Specific Binding of Calcium to Soluble Chromatin

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Equilibrium dialysis studies and sedimentation experiments with soluble rat liver chromatin and radioactive ^{45}Ca support the hypothesis that there is a specific binding of Ca^{2+} to chromatin molecules. At low ionic strength the binding constant is about 12 l/mM indicating that more than half of the negative charges of the chromatin molecules are neutralized due to the binding of Ca^{2+} at appropriate concentrations. This effect possibly accounts for low concentration of Ca^{2+} and other divalent cations being effective in inducing compact higher order structures of chromatin. Monovalent cations are not specifically bound, but high concentrations cause unspecific shielding of the charges on the chromatin molecules and thus structural transformation. Even under these high salt conditions a specific binding of Ca^{2+} to chromatin occurs. The binding constant is, however, only 0.4 l/mM due to the lower effective concentration of the charged chromatin molecules which is reduced by unspecific neutralizing.

Introduction

It is a well established fact that chromatin at very low ionic strength and in the absence of divalent cations has a somewhat extended structure. This so-called tertiary structure is the chain of nucleosomes which appears like "beads on a string" [1–6]. This unravelled structure can be changed into a more compact one, the quaternary structure with threefold diameter and a sixfold reduction in length which is described as a solenoid [3–11] or as an arrangement of superbeads [12, 13]. The structural transformation can be induced by high concentrations (20 mM to 100 mM) of monovalent cations [3–5, 8, 9, 14–19] or by divalent cations [3, 5, 6, 14–16, 18], especially Mg^{2+}. The divalent cations are effective at very low concentrations in the range of 0.2 mM. At higher salt concentrations chromatin condensation increases and the material aggregates, becomes insoluble and finally precipitates [14, 15, 20–22]. This process can be initiated by monovalent cation concentration of about 150 mM or by divalent cations of about 1 mM [14, 15, 20].

The higher efficiency of divalent cations for both effects, structural change and precipitation of soluble chromatin, cannot be solely explained by differences in ionic strength efficiencies. An effect peculiar to divalent cations must be involved, *e.g.* a specific interaction between divalent cations and the negatively charged chromatin molecules.

This paper describes equilibrium dialysis studies and sedimentation experiments with soluble rat liver chromatin and Ca^{2+} ions, containing radioactive ^{45}Ca. The results support specific interaction of Ca^{2+} with chromatin molecules which can be interpreted as a specific binding or association. Quantitative analysis indicates a binding constant K, the value of which depends on the ionic strength of the solvent. The interpretation of the association effect and the binding constant provides a possible explanation for the specific efficiency of Ca^{2+} and perhaps other divalent cations on the structural transformation and precipitation of soluble chromatin.

Materials and Methods

Preparation of chromatin

Soluble chromatin was isolated from rat liver cell nuclei as described in the preceding paper [23]. The yield from 35 g liver was about 4 ml chromatin solution of A_{260} near 100. Before undertaking equilibrium dialysis experiments, this stock solution was diluted and dialyzed against buffers of pH 7.5 containing 1 mM Tris/HCl and a suitable monovalent cation concentration to eliminate residues of EDTA and EGTA.

Equilibrium dialysis and ^{45}Ca measurements

Equilibrium dialysis was carried out with double-chamber cells made out of PTFE of 2×1 ml volume with a membrane of 4.5 cm^2 area. Different mem-

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branes were used without obtaining differing results (Spectrapor type 2 from Spectrum-Medical Industries Inc., Los Angeles and Visking from Serva, Heidelberg, FRG). One chamber was carefully filled with 1 ml chromatin solution of appropriate concentration and buffer composition. Only the buffer composition and a defined Ca concentration including ^{45}Ca were introduced in the other chamber. Several dialysis cells were stacked and rotated slowly to cause gentle agitation of the solutions in the chambers. After dialysis overnight at 4 °C, equilibrium was reached and aliquots were taken from each chamber to determine the Ca concentrations. This was done by measuring the radioactivity of ^{45}Ca with a liquid scintillation counter (MR 300 DPM from Kontron analytical, Eching, FRG) using an adapted energy discrimination.

