Chromatin Aggregation Depends on the Anion Species of the Salts*

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The effects of anions on chromatin aggregation may be classified into three categories. First, monovalent anions, glutamate, acetate, chloride, and thiocyanate, follow the lyotropic series in their effects on both H1 histone displacement and chromatin aggregation. Second, alkyl carboxylates and dicarboxylates differ in their ability to induce chromatin aggregation depending on charge density, suggesting possible interference by bulky alkyl chains with neutralization (screening) of closely spaced positive protein charges. Third, the multivalent anions, citrate\(^{3-}\) and \(\text{SO}_{4}^{2-}\), bind tightly to histone and disrupt nucleosomes and thus interfere with chromatin aggregation. Substantial differences in chromatin aggregation were observed with different species of anions. At salt concentrations of 0–500 mM and pH 7.0, as much as 70% of the chromatin could be induced to aggregate by monosodium glutamate and sodium acetate, whereas only 10% or less was precipitated by NaSCN, \(\text{Na}_{2}\text{SO}_{4}\), and Na\(_2\)citrate. The physiological anion composition of the nucleus is not known; however, the anion effects discussed in the present work suggest a potential for regulation of chromatin condensation in higher eukaryotes.

Chromatin aggregation in the presence of salt is thought to be caused partly by the delocalized screening of DNA phosphate charges by cations and to be driven entropically by the release of counterions (10–12). Studies of chromatin aggregation induced by multivalent cations, however, seem to indicate that these cations may not induce aggregation only by charge screening and counterion release but also by direct binding to create cross-links between DNA strands in adjacent chromatin fibers (7, 13). The possibility that anions might induce chromatin aggregation has been neglected. Probably an effect of anions on chromatin would reside in the interaction of anions with chromosomal proteins rather than through interaction with DNA. Interaction of anions with chromosomal proteins could affect the direct binding of these proteins to DNA and also influence the binding of one nucleosomal strand to another, thus inducing higher or lower levels of aggregation depending on the nature of the anion. The equilibrium association constant of lac-repressor to its operator showed substantial dependence on anion species at constant salt concentrations (14). Recently, it was reported that the H\(_{\text{Glu}}\)\(^{1-}\) could promote protein-DNA interactions more than Cl\(^{-}\) (15). Site-specific protein-DNA interactions in the RNA polymerase system were enhanced 10–30-fold under in vitro assay conditions by replacement of KCl with KH\(_{\text{Glu}}\) (15). Furthermore, a higher and broadened optimal salt concentration was found for KH\(_{\text{Glu}}\) than for KCl in measurements of RNA polymerase activity and protein-DNA complex formation (15). This higher range of K\(^{+}\) and H\(_{\text{Glu}}\)\(^{+}\) was considered to be relevant to the physiological ion environment; H\(_{\text{Glu}}\)\(^{+}\) is not only a major member of amino acid pool, but it also constitutes a significant fraction of the total anion in Escherichia coli (16, 17). In any case, the effect of glutamate on several protein-DNA interactions is consistent with complex formation that is driven by preferential anion release, and such a mechanism is supported by the observation that anions differ in their effects according to their position in the lyotropic series (18, 19). Since the lyotropic series correlates with the effectiveness of anions in salting out proteins and other macromolecules, it seemed possible that the solubility of nucleoproteins such as chromatin might vary according to the nature of the anions that accompany the chromatin in solution. The work about to be discussed confirms this prediction. Moreover, the results suggest that counterion exclusion, direct electrostatic interactions, and steric hindrance (and therefore charge density of counterions) all play important roles in chromatin aggregation. These anion effects indicate that those who study the physical structure of chromatin in vitro need to recognize that any discrepancy between physiological conditions and theirs might give misleading results.

The abbreviations used are: H\(_{\text{Glu}}\), monohydrogen glutamate; Ac, acetate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

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Fig. 1. Chromatin aggregation by NaCl, NaAc, and NaHGlu. Chromatin (A_{260} = 6.0) at pH 7.0 was incubated in various salt concentrations at room temperature for 2 h. Soluble chromatin in the presence of NaCl (○), NaAc (□), and NaHGlu (Δ) was then separated from aggregated chromatin by microcentrifuge (2 min).

MATERIALS AND METHODS

Preparation of Nuclei—All steps in the sample preparation were performed at 0–4°C. Cell culture and nuclei isolation were as described previously (9). Nuclei were washed and stored in Buffer A (50 mM Tris-HCl, 25 mM KCl, 5 mM MgCl₂, 0.34 M sucrose, 1.0 mM phenylmethylsulfonyl fluoride, pH 7.5) before chromatin preparation.

