydrogen bonds between the charged ε-amino group of the H4-Lys16 and oxygen atoms of the peptide carbonyl in the H2B Arg96-Leu99 pocket$^{22}$. This crucial contact can be disrupted either by H4-K16 acetylation or by coordination of K$^+$ (or similar Rb$^+$ and Cs$^+$) with the H2B Arg96-Leu99 pocket. The specific affinity of K$^+$ (or Rb$^+$ and Cs$^+$) for the oxygen of peptide carbonyl (as compared to Na$^+$) has been experimentally observed$^{36}$.

It follows from our work that in the cell nucleus, where K$^+$ is the major cation, the specific tightest NCP-NCP stacking might be inhibited despite the presence of Mg$^{2+}$. It would be interesting to investigate if this unfolding effect of K$^+$ is observed for arrays with different nucleosome repeat length and if other factors (e.g. presence of linker histones), can recover H4-histone-tail-mediated NCP stacking even in the presence of K$^+$.

Self-association of 12-177-601 arrays in mixed-salt solutions. Monovalent cations cannot induce self-association of the wild type 12-177-601 array$^{3,22}$ whereas addition of divalent Mg$^{2+}$ leads to complete precipitation. Mg$^{2+}$-induced precipitation assay is widely-used to assess the balance of repulsive and attractive interactions in self-association of chromatin fibres as a function of a variety of factors such as nucleosome repeat length$^{36}$ or modifications in the histone tails$^{17,18,22,35}$.

To test whether monovalent ions affect the efficiency of the Mg$^{2+}$-induce inter-array aggregation, a series of Mg$^{2+}$ titrations were car-
NaCl produces a similar effect on the EC 50 values: less Mg 2+ EC50 values found in titration without added salt. Addition of KCl or NaCl is required to precipitate the array. The precipitation assay curves (Fig. 3) in the presence of KCl and NaCl are rather broad and some array aggregation is observed already at 1 mM Mg 2+. It is plausible that this fraction of aggregated arrays makes an influence on the AUC measurements causing broadening of the van Holde-Weischi curves (Fig. 1) and larger error bars (Fig. 2).

In the presence of 100 mM salt, the EC50 values are close to 1 mM, which explains why aggregation of the arrays limits application of the AUC-SV method in mixed solution at high monovalent salt. Array precipitation by Mg 2+ occurs in a quite broad range of added Mg 2+ (Fig. 3) and one needs to be cautious in interpretation of the EC50 data. However, the EC50 values in the presence of NaCl salt are typically lower than the corresponding ones determined in KCl. The somewhat higher efficiency of Na+ ions relative to K+ might originate from preferential binding of Na+ (relative to K+) to the phosphate groups of DNA 39 and to the carboxylic groups 40 (important for screening the H2A acidic patch on the HO surface) 22. These specific interactions can increase quenching of the negative charge of the array and facilitate its self-association.

**Conclusions.** Both nucleosome array folding and self-association are determined by electrostatic interactions with the repulsion between the negatively charged DNA as the major force that drives the formation of an extended “beads-on-a-string” structure at low monovalent salt concentrations, while the nature of the cation is of minor importance.

In the folded/aggregated state, the detailed molecular structure of the chromatin and the interplay between intra- (formation of the folded 30-nm fiber) and inter-array condensation is dependent on the DNA molecular structure and sequence, NRL, the histones, as well as nature and amount of salt cations. E.g., the NRL will profoundly influence the ability of folding in the absence of linker histone 28,29. The most important structural element of maximally folded chromatin is the NCP-NCP stacking and in vitro and in vivo studies indicate that there exists multiple nucleosome stacking conformations. A major conclusion from Fig. 2 is that for the 12-177-601 array, the K+ and Na+ ions have a different influence on the array folding, especially at physiologically relevant K+/Na+ and Mg 2+ concentrations; one ion (Na+) promotes stacking while the other (K+) abrogates it.