Sedimentation

In addition, binding of Ca^{2+} to soluble chromatin was controlled by sedimentation experiments. Samples of 0.2 ml chromatin solutions in buffers containing a defined concentration of Ca (including ^{45}Ca) were centrifuged for 4–5 h at $160{,}000 \times g$ in an airfuge (Beckman Instruments, Munich, FRG). Sedimentation of the chromatin molecules was controlled by measuring A_{260} of the supernatant. Ca^{2+} concentration in the solution before and in the supernatant after sedimentation was determined by measuring the radioactivity of ^{45}Ca as described above.

Donnan effect

Chromatin molecules are negatively charged macromolecules which cannot permeate a dialysis membrane in the way small ions of salt and buffer components do. If the chromatin concentration is high and the amount of the permeating ions low, the Donnan effect (a higher concentration of positive ions in the chromatin chamber) results due to the necessary equilibrium of the chemical potentials of all components and charge neutrality on both sides of the membrane. The Donnan ratio r_D is the ratio of the concentrations of any free cation on both sides of the membrane, while the anion concentration ratio is reciprocal. The value of r_D depends quantitatively on the concentrations of charged macromolecules and other ionic components, it can easily be calculated [24].

Effective ways suppressing the Donnan effect are to raise the salt concentration or to use very low chromatin concentrations. However, the Donnan effect must be considered in all experiments under low ionic strength conditions when the concentration of chromatin-bound Ca and of free Ca^{2+} are calculated from the measured values of total Ca concentration present at each side of the membrane.

Determination of chromatin-bound and free Ca^{2+}

The Donnan effect could be ignored when equilibrium dialysis experiments were undertaken with chromatin solutions in solvents of nearly physiological ionic strength. The concentration of free Ca^{2+} was nearly identical in both chambers of the dialysis cell and proportional to the radioactivity measured in the chromatin-free chamber. Radioactivity in the chromatin chamber was proportional to the sum of the concentrations of free Ca^{2+} and chromatin-bound Ca.

In solvents of lower ionic strength, calculation of the Donnan ratio r_D was essential to ascertain the ratio of the free Ca^{2+} concentrations on both sides of the membrane. From these values, and the measured radioactivities in both chambers, the concentrations of free Ca^{2+} and of chromatin-bound Ca in the chromatin chamber were determined.

In sedimentation experiments, the concentration of free Ca^{2+} was proportional to the radioactivity in the supernatant after centrifugation. The difference between this and the radioactivity in the solution before centrifugation determined the amount of chromatin-bound and sedimented Ca. In solutions of lower ionic strength it must be remembered that negatively charged sedimented chromatin molecules unspecifically adsorb cations, such as Ca^{2+}, because of charge neutrality. As with the Donnan effect in equilibrium dialysis experiments, sedimentation studies must be corrected before calculating the concentration of free Ca^{2+} and chromatin-bound Ca at lower ionic strength.

Results

Equilibrium dialysis experiments at ionic strength conditions of 60 mM in both chambers were not remarkably influenced by the Donnan effect. However, radioactivity – and thus Ca concentration – was not identical in both chambers. On the contrary, it was significantly higher in the chamber containing

the chromatin solution. This demonstrated very clearly that under these conditions a specific binding between Ca^{2+} and negatively charged chromatin molecules occured, reducing the free Ca^{2+} concentration which was equal on both sides of the membrane. As Table I shows, this effect increased with increasing chromatin concentration as one would expect.

The term specific binding is used in the sense of a special interaction between Ca^{2+} and the negative charges on the chromatin molecules. They are localized at the phosphate groups of the outside DNA parts which are not neutralized by internal histone-DNA interactions.

The specific binding of Ca^{2+} with chromatin molecules can be formulated much in the same way as with chelating agents by the following association reaction ($Chr.^{2-}$ is a twofold negatively charged segment of the chromatin macromolecule):

$$Ca^{2+} + Chr.^{2-} \rightleftharpoons Chr.Ca$$

An association or binding constant K can then be calculated as follows:

$$K = \frac{[Chr.Ca]}{[Chr.^{2-}] \times [Ca^{2+}]} \text{ l/mM}.$$

For quantitative calculations, a concentration of the negatively charged chromatin and the Ca-chromatin complex must be used in molar dimensions. The conversion of mg/ml chromatin to molarity produces an equivalence of 1 mg/ml chromatin (about 0.5 mg/ml DNA) to 1.6 mM on single negatively charged nucleotides. Within chromatin the negative charges of the DNA are partly neutralized by interaction with the histones and quantitative approximations are between 50% [25] and 15% [26]. The mean value of the residual unneutralized charges in macromolecular chromatin produces an equivalence of 1 mg/ml to about 0.5 mM on twofold negatively charged segment of the chromatin molecules.

