Reversibility of the low-salt transition of chromatin core particles

L.J.Libertini and E.W.Small

Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331, USA

Received April 14, 1987; Revised and Accepted July 16, 1987

ABSTRACT

The low-salt transition of chromatin core particles is reversible if the monovalent cation concentration is kept above 0.2 mM. Exposure of the particles to salt concentrations below this value results in a nonreversible secondary transition. The nonreversible changes are relatively slow with a half-time of about 15 minutes. Once exposed to such low ionic strength, the particles then begin to refold with increasing salt in at least two steps over a much higher ionic strength range than is required for the usual low-salt transition. The refolding is very fast, with a half-time less than a minute. Small differences between particles which had or had not been exposed to very low salt persist even when the particles are returned to near physiological ionic strengths.

INTRODUCTION

Chromatin is made up of repeating units, known as nucleosomes, which consist of about 200 bp of DNA wrapped twice about a protein core containing two each of the four inner histones, H2a, H2b, H3, H4 [1]. Bound to these subunits in varying proportions are a number of other proteins including histone H1 and the high-mobility-group (HMG) proteins, the best known of the nonhistone proteins in chromatin [2].

To prepare nucleosome monomers, chromatin is digested with micrococcal nuclease in conjunction with various extraction techniques which remove essentially all proteins except the inner histones. Depending on the methods used, the nucleosomes may contain DNA ranging from about 145 bp up to about 200 bp in length. Nucleosomes containing 145 bp of DNA are relatively resistant to further digestion and are commonly referred to as chromatin core particles [1].

Important biological functions of chromatin, such as transcription, replication and repair, must require transient disruption of the nucleosome structure. For this reason considerable interest has centered on the conformational changes that core particles and nucleosomes can undergo in solution. When exposed to sufficiently low ionic strengths, chromatin core particles and nucleosomes undergo one or more structural transitions characterized by changes in sedimentation coefficient [3-6], tyrosine fluorescence intensity and anisotropy [6,7], transient electric dichroism [8], translational

diffusion coefficient [9], radius of gyration [10], and histone crosslinking and iodination patterns [5,11]. This low-salt transition is thought to be driven by electrostatic repulsion arising from the high overall negative charge (> 140) on the particles [7,8]. As the ionic strength of the solution is decreased, the magnitude of this internal repulsion increases until the weakest interactions within the particle are disrupted, causing the particle to expand to a form in which the strength of the repulsion is diminished. Hydrodynamic measurements suggest the shape change may be quite dramatic [8].

The low-salt transition has commonly been assumed to be reversible in order to analyze results in terms of cation binding [7,8,12]. We present here an examination of this assumption. Results suggest that the transition is reversible as long as the monovalent cation concentration is maintained above 0.2 mM, but below this concentration the core particles undergo a nonreversible secondary transition which affects the ionic strength required for refolding to the high-salt form. This effect may help to explain disagreements in the literature on the ionic conditions required to induce the low-salt transition.

MATERIALS AND METHODS

Preparation of chicken erythrocyte core particles was as described [6]. For low salt experiments core particle stocks at A_{260} of at least 40 were dialyzed extensively against 0.5 mM potassium phosphate, pH 7 and 0.5 mM 2-mercaptoethanol [7]. Samples at varying ionic strengths were then obtained as before by dilution of this stock into appropriate salt solutions. The monovalent cation concentration contributed by the stock and by any buffers added was estimated and taken into account when $[M^+]$, the total monovalent cation concentration, was estimated.

In some experiments the dialysis to low salt included 0.05 mM EDTA to chelate multivalent ion impurities. Inclusion of EDTA did not significantly affect the shapes or midpoints of the transitions reported here.

Tyrosine fluorescence intensity and anisotropy were measured as described [7]. Circular dichroism measurements were done on a JASCO J-41A spectrophotometer. Sedimentation coefficients were determined on a Beckman Model E ultracentrifuge operated at 40,000 rpm. All measurements were done at 20° C.

RESULTS

When chromatin core particles are exposed to salt concentrations below 10 mM by diluting from higher salt (a procedure which will be referred to as a "back" titration), they undergo a transition which is accompanied by changes in tyrosine fluorescence intensity and anisotropy [7]. This is illustrated by the open circles in Fig 1, results which are very similar to those reported previously.

