The Switch in the Helical Handedness of the Histone (H3-H4)$_2$
Tetramer within a Nucleoprotein Particle Requires a Reorientation
of the H3-H3 Interface*

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It has recently been proposed that the histone (H3-H4)$_2$ tetramer undergoes structural changes, which allow the particle to accommodate both negatively and positively constrained DNA. To investigate this process, we modified histone H3 at the H3-H3 interface, within the histone (H2A-H2B-H3-H4)$_2$ octamer or the histone (H3-H4)$_2$ tetramer, by forming adducts on the single cysteine of duck histone H3. We used three sulfhydryl reagents, iodoacetamide, N-ethylmaleimide, and 5,5'-dithiobis(2-nitrobenzoic acid). Torsionally constrained DNA was assembled on the modified histones. The H3 adducts, which have no effect on the structure of the nucleosome, dramatically affected the structural transition that the (H3-H4)$_2$ tetrameric nucleoprotein particle can undergo. Iodoacetamide and N-ethylmaleimide treatment prevented the assembly of positively constrained DNA on the tetrameric particle, whereas 5,5'-dithiobis(2-nitrobenzoic acid) treatment strongly favored it. Determination of DNA topoisomer equilibrium after relaxation of the tetrameric nucleoprotein particles with topoisomerase I demonstrated that the structural transition occurs without histone dissociation. Incorporation of H2A-H2B dimers into the tetrameric particle containing modified or unmodified cysteines allowed nucleosomes to reform and blocked the structural transition of the particle. We demonstrate the importance of the histone H3-H3 contact region in the conformational changes of the histone tetramer nucleoprotein particle and the role of H2A-H2B in preventing a structural transition of the nucleosome.

The nucleosome, the repeated structural unit of chromatin, consists of two each of the core histones H2A, H2B, H3, and H4 (building up an “octamer”), around which is wrapped 146 bp$^1$ of DNA in a left-handed superhelix (1). Several lines of evidence have suggested that the arginine-rich histones H3 and H4, which interact in solution to form a stable (H3-H4)$_2$ tetramer, play an important role in the establishment of nucleosomal structure (2–5). The two H2A-H2B dimers complete the nucleosome by binding on both sides of the (H3-H4)$_2$-DNA structure (6). The interaction of the histone H2A-H2B dimer with the histone (H3-H4)$_2$ tetramer involves mostly hydrogen bonds and is significantly weaker than the interaction between H3 and H4 within the tetramer. This supports the model of a tripartite organization of the core histone octamer, the assembly of which is governed by positive cooperativity and reversibility (7, 8). In addition, histone H2A-H2B dimers are more easily released and interchanged from chromatin than (H3-H4)$_2$ tetramers (9); the lability of the H2A-H2B dimer interaction within the nucleosome appears to additionally depend on RNA polymerase activity (10–12).

Posttranslational modifications of the core histones, such as ubiquitination, methylation, phosphorylation, ADP-ribosylation, and acetylation, probably result in subtle changes in nucleosome structure. Among these modifications, the reversible acetylation of ε-amino groups of lysine residues present in the amino-terminal domains, or “tails,” of the core histones, is the most strongly linked with transcriptional activity. Certain transcriptional coactivators have acetyltransferase activity, and they can be targeted to specific promoters by transcription factors (13–15). More recently, it has been demonstrated that histone deacetylase can also be targeted by transcriptional repressors (reviewed in Ref. 16). Targeted histone acetylation/deacetylation is likely to play a major role in inducing nucleosome structural changes, thus allowing transcription complex assembly/disassembly and/or RNA polymerase tracking.

The structural complexity of the core histones makes the nucleosome a master molecule that governs biochemical processes implicating chromatin. Mechanisms that modify the conformation of chromatin in living cells are of considerable interest because they are likely to determine the efficiencies of transcription, replication, recombination, and DNA repair. Gene activation is accompanied by alterations in the conformation of chromatin, which for highly transcribed genes are revealed by an increased sensitivity to cleavage by nucleases. The nature of these alterations at the level of nucleosomal structure is not known, but it is clear that both histone-DNA interactions and histone content are modified. One possible way to spread alterations along a chromatin fiber is through the torsional stress generated by RNA polymerase during transcription (17, 18). Garrard and co-workers (19, 20) have proposed that transient positive supercoils downstream of a tracking RNA polymerase result in nucleosome splitting. A similar conclusion was reached from an investigation of the influence of positive stress on nucleosome assembly (9, 21, 22). Recently, it was also shown that the histone (H3-H4)$_2$ tetramer can associate with positively supercoiled DNA with a high affinity, probably as a result of a change in tetramer topology (23). Based on these observations, it has been proposed that a slight shift in the dimer-dimer interface may be all that is needed for the DNA to wrap the (H3-H4)$_2$ tetramer in a right-handed helix (24).