Preparation of Chromatin—Nuclei in Buffer A were adjusted to A_{260} = 50 and incubated at 37°C for 5 min before a mixture of CaCl₂ and micrococcal nuclease was added to the nuclei to give a final concentration of 1 mM CaCl₂ and 20 units/ml micrococcal nuclease. After 3–5 min of incubation at 37°C, the digestion was stopped by EGTA (1 mM). Chromatin extraction and dialysis were as described previously (9). Chromatin so prepared was used within a week of nuclei preparation.

Anion Dependence of Chromatin Aggregation—Chromatin solutions were adjusted to pH 7.0 by the addition of small aliquots of NaOH or HCl (0.1–1.0 N) at 20°C in all aggregation and H1 displacement experiments with a final concentration of chromatin at A_{260} = 6. Sodium salts were made to 1.0 or 2.0 M and pH 7.0 before use. Mixtures of chromatin and salt at the desired concentrations were incubated at 20°C for 2 h, and aggregated chromatin was separated from soluble chromatin by microcentrifuge for 2 min. Solubility of chromatin was calculated as the percentage of total starting material (A_{260}) remaining in the supernatant. The A_{260} was measured in 0.1 N NaOH.

H1 Histone Displacement and Protein Analysis—Mixtures of chromatin and salt at desired salt concentrations were incubated at 20°C for 2 h before subjecting them to ultracen trifugation (100,000 × g, 4°C, 20 h). The protein compositions of supernatants and pellets were analyzed by electrophoresis through 14.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (20). The proteins were quantitatively estimated by scanning Coomassie Blue-stained gels in an Ultrascan laser densitometer, model 2202 (Pharmacia LBK Biotechnology Inc.).

RESULTS AND DISCUSSION

The Effect of NaHGlu and NaAc on the Binding of H1 Histone to DNA—Arakawa and Timasheff (21, 22) concluded that the formation of complexes between proteins and other macromolecules was driven entropically by the exclusion of counterions from the macromolecular surface. In an extension of this notion, Leirmo et al. (15) reported that HGlu⁻ strengthened protein-DNA interactions more than did Cl⁻, on the basis of experiments with various site-specific protein-DNA interaction systems such as RNA polymerase and restriction endonucleases (15). We decided to learn if the same was true for the binding of H1 histone to DNA. It has been known for a long time that H1 histone can be selectively extracted from chromatin by 0.6 M NaCl. When NaHGlu and NaAc were compared with NaCl in their ability to displace the H1 histone from chromatin, it became clear that the concentration of Na⁺ was not the only factor affecting the release of H1. Whereas 0.6 M NaCl released H1 completely, 0.6 M NaAc released only three-quarters and 0.6 M NaHGlu only half of the H1. Clearly then, the nature of the anion affected H1-DNA binding. That HGlu⁻ enhanced H1-DNA interactions more than did Ac⁻, which in turn was more powerful than Cl⁻, correlates with the lyotropic series and is consistent with the conclusion of Record et al. (12) that anion exclusion drives the formation of complex, in our case, between H1 histone and DNA.

Chromatin Aggregation by NaHGlu, NaAc, and NaCl—A question now arises as to how various salts would compare in their effects on chromatin in a lower range of concentrations in which NaCl induces an extensive complex formation (i.e., gross aggregation) between the nucleoprotein fibers of chromatin rather than the release of H1. Preferential anion exclusion might enhance this type of complex formation, but it seemed conceivable that a firmer binding of H1 to DNA in the presence of NaHGlu instead might inhibit gross aggregation if the latter depended on having H1 bridges between chromatin fibers. Differential aggregation by NaCl, NaAc, and NaHGlu is shown in Fig. 1, in which the solubility of chromatin, determined by A_{260} of supernatant fractions, was plotted as a function of salt concentration at pH 7.0. The shapes of the aggregation profiles for the three salts are similar as are the optimal concentrations of each salt for inducing aggregation, but the magnitude of aggregation differed from one anion to another when other conditions were equal. These results clearly show that there is an anion effect on chromatin aggregation. The occurrence of an optimal salt concentration for maximal aggregation in each of these profiles suggests that two salt-dependent phenomena come into play. The first is an aggregation of chromatin promoted by the salt and the second an inhibition of aggregation by the salt. Below the optimal salt concentration, the first phenomenon clearly dominated; but as the salt concentration rose above the optimum, a second process competed with the first with increasing success. At salt concentrations below the optimal concentration, HGlu⁻ was clearly more powerful than Cl⁻ in the induction of aggregation. Above the optimum concentration, it appears that disaggregating forces found...
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Fig. 2. Sodium glutamate-induced chromatin aggregation was changed by addition of either NaCl (panel A) or NaAc (panel B). Concentrations (in mM): 0 (○); 50 (□); 75 (△); 150 (▲).