Figure 1. Nonreversibility of the low salt transition for core particles. The data given by the open circles (dotted lines) were obtained by diluting a concentrated stock of core particles ($A_{260} = 46$, dialyzed as described in MATERIALS AND METHODS) into unbuffered salt solutions to give a final A_{260} of 1.0 and the indicated total monovalent cation concentrations. The data given by the filled circles (solid lines) were obtained by diluting the stock solution into water to a final A_{260} of 1.1, waiting at least one hour (time dependence of the nonreversible changes is discussed later), then mixing the diluted stock with 1/9 volume of unbuffered salt solutions to give the indicated total monovalent cation concentrations. In both cases the tyrosine fluorescence was measured about one hour after the sample was completed. The pH in this experiment was determined mainly by dissolved carbon dioxide and was about 6 as determined on collected samples during the measurements. (A) Anisotropy, (B) Intensity.

If the core particles are first diluted to very low salt ($[M^+] \approx 0.05$ mM) and subsequently adjusted to higher salt (referred to below as a "forward" titration), the results given by the filled circles in Fig. 1 are obtained. Both the intensity and anisotropy indicate changes which occur in two steps. The first (transition 1) occurs over a range of $[M^+]$ which is similar to that observed on dilution from higher salt while the second, larger step (transition 2) is at a much higher $[M^+]$. Curiously, the latter change occurs at different positions for the intensity as compared to the anisotropy, suggesting that two separate processes are responsible. It is apparent that the mechanism involved in refolding the core particle during a forward titration is not necessarily the reverse of that by which the particle opens up when back titrated.

Figure 2. Effect of the salt concentration during unfolding on the reversibility. The concentrated core particle stock was diluted to the indicated $[M^+]$ (including that contributed by the stock) and held at 20° for at least 1 hour (pH 6). Then 1/9 volume of a salt solution sufficient to raise the total $[M^+]$ to 1 mM was added and the tyrosine fluorescence measured about 1/2 hour later. A similar result was obtained when the period after diluting to the indicated $[M^+]$ was extended to at least 5 hours before adjusting to 1 mM.

With increasing pH the low-salt transition (back titration) shifts significantly to higher ionic strength and broadens somewhat while the overall change in tyrosine fluorescence anisotropy remains about the same [7]. To examine the effect of pH on reversibility, the experiment in Fig. 1 was repeated using 0.2 mM Tris base in place of water, a procedure which resulted in a pH of about 7.6 at the time the fluorescence was measured. Under these conditions the back titration curve was shifted, as expected, to about 2-fold higher salt concentration; however, now the results on forward titration (after dilution into 0.2 mM Tris base) were indistinguishable. To determine whether the reversibility at higher pH was due to an effect on the unfolding at low salt or on refolding during the forward titration, core particles were first diluted into water (as for Fig. 1, pH about 6) then adjusted to higher salt and 0.2 mM Tris in one step. The resulting forward titration then suggested a two-step process. Transition 1 was less distinct because it was shifted to higher salt (compared to the filled data in Fig. 1) and was much closer to transition 2, which again constituted the major part of the overall change in anisotropy and showed little if any shift. Thus the effect of the higher pH was to prevent nonreversible changes during the unfolding process.

The reversibility at pH 7.6 could result either from the higher pH at the time the core particles were exposed to very low salt or from the fact that the buffer required to maintain that pH restricts the minimum $[M^+]$ attainable to somewhat over 0.2 mM. To check the latter possibility the experiment in Figure 2 was done. Core particles were diluted to varying $[M^+]$ without buffer and then forward titrated to 1 mM. The results suggest that, as $[M^+]$ during unfolding is increased over the range 0.05 to 0.3 mM, the

Figure 3. Time dependence of the nonreversible step in the low-salt transition of core particles (A) and of the refolding at 1 mM (open circles) and 10 mM (filled circles) after exposure to very low salt (B). In (A) the concentrated core particle stock was diluted into water and at the indicated times aliquots were adjusted to 1 mM $[M^+]$. One hour later the tyrosine fluorescence was measured. In (B) core particles which had been diluted into water about 2 hours earlier were adjusted to either 1 or 10 mM $[M^+]$ and the tyrosine fluorescence measured at the indicated times.

proportion of core particles which undergo nonreversible changes decreases. This interpretation is supported by the observation that, when the $[M^+]$ during unfolding is increased, the positions of the two transitions remain the same with transition 1 making up a greater proportion of the total change in fluorescence intensity and anisotropy. Thus it seems likely that the reversibility at pH 7.6 is an artifact of the addition of buffer to maintain the pH. Once the core particles are exposed to sufficiently low salt, increasing the pH along with the salt concentration does not enhance refolding.