To address the question of how the (H3-H4)$_2$ tetramer can
undergo a structural change allowing it to accommodate either negatively or positively supercoiled DNA, we modified the cysteine located at position 110 within the H3 molecule. X-ray crystallography studies have demonstrated that modifications of this cysteine do not change the histone structure (25, 26). Because the two cysteines face each other at a distance of 6.2 Å, this should create steric hindrances at the H3-H3 interface within the (H3-H4)2 tetramer. Adducts on the cysteine were formed by treating histone octamers and (H3-H4)2 tetramers with three different sulfhydryl reagents. We used these treated histones to reconstitute nucleoprotein particles on DNA minicircles of negative and positive superhelicities. These experiments, together with an analysis of the topoisomerase I relaxation of the particles, demonstrate that the (H3-H4)2 tetramer can flip from the left-handed conformation it adopts within the nucleosome to a right-handed conformation. This flipping requires a reorientation of the H3-H3 interface, because adducts on cysteine 110 allow the freezing of the tetrameric particle in left- or right-handed conformations. The presence of histones H2A-H2B prevents this transition by blocking the tetramer in a left-handed structure. Such a conformational flexibility of the (H3-H4)2 tetramer could provide a fine-tuned mechanism for modulating the accessibility of DNA sequences within the nucleosome.

MATERIALS AND METHODS

Preparation of Topologically Constrained Mini-circles

The murine mammary tumor virus-long terminal repeat 359-bp fragment used to generate mini-circles spans the promoter from −280 to +72. It was amplified by polymerase chain reaction using the following primers, which contain an EcoRI site: primer 1, 5′-GGCAATTCTAGAAGATTATCGAAA-3′, and primer virus 2, 5′-GGGATTCGACGATCCGTGACGAGCGGAGA-3′. The resulting DNA fragment was digested with EcoRI and fractionated by electrophoresis on polyacrylamide gels. The 359-bp band was eluted from the gel. The DNA fragment was ligated to linearized dephosphorylated pUC18 under conditions whereby two tandemly repeated fragments were inserted in the vector (construct pUC359.2m). Negatively and positively supercoiled DNA topoisomers were prepared from the above fragment, after purification by gel electrophoresis and 32P end-labeling by circularization in the presence of ethidium bromide and netropsin, respectively. The different DNA topoisomers were separated by electrophoresis on acrylamide gels, the bands were excised, and the DNA was eluted (27).

Histone Purification

Duck erythrocyte nuclei were prepared according to the method described in Ref. 28, and histone octamers (1–2 mg/ml) were prepared as described previously (29). Long soluble chromatin obtained from micrococcal nuclease digestion of nuclei was adsorbed onto a hydroxyapatite column as described in Ref. 30. Non-histone proteins histone H1 and histone H3, which are eluted first, and the core histones were eluted second by sequential washes with 0.65 M and 2 M NaCl (in 0.1 M potassium phosphate, pH 7.2, 0.25 mM phenylmethylsulfonyl fluoride, and 1 mM 2-mercaptoethanol), respectively. To isolate H2A-H2B and H3-H4 histones separately, after the 0.65 M NaCl wash, a stepwise elution with 0.93 M NaCl allowed recovery of most of the H2A-H2B histone dimers without any contamination with (H3-H4)2 histone tetramers. Samples were dialyzed against 2 M NaCl, 10 mM potassium phosphate (pH 7.6), and 0.25 mM phenylmethylsulfonyl fluoride. Histone H2A-H2B dimers and histone (H3-H4)2 tetramers were concentrated by centrifugation in Centricon-10 and Centricon-30 microconcentrators, respectively. Histone preparations were aliquoted and stored at −80 °C until use.

Treatment of Histones with Sulphydryl Reagents

5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) Treatment—DTNB was prepared as a 10 mM stock solution in 100 mM potassium phosphate (pH 8.0). Histone octamers and (H3-H4)2 tetramers in 10 mM potassium phosphate (pH 7.6), 2 mM NaCl (in a final thiol group concentration of 30 mM) were incubated with 0.25 mM DTNB under the conditions described under “Results.” The reaction was followed by recording absorbance at 412 nm. Product yield was calculated from its extinction coefficient, ε412 = 13.6 mM−1 cm−1 (Ellman’s reagent; Ref. 31). Treated histone octamers and tetramers were dialyzed to remove the excess of reagent and stored at −80 °C.

N-Ethylmaleimide (NEM) Treatment—NEM was prepared as a 1 M stock solution in methanol. Histones were treated at room temperature with 1 mM NEM in 2 mM NaCl and 10 mM phosphate buffer, pH 7.6. Histones were added directly to this solution in a 1-mL cuvette, and the reaction was followed by measuring the decrease of the absorbance at 305 nm (32). The extinction coefficient value ε305 = 0.620 mM−1 was used for the calculations of reaction rate.

Iodoacetamide (IA) Treatment—IA was prepared as a 1 M stock solution in water. Histones were treated with 1 mM IA for 2–3 h at room temperature.