The obtained values of the constant K, listed in Table I, were nearly constant. The mean value ± S. D. was 0.42 ± 0.05 l/mM and independent of the chromatin concentration.

The same value was obtained when the Ca concentration was varied at constant chromatin concentration and ionic strength of 62 mM as shown in Table II. The independence of the association constant K from the concentration of the two association partners demonstrates that the above postulated binding model of Ca^{2+} to negatively charged chromatin molecules corresponds well with the experimental results, although it is only a fairly rough approximation.

In equilibrium dialysis experiments at lower ionic strength conditions, the Donnan effect could not be ignored. The Donnan ratio r_D and the asymmetrical distribution of Ca^{2+} between the two dialysis chambers had to be calculated. In Table III, both anticipated and the measured values are listed, the latter were always higher indicating a specific binding of Ca^{2+} to chromatin. It is remarkable that with decreasing ionic strength the binding constant K increased considerably.

Additional experiments with varying chromatin concentrations in the range of 0.014 to 1.5 mg/ml at very low ionic strength indicated (not shown) that

Table I. Equilibrium dialysis with various chromatin concentrations. Specific binding of Ca^{2+} to chromatin with a binding constant K independent of the chromatin concentration is indicated by the increasing content of Ca in the chromatin chamber being dependent on increasing chromatin concentration. Ionic strength was 62 mM and the initial Ca^{2+} concentration in the buffer was 0.2 mM.

Chromatin concentration [mg/ml]	% Radioactivity of ^{45}Ca in the chromatin chamber	Binding constant K [l/mM]
0.125	50.8	0.50
0.625	53.2	0.44
1.25	55.3	0.37
1.25	55.6	0.40
1.40	56.6	0.43
1.50	57.2	0.44
1.50	56.9	0.42
2.50	61.4	0.46
3.95	63.0	0.34

Table II. Equilibrium dialysis with various Ca^{2+} concentrations. The binding constant K is independent of the initial Ca^{2+} concentration which was introduced into the buffer chamber. Ionic strength was 62 mM and the applied chromatin concentration 1.4 mg/ml.

Initial Ca^{2+} concentration in the buffer chamber [mM]	Binding constant K [l/mM]
1.3×10^{-5}	0.40
2.0×10^{-4}	0.43
2.0×10^{-3}	0.36
2.0×10^{-2}	0.44
2.0×10^{-1}	0.43

Table III. Equilibrium dialysis at various low ionic strength conditions. The Donnan effect has to be considered. The expected higher Ca^{2+} concentrations in the chromatin chamber caused by the Donnan effect are listed. They are significantly lower than the measured values. For experiments under similar conditions mean values ± S. D. are given. The binding constant K ceases to be constant and increases with decreasing ionic strength indicating that the specific binding of Ca^{2+} to chromatin rises at lower ionic strength. The applied chromatin concentration was in the range of 1.1 to 1.5 mg/ml, the initial Ca^{2+} concentration 0.2 mM.

Ionic strength [mM]	% Radioactivity of ^{45}Ca in the chromatin chamber expected	measured	Binding constant K [l/mM]
62.0	50.3	56.3 ± 0.8	0.41 ± 0.3
32.0	50.5	59.8 ± 0.5	0.85 ± 0.1
11.9	51.3	68.7	2.2
6.8	52.3	75.5 ± 1.5	3.9 ± 0.5
3.7	56.5	82.9 ± 6.0	5.4 ± 3.0
3.4	55.1	75.9	3.9
2.5	57.0	85.2 ± 2.5	8.2 ± 2.2
2.4	58.0	81.9	5.0
2.1	59.3	83.9 ± 1.2	5.9 ± 0.8
2.1	63.4	92.7 ± 0.7	10.6 ± 1.2
2.0	63.0	94.3 ± 0.9	17.1 ± 2.9
1.8	61.1	88.9 ± 3.8	10.0 ± 3.7

the binding constant K was very high but independent of chromatin concentration. The mean values ± S. D. of K from several data were 12.4 ± 1.8) l/mM at 1.4 mM and 10.8 ± 3.5 l/mM at 2.0 mM ionic strength.