On dilution, independent of the pH, transition of core particles from the high-salt to the low-salt form occurs with a half-time of about 5 min [7]. The rate at which particles, once diluted to very low salt, undergo the transition to a nonreversible form can be determined by diluting core particles into water and then adjusting samples to 1 mM salt at various times. Results, shown in Fig. 3A, suggest a somewhat slower process with a half-time of about 15 min. In contrast, once particles have been nonreversibly unfolded at very low salt, the rate of the changes which occur when the ionic strength is increased is much faster as shown in Fig. 3B. For final salt concentrations of 1 and 10 mM the half-time for the anisotropy changes appear to be less than 1 min. Also, at 1 mM salt a secondary, very slow change in the anisotropy follows the fast change. The nature of this slow change has not been further investigated except that a forward titration like that shown in Fig. 1 (filled circles) was done and the fluorescence measured at 1 and 24 hours. The shapes of the resulting curves were very similar to those in Fig. 1A except that the proportion of the overall

Table 1. Effect of various ionic strengths, with or without exposure to very low salt, on core particle sedimentation coefficient, CD and tyrosine fluorescence.

$[M^+]_{\min}^1$ (mM)	$[M^+]_{\text{final}}^1$ (mM)	$S_{20,w}$	$[\theta]_{283}^2$	I_{tyr}	r_{tyr}
0.05	0.05	6.0	3700	1.30	.151
----	0.15	8.5	2600	1.20	.162
0.05	0.15	7.9	2900	1.24	.157
----	1.0	10.2	2000	1.06	.175
0.05	1.0	9.3 ³	2600	1.18	.160
----	10.	10.5	1900	1.00	.179
0.05	10.	10.5	2500	1.13	.178
----	100.	11.1	nd ⁴	0.91	.181

¹ $[M^+]_{\min} = 0.05$ mM indicates that the core particle stock was first diluted into water before being adjusted to $[M^+]_{\text{final}}$. Otherwise the dilution was directly to $[M^+]_{\text{final}}$.

²Units are degrees-cm²/decimole DNA phosphate.

³For core particles diluted first to 0.05 mM, then to 1 mM $[M^+]$, the sedimentation boundary was considerably broadened and gave evidence of two components with $S_{20,w}$ values of about 8 and 10 S in roughly equal amounts. The value given (9.3 S) was obtained if this multicomponent behavior was not taken into account.

⁴nd = not done.

anisotropy change making up transition 1 increased from about 30% at 1 hour to 60% at 24 hours.

Circular dichroism in the wavelength range 260-300 nm, which reflects properties of the DNA, is also sensitive to the low-salt transition; the CD signal observed near 280 nm changes on back titration in a manner very similar to the tyrosine anisotropy [13]. As indicated in Table 1, the ellipticity measured at 1 mM salt, like the tyrosine fluorescence, reflects differences in core particles depending on whether the final ionic strength is reached in a forward or back titration. CD also shows a difference at 10 mM salt depending on the path of dilution, in agreement with the tyrosine fluorescence intensity, whereas the anisotropy suggests essentially complete refolding at 10 mM. For wavelengths below 250 nm core particle CD is dominated by secondary structure of the histone component. However, no significant differences were seen in the CD between 220 and 250 nm, even when the particles were diluted to very low salt.