Chromatin Reconstitution

To generate nucleoprotein particles, histone octamers or tetramers were assembled on topologically constrained DNA circles according to the “salt jump” method (33) as described in Ref. 29. The procedure involved the addition of 32P-labeled DNA topoisomer to the plasmid DNA (form I) from which the fragment originated (final DNA concentration, 200 mg/ml), followed by the suitable amount of histones (histone/DNA weight ratio = r, in 2 mM NaCl, 10 mM Tris-HCl (pH 7.5), 100 mg/ml bovine serum albumin. The mixture was first incubated for 10 min at 37 °C, diluted to 0.5 mg/ml DNA and 0.5 mM NaCl, incubated at the same temperature for 30 min, and finally dialyzed at 4 °C against 10 mM Tris-HCl (pH 7.5), 1 mM EDTA for at least 1.5 h. For the experiments involving measurement of the DNA topoisomer:histone ratio (two-dimensional gel electrophoresis), nonradioactive topoisomer was substituted to the carrier plasmid DNA.

Assembly of histones H2A-H2B on tetrameric nucleoprotein particles was performed as follows: increasing amounts of purified H2A-H2B dimers were added to particle preparations; adjusted to 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 50 mM KCl, 5 mM MgCl2, 100 mM bovine serum albumin, diluted to 250 mg/ml DNA (form II), in 0.15 M NaCl, 0.1 M Tris-HCl, pH 7.5, 0.1% bovine serum albumin, and the DNA was relaxed by incubation with 800–1000 units of calf thymus topoisomerase I (Life Technologies, Inc.) per ml at 37 °C for 1 h (34).

Gel Electrophoresis

Octameric and tetrameric nucleoprotein particles were electrophoresed at room temperature in 4% polyacrylamide (acrylamide/bisacrylamide, 29:1, w/v) slab gels (15 × 17 × 8 cm) in TE buffer (10 mM Tris base, 1 mM EDTA). Gels were pre-electrophoresed for 1 h at 200–250 V and electrophoresed at the same voltage for 3–4 h with extensive buffer recirculation. Gels were dried and autoradiographed at −80 °C. For the two-dimensional gel analysis, after the first dimension run in TE buffer (see above), the bands were excised an loaded onto a second dimension 18% acrylamide denaturing gel (35). DNA and histones were visualized by silver staining (36). In preparative relaxation experiments, “chromatin” gels were dried without heating to allow the reswelling of the excised gel slices and the elution of the DNA. Unless otherwise stated, naked DNA was electrophoresed at room temperature in 4% polyacrylamide (acrylamide/bisacrylamide, 20:1, w/v) slab mini-gels (0.15 × 10 × 8 cm) for 2 h at 100 V in 20 mM sodium acetate, 2 mM EDTA, 40 mM Tris acetate (pH 7.8). When required for the separation of DNA topoisomers, chlorelcine (125 μM) was included. The radioactivity in the bands was quantitated in the dried gels using a phosphorimager (Fuji PC-Bas).

Topoisomer Identification, Linking Number Reduction Associated with Single Nucleoprotein Particle Formation; Topological Constraint in the Loop

Topoisomer linking difference relative to its most probable configuration is given by the equation,

\[ \Delta \text{Lk} = \text{Lk} - \text{Lk}_0 \]  

(Eq. 1)

in which Lk and Lk0 are the linking numbers of the topoisomer and of its most probable configuration, respectively (37). \( \Delta \text{Lk} \) is also known as the constraint (38) or as the number of titratable superhelical turns (39). Lk0 is the linking number at the center of the topoisomers equilibrium distribution, obtained upon DNA relaxation. It can be estimated, more conveniently, from the helical periodicity of the DNA, h0, through the equation,
where \( N \) is the ring size \((40)\). 

\[ \Delta L_k = \Delta L_{k0} + \frac{1}{2} \mu \rho \Delta L_k \]  

(Eq. 5)

in which \( \Delta L_k \) and \( \Delta L_{k0} \) are given by Eqs. 1 and 3. This implies that the loop is an independent topological domain of constant size delimited by the clamping of DNA to the histones.