We may question how relevant the findings of the present study are to the situation in vivo? We argue that the range of K+, Na+ and Mg 2+ activities applied in the AUC-SV and PA studies are in the range of ionic activities of the eukaryotic cell cytoplasm. To support this claim we would like to note that low molecular weight species (with molecular mass below 50 kDa) can freely exchange between the nucleus and cytoplasm through nuclear pores and as a consequence the small and highly mobile cations K+, Mg 2+, Na+ and Ca 2+ are in thermodynamic equilibrium within the entire cell. Therefore, activities of these cations are equal in the cytoplasm and in the nucleoplasm but their amounts and concentrations in various cell organelles with significant binding to the biomolecules, are expected to be very different. This reflects the variable amount and different cation affinities of the anionic groups present in the given organelle or biomolecule. E.g., the average concentration over the whole cell volume is estimated at 7–9 mM for Ca 2+ in interphase cells 41. However, the activity or concentration of “free” Ca 2+ is strictly regulated and maintained at micromolar levels by the presence of a large amount of Ca-selective proteins (e.g. calmodulin). The contribution of Ca 2+ to chromatin properties is therefore expected to be insignificant, since the Ca 2+ affinity to DNA is negligible in comparison to the binding constants of the specialized proteins. Data on the amount or total concentration of K+, Na+, Mg 2+ inside the eukaryotic cell and its compartments varies significantly. As reported 1, the local concentration (not activity) of Mg 2+ vary in the range 2–32 mM depending on the cell type, cell cycle and cell organelle. In a relevant book 42, the concentration of “free” Mg 2+ in
the cell is estimated at about 0.5 mM, while the “total” concentration might be as high as 40 mM. Regarding average concentrations of K⁺ and Na⁺, X-ray microanalysis does not report absolute values, but give data for the K⁺ enrichment in the nucleus. The distinction between the ionic concentration and ion activity of K⁺ and Na⁺ in the cytoplasm is rarely acknowledged and reported. In a relevant paper, the concentrations and activities of K⁺, Na⁺ and Cl⁻ in Ehrlich ascites tumor cells were measured and the following concentration/activity values were obtained: Na⁺: 25.3/4.6 mM; K⁺: 165.1/68.3 mM; Cl⁻: 65.3/43.6 mM. The observations and arguments presented above justifies the relevance of our experimental setup that obviously simplifies the situation in vivo, but nevertheless exhibits an essential feature: the mixing of monovalent K⁺ or Na⁺ (at concentrations of 100 mM or below) with Mg²⁺ at millimolar concentrations is physiologically relevant. An important conclusion is that one needs to be cautious in extrapolation of results of in vitro studies of chromatin routinely carried out in sodium salts to the in vivo conditions where potassium cations are dominant.

**Methods**

**Histone and DNA preparation.** The DNA template containing the 12 tandem 177 bp repeats of the high-affinity Widom ‘601’ nucleosome positioning sequence (abbreviated as 12-177-601) was used. The pWM30 plasmid vector carrying 12-177-601 sequence was transformed to E. coli HB101 competent cell and amplified and purified as described previously. Xenopus laevis histones H2A, H2B, H3, and H4 were individually expressed in E. coli BL21 DE3 PlysS and purified using AKTA FPLC system (Uppsala, Sweden) in two stages: by gel filtration on Sephacryl S200 column followed by cation exchange chromatography using Resource HR 10/30 column. Histone octamer (HO) was refolded using 1:1:1:2:1:2:1 10 mM Tris-HCl, pH 7.5, 10 mM KCl, 0.1 mM EDTA followed by purification by MgCl₂ precipitation and redissolution in TEK buffer as described previously. Briefly, small-scale nucleosome array construction trials were carried out to determine an optimal HO:DNA stoichiometry. The optimal HO:DNA ratio (12.5 moles of HO per one mole of 12-177-601 DNA) was used for large scale array preparation. It included step-wise dialysis of the of the mixture of HO, 12-177-601 DNA, and competitor 145 bp DNA from 2 M KCl to TEK buffer (10 mM Tris-HCl, pH 7.5, 10 mM KCl, 0.1 mM EDTA) followed by purification by MgCl₂ precipitation and redissolution in TEK buffer as described previously. The competitor DNA was obtained from nuclease-digested calf thymus chromatin and was about 145 bp length. The concentration of competitor DNA was 0.6 fragments per one molecule of 12-177-601 DNA. The reconstituted arrays were digested by Scal (a Scal restriction site is at linker DNA of each of the 12 repeats) and analysed by 6% native polyacrylamide gel electrophoresis (PAGE) to check saturation of the array with HO (see for details). Stocks of 12-177-601 DNA were prepared from the recombinant 12-177-601 DNA template and the histones as described previously. Briefly, small-scale nucleosome array construction trials were carried out to determine an optimal HO:DNA stoichiometry. The optimal HO:DNA ratio (12.5 moles of HO per one mole of 12-177-601 DNA) was used for large scale array preparation. It included step-wise dialysis of the of the mixture of HO, 12-177-601 DNA, and competitor 145 bp DNA from 2 M KCl to TEK buffer (10 mM Tris-HCl, pH 7.5, 10 mM KCl, 0.1 mM EDTA) followed by purification by MgCl₂ precipitation and redissolution in TEK buffer as described previously. The competitor DNA was obtained from nuclease-digested calf thymus chromatin and was about 145 bp length. The concentration of competitor DNA was 0.6 fragments per one molecule of 12-177-601 DNA. The reconstituted arrays were digested by Scal (a Scal restriction site is at linker DNA of each of the 12 repeats) and analysed by 6% native polyacrylamide gel electrophoresis (PAGE) to check saturation of the array with HO (see for details).