Table 1 also lists the affect of low salt concentrations on the sedimentation coefficient of core particles. The S value decreases from about 11 S at 100 mM ionic strength to 6 S at very low salt, suggesting dramatic changes in the particles. When core parti-

Figure 4. Effect of using a divalent cation on the low-salt transition and reversibility. (A) Anisotropy, (B) Intensity. The core particle stock was either diluted directly to the indicated $[Mg^{++}]$ (open circles) or first diluted to very low salt (~ 0.05 mM M^{+} ; filled circles) in a manner equivalent to that used for Fig. 1. (C) Onset of the nonreversibility as a function of $[Mg^{++}]$. The core particles were first diluted to an A_{260} of 1.1 and the indicated magnesium concentrations. About 1.5 hours later aliquots were mixed with 1/9 volume of magnesium chloride solution sufficient to give 10 μ M final concentration. The tyrosine fluorescence was then measured 1 hour later.

cles were forward titrated to 1 mM salt, there was evidence of two components with the estimated S value for the faster sedimenting component similar to that for particles back titrated to 1 mM; this result again suggests that transition 1 represents refolding of core particles which had not experienced nonreversible changes.

When a divalent cation is used in place of the monovalent salt, the low-salt transition is observed at a considerably lower ionic strength [7,8]. This is illustrated by the back titration in Fig. 4 (open circles). The filled circles show the effect of first diluting the core particles to very low salt before titrating the effect of $[Mg^{++}]$. Again, in a manner similar to the monovalent salt results (Fig. 1), two separate transitions are obtained; transition 2 is shifted to higher cation concentrations while transition 1 is a smaller part of the overall change and occurs over about the same range of $[Mg^{++}]$ as in the back titration. Also, in contrast to the sharp change in anisotropy centered just above 10 μ M Mg^{++} , a broad change in intensity is observed on forward titration centered near 100 μ M which again suggests two separate processes. (The behavior above 500 μ M

Mg^{++} is possibly related to the observation that Mg^{++} is capable of aggregating chromatin above 500 μM .)

The ability of low concentrations of magnesium to protect core particles from the nonreversible changes when diluted to very low salt is shown in Fig. 4C. The result is much like that for monovalent cation (Fig. 2) in that the range of $[Mg^{++}]$ over which the nonreversible changes are seen is similar to, but somewhat lower than, the range for the low-salt transition itself (Fig. 4A, open circles).

DISCUSSION

The results presented here show that when chromatin core particles are exposed to a sufficiently low ionic strength, they undergo a nonreversible transition. It remains uncertain whether a low pH near 6 is also required since the quantity of buffer needed to overcome the effects of dissolved carbon dioxide in the water raises the ionic strength enough to minimize the nonreversible effects. However, increasing the pH after exposure to very low salt does not lead to reversibility. When core particles have been exposed to the lowest salt concentrations which were used here (estimated at about 0.05 mM monovalent cation) the nonreversibility shows up by an increase in the ionic strength required to return several parameters which are affected by the low salt (sedimentation coefficient, circular dichroism of the DNA, and tyrosine fluorescence intensity and anisotropy) to near normal values.

When magnesium is used in place of a monovalent cation, the low-salt transition requires a much lower ionic strength. This result (along with the fact that a change to divalent anions such as sulfate does not affect the transition) has been taken to indicate that, in addition to effects of decreased electrostatic shielding at low salt, physical binding of cations to the core particle is involved [7,8]. Similarly, comparison of Fig. 2 and Fig. 4C leads to the conclusion that cation binding is also involved in the nonreversible transition.

The low-salt transition occurs at pH 7.6 where no nonreversible changes are indicated. Also, the apparent midpoint of the effect of salt on the onset of the nonreversible transition (Figs. 2 and 4C) is indicated at about 2-fold lower M^+ concentration and about 1.5-fold lower Mg^{++} than for the corresponding low salt transition. Thus, it is apparent that the low-salt transition and the nonreversible transition at pH 6 are not the same. It is uncertain whether the low-salt form of the core particle is an obligatory intermediate for the nonreversible changes.

The ionic strength required for the low-salt transition of core particles and nucleosomes has been examined by a number of techniques [3-11] and the results have varied rather widely [14]. Some of the disagreement could arise from the nonreversible