**RESULTS**

**Titration of Histone H3 Cysteine within Histone Tetramers or Octamers with Sulphydryl Reagents**—We have used sulphydryl reagents to investigate the structure and the dynamics of the nucleosome. Titration of the unique histone H3 cysteine by DTNB has been reported \((43)\). The authors of that study concluded that cysteine accessibility is different in histone (H3-H4)\textsubscript{2} tetramers (two cysteines, corresponding to the two H2 molecules, are titrated) than in histone tetramers (only one cysteine was titrated). To ensure that under our experimental conditions the two cysteines were reacted with DTNB in both histone tetramers and octamers, we treated them with DTNB at 25 °C for different periods of time in the presence of 2 M NaCl to preserve their oligomeric structure. Fig. 1 presents the result of such an experiment. The two histone H3 cysteines were rapidly titrated by DTNB within the histone tetramer. In contrast, within histone octamers, titration of one and two cysteines was achieved only after a treatment time of 25–30 min and 2–3 h, respectively. During that time, spontaneous hydrolysis of the DTNB was negligible. To confirm that the maximal absorbance reflected a complete reaction of the two cysteines with DTNB, we performed the following controls. After each reaction had reached its maximal value, an aliquot of the mixture was brought to a final concentration of 5 M urea to allow complete accessibility of the cysteines and incubated for 10 min at 37 °C with DTNB, and the absorbance was measured. There was no increase in the absorbance, compared with the samples untreated with urea. This allowed us to conclude that the two cysteines were titrated in both histone tetramers and octamers. In addition, samples containing untreated histone tetramers or octamers were brought to a final concentration of 5 M urea and then incubated for 15 min at 37 °C with DTNB, and the absorbance of the samples was measured. This absorbance was identical to that obtained in the previous experiment. Taken together, these results demonstrate that providing the reaction is left to proceed long enough, DTNB can react with the two histone H3 cysteines in both the histone tetramer and octamer. The same results were obtained for the titration of the cysteines with N-ethylmaleimide (not shown).

**Influence of IA, NEM, or DTNB Cysteine Adducts on Nucleoprotein Particle Assembly**—We used torsionally constrained DNA mini-circles to reconstitute nucleoprotein particles on histone octamers or tetramers that were treated or not treated with sulphydryl reagents. Fig. 2 presents the results of an experiment in which histone octamers were treated with either NEM, IA, or DTNB. The DNA topoisomers used to reconstitute a nucleosome had a linking number difference of \( \Delta L_k = -1 \) (Fig. 2, lanes 1–5) or +1 (lanes 6–10). Lanes 1 and 6 show the naked DNA. Lanes 2 and 3 demonstrate the assembly of the untreated histone octamer particle on topoisomerase I, resulting mainly in the formation of nucleosomes \((O_p)\) but also in the formation of some dinucleosomes \((D_i)\). The amount of dinucleosomes assembled with the mini-circles increased with the increase of the histone:DNA ratio. The different dinucleosome bands correspond to different positions of the two nucleosomes on the DNA. Treatment of the histone octamers with the sulphydryl reagents \((Fig. 2, lanes 4–5)\) did not prevent the assembly of the nucleosome on DNA topoisomerase I. However, the formation of dinucleosomes was completely abolished, independent of the reagent used, even at the highest histone:DNA ratio. Lanes 7 and 8 show the assembly of the DNA topoisomer +1 on histone octamers. The migration of the DNA-protein complex was slower than that of the naked circle and corresponds to a histone tetrameric particle, illustrating the previously described release of H2A-H2B from histone octamers upon assembly on positively supercoiled DNA \((23)\). In contrast, histone octamers treated with the sulphydryl reagents were unable to assemble with positively supercoiled mini-circles. Reaction of the cysteine with these reagents probably induces a conformational change of the histone (H3-H4)\textsubscript{2} tetramer that does not allow it to accommodate positively supercoiled DNA.

To test this hypothesis, negatively or positively supercoiled DNA mini-circles were assembled on (H3-H4), tetramers treated with these reagents. Fig. 3 shows the result of such an experiment. Lanes 2, 3, 7, and 8 demonstrate that untreated histone tetramers efficiently assemble on either negatively \((lanes 2 and 3)\) or positively \((lanes 7 and 8)\) constrained DNA, resulting in the formation of tetrameric nucleoprotein particles \((T_p)\). Histone tetramers treated with sulphydryl reagents were able to associate on negatively constrained circles \((lanes 4 and 5)\). However, these tetrameric particles migrated faster than
the control particles, suggesting differences in conformation and/or composition. As observed for the histone octamer, positively supercoiled DNA was unable to assemble on histone tetramers treated with either NEM or IA (Fig. 3, NEM and IA, lanes 9 and 10). Two-dimensional gel electrophoresis allowed us to confirm that the band migrating at the same position as DNA topoisomer 1 (lanes 1-5) or +1 (lanes 6-10) was assembled on histone octamers untreated (lanes 2, 3, 7, and 8) or treated with sulfhydryl reagents (lanes 4, 5, 9, and 10). Lanes 1 and 6, naked DNA topoisomers −1 and +1, respectively. OC, open circles; OP, and TP, circles with an octameric and a tetrameric particle, respectively; Di, circles with dinucleosomes.