Chromatin aggregation by fatty acid salts—We speculated that because the differential interactions of anions with chromatin would likely be directed at the proteins, hydrophobicity of the anions might play some role in their interaction with chromatin. This effect was examined by studying chromatin aggregation induced by the sodium salts of a series of fatty acids.

To test for possible competition or additivity of anions, chromatin aggregation was assayed in the presence of various combinations of NaHGluc with either NaCl or NaAc as shown in Fig. 2. In the promotion phase, the amount of chromatin aggregated by a combination of HGluc and Cl\(^-\) was roughly equal to the sum of the amounts precipitated by the individual anions alone at the same concentrations (Fig. 2A). The same was true for combinations of HGluc\(^-\) and Ac\(^-\) except of course that Ac\(^-\) was more powerful than Cl\(^-\) (Fig. 2B). In contrast, in the disaggregation phase, the solubility of chromatin was a compromise between the solubilities found for the individual salts used alone at the same concentrations (Fig. 2). This suggests that although anions directly enhance the forces that promote aggregation, they have little or no effect directly on disaggregating forces. A major process leading to aggregation, and therefore to stabilization of the aggregates, seems to be the differential release of anions from the chromosomal proteins. It would not be surprising to find additive effects of anions in this process. The disaggregating forces might be driven largely by cations, perhaps as they compete with H1 histone for DNA-binding sites. If such were the case, the disaggregating force itself would not be expected to be affected by the nature of the anion even though the anion could affect the net amount of aggregation by its effect on the stability of the nucleoprotein complex.
acids. With the exception of formate, an increase in hydrophobicity from acetate to heptanoate hindered aggregation (Fig. 3). In fact, it was especially the disaggregation phase of the solubility profile which was affected by the size of the fatty acid salts. We considered the possibility that with increasing length of alkyl chains the fatty acids would be more effective in releasing H1 histone from chromatin or even that the hydrophobic nature of these salts might disrupt the core of the nucleosome and thus increase the solubility of the chromatin. The possibility of the latter was suggested by the report that deoxycholate selectively removes histones H3 and H4 from chromatin (23). When the ability of fatty acid salts to release histones from chromatin was tested directly, however, we found that acetate, butyrate, and hexanoate have about the same effect (Fig. 4). Furthermore, at least up to six carbons in length, fatty acids do not seem to interrupt the nucleosome since little or none of the core histone proteins was released along with the release of H1 which was observed in the presence of up to 800 mM salts (data not shown).

The explanation we favor for the hindrance to chromatin aggregation by increasing size of the alkyl side chain is that the low charge density of the larger salts results in only partial neutralization of the positive charges of chromatin even when the screening anions are maximally crowded around the chromatin fibers. Electrostatic repulsion would therefore hinder the packing of nucleoprotein into aggregates. One prediction of this notion is that carboxylic acid salts of similar charge density ought to have similar effects on chromatin aggregation, independent of their size. A series of dicarboxylate ions was therefore tested for effects on chromatin aggregation.

As may be seen in Fig. 5, when any dicarboxylate was compared on the basis of molarity with the monocarboxylate of corresponding size, the dicarboxylate was more effective than the monocarboxylate, but when compared on the basis of normality rather than molarity, the chromatin solubilities were similar. It is significant that the difference between a dicarboxylate and a monocarboxylate ion of the same size was not 4-fold as one would expect for an effect of ionic strength; instead it was 2-fold as one would expect of the effect of charge density. We conclude then that when other factors are equal, bulky anions interfere with aggregation and that this effect manifests itself in alkyl chain sizes greater than three carbon atoms per charge. Intriguingly, for anions of high charge density, increasing size enhanced chromatin aggregation as shown in Fig. 6 in which the amount of aggregation by either monocarboxylates or dicarboxylates at constant ionic strength was plotted against the number of carbons in the anions. Similarly, enhancement in the equilibrium association constant of lac-repressor to its operator by acetate was reported to be greater than the enhancement by formate (14). If these small anions can pack together tightly enough to screen all the positive charges of the nucleoprotein fibers with roughly equal effectiveness, the interactions of their hydrophobic side chains could enhance aggregation to various degrees. The fact that there was an optimal charge density in inducing chromatin aggregation supports the notion that a balance of electrostatic interactions and hydrophobicity of the anions determines the overall effectiveness of salts in promoting complex formation.