In contrast to these values the binding constant of Ca-EDTA is several magnitudes higher implying that Ca^{2+} is quantitativly bound in a complex when EDTA is present. Equilibrium dialysis control experiments with additional 1 mM EDTA containing buffers of high ionic strength resulted in a uniform distribution of the Ca-radioactivity between the two chambers – as one would expect. At low ionic strength conditions, the Donnan effect caused an asymmetrical distribution of the Ca-EDTA complex. Being a negatively charged complex, it was enriched in the buffer chamber. With a chromatin concentration of 1.5 mg/ml at 4 mM ionic strength the expected radioactivity of ^{45}Ca in the chromatin chamber due to the Donnan effect should have been only 44.6%, the mean value actually obtained from several experiments was 45.2 ± 1.0%.

To test the effect of Mg^{2+} on the association of Ca^{2+} to chromatin experiments were carried out with additional Mg^{2+} of various concentrations in the buffer. As Table IV shows, the presence of Mg^{2+} had almost no effect on the binding of Ca^{2+} to chromatin. There was an apparent reduction in the value of the Ca binding constant only when the concentration of Mg^{2+} was at its highest.

Table IV. Equilibrium dialysis with various Mg^{2+} concentration in the buffer. The binding constant K appears to be independent of the Mg^{2+} concentration. Chromatin concentration was 1.25 mg/ml, initial Ca concentration 0.001 mM, ionic strength 62–64 mM.

Initial Mg^{2+} concentration in the buffer chamber [mM]	Binding constant K [l/mM]
0.001	0.39
0.02	0.41
1.0	0.34

The sedimentation experiments under different conditions (various Ca^{2+} concentrations, ionic strengths and Mg^{2+} concentrations) demonstrated that a certain amount of Ca sedimented together with the chromatin due to the specific association. Control experiments showed that this effect was completely prevented by EDTA. Quantitative determination of the binding constant K was a more approximation when sedimentation rather than dialysis data were used. At high ionic strength of about 60 mM, constant K was 0.27 ± 0.05 l/mM independent of the Ca^{2+} and Mg^{2+} concentrations.

Unspecific adsorption of Ca^{2+} to the sedimented chromatin at low ionic strength was an additional source of error, and quantitative determination of K was more difficult. The sedimentation data also showed that the constant K increased with decreasing ionic strength, but in comparison with the values determined from equilibrium dialysis experiments, this effect was not so marked.

Discussion

For isolated DNA various effects as thermal melting or helix-coil transition depending on the ionic components of the solvents have been investigated in many cases. In particular, effects caused by interaction of DNA with monovalent and divalent cations are reported and discussed in detail [27–29]. On the one hand chromatin is somewhat similar to DNA as being a polyanionic macromolecule, on the other hand it is rather different mainly in the structural sense due to intermolecular interactions of histones with DNA.

All experimental results of this paper support a specific binding of Ca^{2+} to chromatin molecules. A binding or association constant K is independent of the concentration of the association partners and can be calculated from equilibrium dialysis and sedimentation data. At higher ionic strength, the values of K determined from both methods generally agree, but at lower ionic strength the agreement is only qualitative in the sense that the values of K are rising.

At low ionic strength, the calculation from data of both methods need corrections implying additional sources of error. The equilibrium dialysis data give more reliable results because they are only influenced by the Donnan effect. Control experiments with EDTA have shown that the quantitative consideration of the Donnan effect is correct. Data from sedimentation experiments also have to be corrected, but the effect of unspecific adsorption of cations to sedimented chromatin can only be roughly approximated.

The results of all the equilibrium dialysis experiments are summarized in Fig. 1 and the mean values and error bars of the binding constant from several experiments are plotted against the ionic strength in logarithmic scale. At higher ionic strength, the Donnan effect is negligible and no corrections are necessary. Also, the values for K vary little. As Fig. 1 shows, the error bars of K increase strongly with decreasing ionic strength. Under these conditions, the Donnan effect influences the data more and more, and the necessary corrections cause increasing deviations.