It was intriguing that histone tetramers treated with NEM or IA associated exclusively with negatively constrained DNA, whereas histone tetramers treated with DTNB associated with both negatively and positively constrained DNA. In addition, as pointed out above, in the case of the negatively constrained circles, the tetrameric nucleoprotein particles migrated faster than the control ones, suggesting an altered conformation and/or histone composition. To investigate the possibility of a change in histone H3 structure induced by the formation of a DTNB adduct on cysteine 110, we compared the behavior of tetrameric nucleoprotein particles containing either one or two modified cysteines (Fig. 1). To obtain such tetramers containing a single modified cysteine, histone octamers were treated with DTNB, because cysteine titration within a histone octamer is much slower than in a tetramer, allowing us to stop the reaction after the titration of either one or two cysteines. Histone H2A-H2B were dissociated and histone tetramers purified. The resulting histone tetramers could be either mostly tetramers with one cysteine adduct or a mixture of tetramers containing zero, one, or two cysteine adducts. In the second hypothesis, after assembly of these treated histone tetramers with DNA topoisomer −1, the particle population containing two adducts per molecule should be visualized, after electrophoresis, by the presence of a fast migrating band. This band was not present (compare the positions of the tetrameric particles in Fig. 4), demonstrating the homogeneity of the tetrameric particles that contain one DTNB adduct. In contrast,
The already described increased electrophoretic mobility of the particles containing two modified cysteines, assembled on negatively constrained mini-circles, was observed (Fig. 4, lane 3).

The particles containing only one modified cysteine displayed a migration pattern indistinguishable from that of the control particles (Fig. 4, compare lanes 2 and 4). If the reaction with DTNB induces a change in histone H3 conformation, the electrophoretic mobility of tetrameric particles containing one cysteine adduct/particle should be altered. Our data support the fact that treatment of histone H3 with DTNB does not change the histone H3 structure, as inferred from the x-ray crystallography data and that the change in tetrameric nucleoprotein particle electrophoretic mobility requires the presence of two DTNB adducts.

To investigate further the mechanisms involved in this change, we compared the sucrose gradient sedimentation profiles of tetrameric nucleoprotein particles containing untreated or modified histones. The two profiles were indistinguishable, suggesting that the histone composition of the particles was identical (not shown).

To confirm the identity of this tetrameric particle, we analyzed it by two-dimensional gel electrophoresis. Fig. 5A presents the first dimension gel. The particle containing DTNB-treated histones has an increased mobility compared with the control (Fig. 5A, compare lanes 4 and 2). The removal of the cysteine adducts by treatment of the nucleoprotein particle with 5 mM dithiothreitol for 1 h at 37 °C allowed recovering an electrophoretic mobility identical to that of the control particles (Fig. 5A, compare lanes 3 and 4). The bands corresponding to the nucleoprotein particle were excised from lanes 2 and 4 were loaded on a second dimension denaturing gel. Fig. 5B presents the silver-stained gel showing both DNA and histones. Comparison of lanes 2 and 3 demonstrates that the DNA/histone stoichiometry is the same for particles containing untreated or DTNB-treated histone H3-H4 tetramers, assembled on DNA topoisomer –1. This demonstrates that the DTNB-induced change in particle electrophoretic mobility is not due to a modification of its protein composition. It allows excluding the stacking of two (H3-H4)2 tetramers in the particles containing DTNB-treated histones.

To investigate further the changes in tetrameric particle structure induced by DTNB treatment of the histones, we asked whether such particles could support the subsequent formation of octameric particles. Nucleosomes were reconstituted by incorporating histones H2A-H2B into these tetrameric particles, which displayed altered electrophoretic mobility. Fig. 6 presents the results of this experiment. Lanes 2 and 3 are controls in which DNA topoisomer –1 was assembled on untreated (lane 2) and DTNB-treated (lane 3) histone octamers. Regardless the presence of DTNB adducts on cysteines, the octameric particles display the same electrophoretic mobility. DNA topoisomer –1 was assembled on histone (H3-H4)2 tetramers that were untreated (lane 4) and DTNB-treated (lane 5). As shown in Figs. 3 and 4, the tetrameric nucleoprotein particles containing DTNB-treated histones migrated faster than those in the untreated control. A faint band just underneath the naked DNA band was identified as a nucleoprotein particle containing a histone hexamer, on the basis of its histone composition as analyzed by two-dimensional electrophoresis (not shown). The formation of this hexameric particle was due to a slight contamination of the histone (H3-H4)2 tetramer preparation used in this experiment with histone H2A-H2B dimers. Increasing amounts of purified H2A-H2B were added to the samples containing the DTNB-treated tetrameric nucleoprotein particles (lanes 6–9). The addition of 1 mol of H2A-H2B dimer per mol of tetrameric particle resulted in the simultaneous formation of hexameric and octameric nucleoprotein particles (lane 6). The hexameric particle was completely converted into its octameric counterpart when the amount of H2A-
H2B dimer was increased over 2 mol/mol of tetrameric particle. These results confirm that the faster migrating band obtained with sulfhydryl-treated histones is a tetrameric nucleoprotein particle, the histone composition of which is unaltered. This is evidenced by its ability to assemble into an octameric particle that displays the same electrophoretic mobility as native nucleosomes. This change in electrophoretic behavior arises from differences in particle flexibility rather than from histone-conformation change. The flexible control tetrameric particles are retarded, whereas the more rigid sulfhydryl reagent-treated particles migrate faster. Reassociation of H2A-H2B dimers on control or modified particles allows reforming nucleosomes with identical electrophoretic mobility. Restricting the tetrameric particle flexibility by increasing the torsional stress in the loop (see particle reconstitution on topoisomer −2 in Fig. 8) also abolishes the electrophoretic migration differences between untreated and treated particles.