Stock solution of the 12-177-601 array (∼2 mg/mL in TEK buffer) was stored at 4 °C and used in AUC and precipitation assay experiments. The quality of array was checked upon reconstitution and after one-two week storage by: a) running both native array and Scal digested on 0.8% agarose and 6% DNA-PAGE gel to check saturation of the array with HO, and b) running AUC-VC measurement at 1 mM MgCl₂. Absence of the 177 bp lane in the Scal-digested array and an s20,w value of 52–53 S indicates a homogeneous and saturated sample. Since this study compares the effect of KCl and NaCl, it may be asked whether the reconstitution nature of the salt influence the properties of resulting stock solution. In our laboratory, fibres were reconstituted in KCl and saturated sample. Since this study compares the effect of KCl and NaCl, it may be asked whether the reconstitution nature of the salt influence the properties of resulting stock solution. In our laboratory, fibres were reconstituted in KCl and NaCl using the same source of DNA and histones. Identical results were obtained for the Mg²⁺-induced compaction, which shows that reconstitution in potassium or sodium ions, results in fibres with identical folding properties.

**Analytical Ultracentrifugation (AUC).** Analytical ultracentrifugation sedimentation velocity (AUC-SV) method provides information about degree of folding of nucleosome arrays and regularly used in studies of chromatin. AUC-SV measurements were carried out using a Beckman XL-A UV-visible ultracentrifuge (Beckman Coulter, Fullerston, CA). The nucleosome arrays were diluted in TEK buffer to optical density A380 = 0.8 cm⁻¹. Samples and references were equilibrated in 12 mm double-sector cells under vacuum, for 30 min at 25 °C and at 70,000 rpm. Forty scans were collected at 12,000 rpm with 10 min interval. The data was analysed using the van Holde-Weischet method by the Ultrascan program and plotted as integrated curves of boundary versus sedimentation coefficient (Fig. 1). The sedimentation velocity coefficient was corrected to s20,w using a partial specific volume of 0.622 mL/g. For each AUC-SV measurement, characteristic mean s20,w
value was calculated as the average of s_{20,w} from 20–80% of the boundary, each characterized by a given standard deviation. Two-three independent AUC-SV runs were performed for each array sample and the mean s_{20,w} value was calculated from the average s_{20,w} (obtained from 20–80% of the boundary) with the standard deviation (SD) obtained as the root mean square of the corresponding SDSs.

**Precipitation assay (PA).** The stock solution of the array was diluted in 10 mM Tri-HCl, pH 7.5 to optical density A_{260} = 2 after which the solution was mixed with equal volume of the same buffer containing twice the final concentration of monovalent and divalent salt. The absorbance of the final solution was A_{260} = 1, similar to the one used in the AUC-SV method. After incubation for 15 min, the samples were spun for 15 min × 20,000 g at room temperature. The absorbance of the supernatant was measured at 259 nm using NanoDrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE); the range of A_{260} absorbance used in the PA corresponds to the best precision of the spectrophotometric measurements. The efficiency of mixed cation system to precipitate the array was characterized by the average Mg^{2+} concentration at 50% precipitation of the array (E_{50}) calculated from at least three measurements.

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**Additional information**

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