effects described here. Unfortunately, the experimental details provided in many cases are insufficient to determine whether the particles used might have been exposed to low enough ionic strength. The earliest studies of the effect of low salt indicated two transitions, at about 1 mM and 7 mM ionic strength [15,16]. This complexity was later attributed to the sample being a mixture of core particles and nucleosomes containing DNA longer than 170 bp with the transition at higher salt assigned to the nucleosomes [17]. However, others have found that, while the low-salt transition of nucleosomes does occur at a higher ionic strength than for core particles, the ionic strength required is about 3 mM [3,14]. Since nucleosomes experience the low salt transition at a higher ionic strength than core particles, it seems reasonable to suggest that nucleosomes may also undergo nonreversible changes like those described here at ionic strengths where core particles are relatively unaffected. Similarly, the ionic strength required to refold such nonreversibly affected nucleosomes could be higher. For core particles it seems unlikely that others have unintentionally exposed their samples to low enough salt to cause the nonreversible transition since, in those studies which included sedimentation results, the lowest *S* values reported were about 9 *S* [3-6, 15] whereas Table 1 indicates that the nonreversible effects are not prominent until the sedimentation coefficient is below 8.5 *S* ($[M^+] < 0.2 \text{ mM}$).

The low-salt transition is of particular interest because the interactions which are disrupted by the increasing electrostatic repulsion as the ionic strength is lowered are likely to be the weakest interactions involved in maintaining the structure of the core particle. When chromatin structure is modified *in vivo* in order to permit replication, transcription or basic repair processes to occur, it is very likely that the structure of the nucleosome is modified in some way. The weakest interactions within the nucleosome are likely to be the ones targeted in whatever processing is required. The nonreversible transition studied here results in metastable conformations of the core particle which are different from the normal structure and may be representative of important intermediates in the rearrangements which occur *in vivo*.

Ionic interactions within the core particle will become stronger as the ionic strength is decreased. However, the most likely mechanism to explain the nonreversibility results requires that the repulsive interactions within the molecule eventually become strong enough to break significant numbers of ionic bonds. The molecule may then rearrange and establish alternate ionic bonds. Once this has happened, the ionic strength required to labilize the new ionic interactions and allow the particle to begin returning to its most stable conformation should be considerably higher than that required to unfold and disrupt the starting structure.

ACKNOWLEDGEMENTS

This work was supported by NIH grant GM25663. We thank Dr. K.E. van Holde for permitting use of the analytical ultracentrifuge and Dr. W.C. Johnson for allowing use of the CD spectrophotometer.

REFERENCES

1. McGhee, J.D. and Felsenfeld G., *Ann. Rev. Biochem.* **49**, 1115-1156 (1980).
2. Johns, E.W. (ed.) (1982) *The HMG Chromosomal Proteins*, Academic Press, New York.
3. Burch, J.B.E. and Martinson, H.G., *Nucleic Acids Res.* **8**, 4969-4987 (1980).
4. Simpson, R.T., *Proc. Natl. Acad. Sci. USA* **78**, 6803-6807 (1981).
5. Martinson, H.G., True, R.J., and Burch, J.B.E., *Biochem.* **18**, 1082-1089 (1979).
6. Libertini, L.J. and Small, E.W., *Nucleic Acids Res.* **8**, 3517-3534 (1980).
7. Libertini, L.J. and Small, E.W., *Biochem.* **21**, 3327-3334 (1982).
8. Wu, H.-W., Dattagupta, N., Hogan, M. and Crothers, D.M., *Biochemistry* **18**, 3960-3965 (1979).
9. Hantz, E., Cao, A., Taillandier, E., Tivant, P., Drifford M., Defer, N., and Kruh, J., *Int. J. Biol. Macromol.* **5**, 130-134 (1983).
10. Mita, K., Zama, M., Ichimura, S., Niimura, N., Kaji, K., Hirai, M. and Ishikawa, Y., *Physica* **120B**, 436-439 (1983).
11. Burch, J.B.E. and Martinson, H.G., *Nucleic Acids Res.* **9**, 4367-4385 (1981).
12. Dieterich, A.E., Eshaghpour, H., Crothers, D.M. and Cantor, C.R., *Nucleic Acids Res.* **8**, 2475-2487 (1980).
13. Libertini, L.J., unpublished observations.
14. Schlessinger, F.B., Dattagupta, N., and Crothers, D.M., *Biochem.* **21**, 664-669 (1982).
15. Gordon, V.C., Knobler, C.M., Olins, D.E., and Schumaker, V.N., *Proc. Natl. Acad. Sci. USA* **75**, 660-663 (1978).
16. Harrington, R.E., *Biopolymers* **20**, 719-752 (1981).
17. Harrington, R.E., *Biochem.* **21**, 1177-1186 (1982).