Topological Analysis of the DNA within Particles Containing Histones Modified with Sulfhydryl Reagents.—The wrapping of closed circular DNA on a histone surface, followed by relaxation of unbound DNA with topoisomerase I and removal of proteins, produces a characteristic DNA linking deficiency, ΔLk. The determination of this value provides information on DNA structural changes due to its association with a protein. Consequently, changes in the protein conformation will provoke a modification of the torsional stress of the DNA. We used this approach to elucidate the nature of the conformation changes in octameric and tetrameric nucleoprotein particles reconstituted in the presence of histone H3 treated or not treated with sulfhydryl reagents.

Fig. 7A presents the results of an experiment in which DNA topoisomerase −3 was assembled on histone octamers, treated or not treated with the sulfhydryl reagents, to form nucleosomes (lanes 3, 5, 7, and 9). We selected DNA topoisomerase −3 because it is absent from the topoisomer equilibrium produced after relaxation by topoisomerase I of the DNA within the particle.

Its complete disappearance demonstrates that relaxation of the DNA is complete. In Fig. 7A, lanes 1–3 are control lanes showing particles assembled with DNA circles of different negative supercoiling used as markers. Treatment with topoisomerase I of particles containing histones treated or not treated with sulfhydryl reagents relaxed the torsional stress of the DNA within the loop. This resulted in a change in the migration pattern of the octameric particles regardless of whether histone H3 was treated or not treated with sulfhydryl reagents. After topoisomerase I treatment, all the particles migrated as octameric particles assembled on DNA topoisomer −1 (compare lanes 4, 6, 8, and 10 to lane 1). This suggests strongly that the conformation of the protein moiety of the octameric particle was unchanged, whether or not histones were treated with...
TABLE I
Lk^p of octameric and tetrameric particles

| SH reagents       | Starting topoisomer | ΔLk^p |
|-------------------|---------------------|-------|
| Octameric particles | None                | −3    |
|                   | −1.15 (±0.03)       |       |
|                   | n = 3               |       |
|                   | NEM                 | −3    |
|                   | −1.16 (±0.03)       |       |
|                   | n = 3               |       |
|                   | IA                  | −3    |
|                   | −1.16 (±0.03)       |       |
|                   | n = 3               |       |
|                   | DTNB                | −2    |
|                   | 1.15 (±0.03)        |       |
|                   | n = 3               |       |
| Tetrameric particles | None                | −2    |
|                   | −0.65 (±0.05)       |       |
|                   | n = 3               |       |
|                   | NEM                 | −1    |
|                   | −0.65 (±0.05)       |       |
|                   | n = 3               |       |
|                   | IA                  | −2    |
|                   | −0.99 (±0.00)       |       |
|                   | n = 3               |       |
|                   | DTNB                | −2    |
|                   | +0.15 (±0.05)       |       |
|                   | n = 3               |       |
|                   | DTNB                | +1    |
|                   | +0.85 (±0.02)       |       |
|                   | n = 3               |       |

sulphhydryl reagents. To ascertain it, the particle linking number difference ΔLk^p was determined. The bands corresponding to the octameric particles (Fig. 7A, lanes 4, 6, 8, and 10) were excised from the gel, the DNA was eluted and purified, and the topoisomer distribution was analyzed (Fig. 7B). It was the same for all samples (Fig. 7A, lanes 4–7), with 94% of topoisomer −1 and 6% of topoisomer −2. The quantification of this DNA topoisomer distribution allowed us to calculate the ΔLk^p for the particle (Table I). This ΔLk^p was the same for all samples (−1.15), demonstrating that histone treatment with sulphhydryl reagents does not result in an alteration of the structure of the nucleosome.