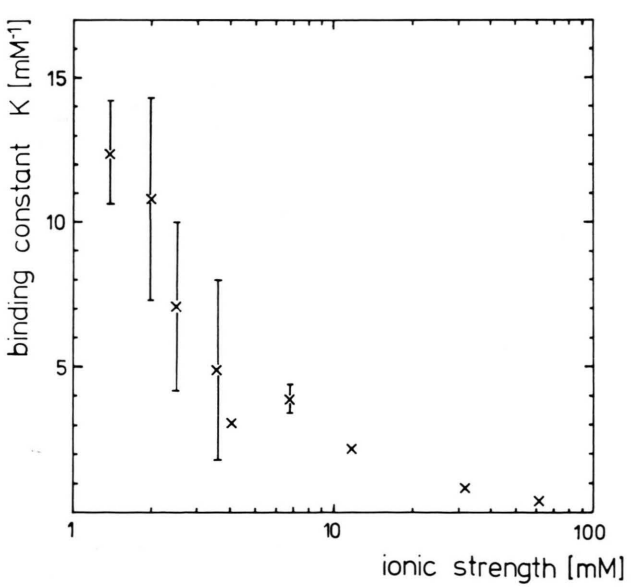

Fig. 1. Plot of the binding constant K of Ca^{2+} to chromatin molecules against ionic strength (logarithmic scale). For experiments under similar conditions, the mean values are given with error bars indicating S. D. Above 30 mM ionic strength, the error bars are too small to be recognized.

In spite of this, it is evident that K increases considerably with decreasing ionic strength. This resembles to the interaction of DNA with mono- and divalent cations [27, 29] or to the condensation and precipitation of chromatin depending on mixtures of Mg^{2+} or Mn^{2+} with Na^{+} [15]. In the range of 1–2 mM ionic strength the binding constant K is about 10 to 15 l/mM. This denotes an association rate of about 55% at a Ca concentration of 0.2 mM and a chromatin concentration of 0.4 mg/ml. More than half of the negative charges of the chromatin molecules are thus neutralized by specific binding of Ca^{2+}. This drastic reduction of the charge density attenuates repelling forces within the chromatin molecules and facilitates further condensation to higher order structures [3, 5, 6, 14–16]. Increasing Ca^{2+} concentration also increases charge neutralization causing a higher compaction and aggregation of the chromatin molecules which finally become insoluble and precipitate [14, 15, 20].

Without any Ca^{2+} or other divalent cations similar effects are also produced by monovalent cations when the concentrations are more than hundredfold higher [3–5, 8, 9, 14–22]. Even at such high concentrations of monovalent cations there is a specific binding of Ca^{2+} to chromatin: at 60 mM the binding constant K has a value of 0.42 l/mM. This indicates an association rate of only about 7% at a Ca-concentration of 0.2 mM and a chromatin concentration of 0.4 mg/ml. The reduced effective concentration (chemical activity) of the negatively charged chromatin molecules due to unspecific shielding or partial charge neutralization by monovalent cations is the reason for the lower binding constant K at these conditions.

Chromatin condensation and formation of higher order structures is generally induced with high efficiency by small amounts of divalent cations, in particular Mg^{2+}. An explanation of this effect, especially with Ca^{2+}, is the specific binding of Ca^{2+} to chromatin molecules. Experiments studying the competition between Ca^{2+} and Mg^{2+} showed that the binding of Ca^{2+} to chromatin is hardly influenced by Mg^{2+} with-

in the tested concentration range. Only in the case of the last value in Table IV (extremely high Mg^{2+} concentration) a competing effect of Mg^{2+} could be conceived which reduces slightly the value of the Ca binding constant. This might be an indication for an alternative binding of Mg^{2+} to chromatin molecules. This is further supported by the efficiency on structural transformation and agrees with data of Ausio *et al.* [14] who studied Mg^{2+} binding to chromatin. Following the above experiments it can be deduced only that a Mg binding constant must be lower than that established for the Ca^{2+} binding. A quantitative determination requires further studies. Theoretically, one would expect that Mg^{2+} and Ca^{2+} act in a similar way, but it is not quite surprising that there are also differences. Such differences have been reported *e.g.* for Mg^{2+} and Mn^{2+} precipitating chromatin in presence of NaCl [15] and for Ca^{2+} being effective on chromatin condensation in a different way than Mg^{2+}, Zn^{2+} and Cu^{2+} [16].

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