The Effect of Tightly Bound Anions on Chromatin Solubility
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FIG. 5. Chromatin aggregation by divalent carboxylate salts. Symbols: malonate (×); succinate (○), glutarate (□), adipate (△), pimelate (●), suberate (■); and sebacate (+).

ity—Having observed the correlation of the lyotropic series with the power of monovalent anions to promote the aggregation of chromatin, it might have been predicted that sulfate and citrate ions would be even more effective than HGlu- and Ac-. Experimental observation contradicted this expectation as shown by the aggregation profiles of Na₃citrate, Na₂SO₄, NaCl, and NaSCN in Fig. 7A. The fact that the monovalent anion SCN⁻ was less effective in inducing aggregation than Cl⁻ fits the lyotropic series, but the effects of the multivalent salts did not. Recognizing that the multiple valences of citrate and sulfate might cause them to bind directly and tightly to the closely spaced positive charges in H₁ histone, we wondered if these anions would be more effective than Cl⁻ in causing the displacement of H₁ from chromatin to render the chromatin soluble. Their ability to displace H₁ was tested in the presence of 300-800 mN sodium salts, and the results are shown in Fig. 7B and C. The fact that in 400 mN salt more H₁ was released by SCN⁻ than by Cl⁻ was consistent with the lyotropic series and with the relative effectiveness of monovalent lyotropic anions in stabilizing nucleoprotein complexes in accordance with the series HGlu⁻ > Ac⁻ > Cl⁻ > SCN⁻ (Fig. 7B). In addition to H₁ displacement, SCN⁻ might have increased solubility of chromatin by the preferential release of core histones H₂a and H₂b which is evident in Fig. 7C. In contrast, citrate³⁻ and SO₄²⁻ did not stabilize the binding of H₁ with chromatin fibers more than Cl⁻ as might have been predicted by the lyotropic series (citrate³⁻ > SO₄²⁻ > Cl⁻ > SCN⁻). The failure of citrate and sulfate ions to fit into the lyotropic series indicates that extra factors were operative here beyond those affecting the induction of chromatin aggregation by monovalent anions. Such a process could be the disruption of the nucleosome by the multivalent anions as is demonstrated in Fig. 7C. Another possible reason that multivalent anions do not fit the lyotropic series for chromatin aggregation might be that these ions bind to the nucleoprotein fibers much more tightly than chloride ions do. An analogy might be seen in the explanation of Record (24) that the tight binding of Mg²⁺ to DNA overrides the general effects of counterion release on protein-DNA binding. In our case, tight binding of citrate³⁻ and SO₄²⁻ to H₁ histone would interfere with chromatin aggregation by adding negative charge to the chromatin fibers even while stabilizing the binding of the proteins to DNA (Fig. 7A).

CONCLUDING COMMENTS

The extent to which the species effect of anions plays a role in the regulation of chromatin aggregation in vivo is not clear.
FIG. 7. Chromatin aggregation and H1 displacement by N-acitrate, Na$_2$SO$_4$, NaCl, and NaSCN. Panel A shows aggregation profiles as described in Fig. 1. Symbols: N-acitrate (A), Na$_2$SO$_4$ (C), NaCl (Δ), and NaSCN (○). Note that the ordinate covers the range 66-100% instead of 0-100% as in previous figures; this scale expansion enhances comparison of the upper curves. Panels B and C show H1 displacement. Chromatin incubated in 300-800 mN salt, pH 7.0, for 2 h at room temperature was centrifuged at 100,000 × g, 20 h at 4°C. Equal volumes of the supernatants were analyzed by electrophoresis in 14.5% polyacrylamide, 0.1% sodium dodecyl-sulfate gel. Panel B, H1 displacement at lower range of salt concentration, 300-500 mN. Panel C, both H1 displacement and nucleosome disruption at a higher range of salt concentration, 500-800 mN. In panels B and C: lanes 1-3, N-acitrate; lanes 4-6, Na$_2$SO$_4$; lanes 7-9, NaCl; and lanes 10-12, NaSCN. Panel B, lanes 1, 4, 7, and 10, 300 mN salts; lanes 2, 5, 8, and 11, 400 mN salts; lanes 3, 6, 9, and 12, 500 mN salts. Panel C: lanes 1, 4, 7, and 10, 500 mN salts; lanes 2, 5, 8, and 11, 600 mN salts; lanes 3, 6, 9, and 12, 800 mN salts.

The presence of a high concentration of glutamate in E. coli was reported by Measures (17) and confirmed by Record and co-workers (15, 16) when they reported the large effect of HGlu$^-$ on the binding of DNA to nuclease and polymerases in vitro. Subsequently it was suggested that in vivo the chromatin might not be very sensitive to changes in the type of ions because histones and other chromosomal proteins might protect the DNA from effect of changing anion composition (16). Our results show that even in the eukaryotic chromosome, HGlu$^-$ enhances DNA-histone binding and promotes aggregation. Whatever the physiological relevance of the present observations might be, they clearly warn of potential artifacts in structural studies of isolated nuclei and chromatin fragments. Most commonly the ion used in such studies is Cl$^-$, which probably does not represent the natural condition well when chromatin condensation is under consideration.

REFERENCES
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24. Record, M. T., Jr. (1975) Biopolymers 14, 2137–2158