The same experiment was performed on histone tetramer nucleoprotein particles, treated or not treated with sulphhydryl reagents assembled on negative or positive DNA topoisomers. We selected DNA topoisomer −2 (Fig. 8A) because it is absent from the final topoisomer equilibrium after DNA relaxation and +1 (Fig. 8C). Fig. 8A, lanes 3, 5, 7, and 9, and Fig. 8C, lanes 2 and 4, show the migration pattern of the DNA samples after the assembly of tetrameric particles. In contrast with the data presented in Fig. 3, obtained with DNA circles less constrained (topoisomer −1), the electrophoretic profiles of the particles assembled on DNA topoisomer −2 were similar whether or not histones were treated with sulphhydryl reagents. After relaxation of the DNA with topoisomerase I, the circles bearing control tetrameric particles (Fig. 8A, lane 4, and Fig. 8C, lane 3) migrated as particles reconstituted on DNA topoisomer −1 or +1. After DNA relaxation, the particles assembled on DNA topoisomer −2 containing histones treated with sulphhydryl reagents migrated at the same positions, but the bands were more disperse (Fig. 8A, lanes 6, 8, and 10). After DNA relaxation, in contrast with the control particles, the particles containing DTNB-treated histones assembled on DNA topoisomer +1 migrated as tetrameric particles assembled on DNA topoisomer +1 (Fig. 8C, lane 5). The bands (including the smeary area) corresponding to the relaxed particles were cut out from the gel, the DNAs were purified, the topoisomers distribution was analyzed (Fig. 8, B and D), and the ΔLk^p values were calculated (Table I). The DNA from the control sample (Fig. 8, B and D, lanes 5) displayed predominantly negative supercoiling (75% of DNA topoisomer −1), with DNA topoisomers 0 (5%) and +1 (20%) also present. As expected for a 359-bp DNA fragment, ΔLk^p was −0.65. For the NEM and IA samples (Fig. 8B, lanes 7 and 8) only topoisomer −1 (100%) was present, and the ΔLk^p was −1.09. This reflects the fact that the majority of these particles adopt a left-handed conformation, accommodating one negative topological turn. For the DTNB-treated particles, after relaxation with topoisomerase I, the topoisomer equilibria were different depending on the torsional stress of the DNA assembled on the (H3-H4)2 tetramers. For particles assembled on topoisomer −2 (Fig. 8B, lane 6) the predominant topoisomer was +1 (61%), with some topoisomer −1 (37%), and the ΔLk^p was +0.15. For particles assembled on topoisomer +1 (Fig. 8D, lane 6), mostly topoisomer +1 was present, with only trace amounts of topoisomers 0 and −1, and ΔLk^p was +0.85. This positive ΔLk^p value indicates that most of these particles adopt a right-handed conformation. As discussed below, we interpret the differences in ΔLk^p values calculated for DTNB-treated histone tetramers assembled on negatively or positively supercoiled DNA as a result of interactions of the nitrobenzoic moieties in different configurations. When they are in close vicinity, nitrobenzoic adducts should interact strongly through dipolar interactions, forming a head to tail stack. Such strong interactions could be favored when the particle is in a right-handed conformation and prevent the re-formation of the left-handed one.

DISCUSSION

Previous studies have shown that the (H3-H4)2 tetramer can accommodate both negative and positive DNA supercoiling (23). This may be due to a change from the left- to the right-handed conformation in the horsehoe structure of the tetramer, resulting from changes in contacts at the H3-H3 interface (the contact area between the two halves of the horsehoe).

To investigate the equilibrium between the two forms of the tetramer, sulphhydryl reagents were used to trap these different conformations. Histone H3 was treated with three different reagents to form adducts on the single cysteine located at position 110. We used it to reconstitute a mononucleosome or a tetrameric particle on topologically constrained DNA mini-circles. We monitored structural changes of these particles in response to variations in DNA supercoiling. Modification of histone H3 by the sulphhydryl reagents had no effect on the nucleosomal structure because the ΔLk^p value (−1.15) was the same as for the control particle. In contrast, these modifications had a dramatic effect on the flexibility of the (H3-H4)2 tetrameric nucleoprotein particle. The DNA topoisomer equilibrium resulting from the relaxation with topoisomerase I of untreated tetrameric particles includes three adjacent DNA topoisomers (topoisomer −1, 0, and +1). In contrast, the equilibrium obtained under similar conditions with mononucleosomes and naked DNA includes only two topoisomers. This demonstrates that histone tetramer interaction with DNA strongly accentuates DNA thermal fluctuations. In this case, the DNA topoisomer equilibrium deviates significantly from the Gaussian distribution normally expected upon DNA relaxation (44, 45). It is especially striking for the topoisomer distribution obtained after relaxation of the tetrameric particle assembled with DNA topoisomer −2. At equilibrium, topoisomer 0 was only a minor component; topoisomers −1 and +1 were the major ones (ΔLk^p = −0.65). We interpret this as the result of the tetrameric nucleoprotein particle oscillation between two conformations, one stable left-handed conformation (represented by topoisomer −1) and a metastable right-handed one (represented by topoisomer +1). The predominance of negative topoisomers in the relaxation equilibrium indicates that the left-handed conformation of the tetrameric nucleoprotein particle is the most stable. At the midpoint between the two conformations, the tetramer is expected to adopt, at least transiently, a flat conformation with no writhing (revealed by a trace of the relaxed DNA topoisomer 0). The same result was obtained using topoisomer +1 instead of topoisomer −2 as a starting topoisomer. As expected, for untreated particles, ΔLk^p did not depend on the starting topoisomer.

In contrast, for tetrameric particles containing DTNB-
treated histone H3, after relaxation, the final DNA topoisomer was strongly dependent on the starting topoisomer. Relaxation of tetrameric particles containing DTNB-treated histones reconstituted on DNA topoisomer $-2$ generated DNA topoisomers $+1$ (61%) and $-1$ (37%), with a $\Delta Lk^{p}$ value of $+0.15$. Although this $\Delta Lk^{p}$ value is close to zero, it does not indicate the absence of writhing in such particles, because a flat conformation of the tetramer should be represented by topoisomer 0. The absence of topoisomer 0 from the final relaxation equilibrium is due to the lack of affinity of the DTNB-treated tetramers for relaxed DNA (data not shown). This $\Delta Lk^{p}$ value reflects the slight prevalence of the right-handed conformation over the left-handed one and the absence of the flat one. In contrast, relaxation of tetrameric particles containing DTNB-treated histones reconstituted on DNA topoisomer $+1$ generated only DNA topoisomer $+1$, resulting in a higher positive $\Delta Lk^{p}$ value ($+0.85$). This indicates that for tetrameric nucleoprotein particles containing DTNB-treated histones assembled on positively supercoiled DNA, the more stable conformation is the right-handed one. This dependence of $\Delta Lk^{p}$ on the starting topoisomer can be explained by different interactions of the two nitrobenzoic acid modifiers arising from changes in protein topology, rather than by a change in histone H3 structure. This hypothesis is supported by the fact that tetrameric nucleoprotein particles containing only one DTNB adduct per histone tetramer are indistinguishable from untreated ones. Similar constraints have been described for histone (H3-H4)$_2$ tetramers treated with N-pyrene maleimide (46). An increase in excimer fluorescence intensity of N-pyrene maleimide-treated tetramers was observed when H2A-H2B dimers were added simultaneously to linear DNA to assemble nucleosomes. This reflects the interaction through stacking of the two N-pyrene maleimide molecules. The excimer fluorescence was reduced by one-half when H2A-H2B dimers were not present, and a dead-end complex DNA-histone (H3-H4)$_2$ tetramer was produced. Such a structure precluded the subsequent incorporation of H2A-H2B, suggesting a change in histone tetramer conformation. We propose that such particles are in a right-handed conformation, as are DTNB-treated tetramers.

An extreme situation was seen with tetrameric particles containing NEM or IA-treated histones that relaxed completely into DNA topoisomer $-1$ with a negative $\Delta Lk^{p}$ value ($-1.09$). This value reflects the lack of affinity of such a tetramer not only for topoisomer $+1$ but also for topoisomer 0, suggesting that it is locked in the left-handed conformation. The possibility that in these particles, two (H3-H4)$_2$ tetramers were stacked, allowing them to wrap twice as much DNA as unmodified particles, was excluded by determining protein/DNA stoichiometry and analyzing the sucrose sedimentation profile. In addition, as described here for the DTNB-modified tetramers, NEM- and IA-treated histone tetramers were able to incorporate H2A-H2B dimers and nucleate into a normal mononucleosome (data not shown).

Histone (H3-H4)$_2$ tetramers assembled on negative or positive DNA topoisomers show similar horseshoe shapes under electron microscopy (23). This supports the hypothesis that the conformational transition of the tetrameric particle might be achieved through a reorientation of the two H3-H4 dimers, due to reshuffling at the H3-H3 interface. This hypothesis is supported by the existence in archaea of a nucleosome-like particle that, although it wraps DNA in a right-handed superhelix (47), displays a structure resembling the histone (H3-H4)$_2$ tetramer (48). Many proteins undergo conformational changes, and the switch between specific conformational isomers is part of the...
mechanism of their function. The intrinsic flexibility of proteins results from the ability of different segments of the protein to move in relation to one another with small expenditures of energy. Protein motion can take two forms: hinge motions in strands, β-sheets, and α-helices that are not constrained by tertiary packing interactions, and shear motions between close-packed segments of polypeptides (for a review, see Ref. 49). Crystallographic data show that H3-H4 histone pairs form tetramers through the interaction of the carboxy-terminal halves of the α2- and α3-helices of H3’ and H3 across the dyad in addition to hydrophobic interactions (26). Two mechanisms may lead to the structural transition of the tetrameric nucleoprotein particle: a hinge motion in α-helices (e.g., catabolic activator protein, CAP) or small shifts and rotations of α-helices at the interface (e.g., citrate synthase). At present, we cannot discriminate between these two possibilities. Simulation work on the 2.8 Å resolution crystal structure of the tetramer within the nucleosome (26) might help provide an answer.

The nucleosome structural changes described here may be of importance for transcription. The induction of positive supercoiling downstream from a tracking RNA polymerase should result in the release of H2A-H2B dimers. This would allow the formation of a flexible tetrameric nucleoprotein particle. Transient histone modifications, such as tail acetylation, might also favor the release of H2A-H2B dimers from the nucleosome. The existence of tetrameric particles, able to easily flip from a left-to a right-handed structure, might result in transient changes in DNA/histone contacts, facilitating polymerase tracking and rapid chromatin reassembly after transcription has taken place.

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