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Modification of the histone tetramer at the H3-H3 interface impacts tetrasome conformations and dynamics

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Nucleosomes consisting of a short piece of deoxyribonucleic acid (DNA) wrapped around an octamer of histone proteins form the fundamental unit of chromatin in eukaryotes. Their role in DNA compaction comes with regulatory functions that impact essential genomic processes such as replication, transcription, and repair. The assembly of nucleosomes obeys a precise pathway in which tetramers of histones H3 and H4 bind to the DNA first to form tetrasomes, and two dimers of histones H2A and H2B are subsequently incorporated to complete the complex. As viable intermediates, we previously showed that tetrasomes can spontaneously flip between a left-handed and right-handed conformation of DNA-wrapping. To pinpoint the underlying mechanism, here we investigated the role of the H3-H3 interface for tetramer flexibility in the flipping process at the single-molecule level. Using freely orbiting magnetic tweezers, we studied the assembly and structural dynamics of individual tetrasomes modified at the cysteines close to this interaction interface by iodoacetamide (IA) in real time. While such modification did not affect the structural properties of the tetrasomes, it caused a 3-fold change in their flipping kinetics. The results indicate that the IA-modification enhances the conformational plasticity of tetrasomes. Our findings suggest that subnucleosomal dynamics may be employed by chromatin as an intrinsic and adjustable mechanism to regulate DNA supercoiling. Published by AIP Publishing. https://doi.org/10.1063/1.5009100

I. INTRODUCTION

The genome of eukaryotic organisms is tightly packed into chromatin, a hierarchical deoxyribonucleic acid (DNA)-protein assembly with a repeating basic unit termed the nucleosome.\textsuperscript{1–3} This fundamental complex consists of 147 base pairs (bp) of deoxyribonucleic acid (DNA) wrapped around a discoidal core of eight histone proteins by 1.7 turns in a left-handed superhelix.\textsuperscript{4–6} The histone octamer comprises two copies of each of the core histones H2A, H2B, H3, and H4, which group into two types of heterodimers by the pairing of histones H2A and H2B, and histones H3 and H4, respectively.\textsuperscript{7,8} Via the four-helix region formed by both H3 histones, the two H3-H4 dimers form a tetramer to which H2A/H2B dimers attach through similar interactions between histones H2B and H4. In the presence of DNA, the (H3-H4)\textsubscript{2} tetramer assembles first into a tetrasome, after which two H2A/H2B dimers bind to form the full nucleosome.\textsuperscript{9} In cells, nucleosome assembly is promoted by histone chaperones, such as Nucleosome Assembly Protein-1 (NAP1) or Chromatin Assembly Factor-1 (CAF1), and energy dependent chromatin assembly factors such as Adenosine triphosphate (ATP)-utilizing Chromatin assembly and remodeling Factor (ACF) or Chromodomain Helicase DNA binding protein-1 (CHD1).\textsuperscript{10,11} In vitro, nucleosomes are reconstituted via salt-dialysis or using purified recombinant enzymes.\textsuperscript{12} This first level of DNA compaction already highly affects and thereby regulates the accessibility of the genome during vital processes such as replication, transcription, and repair. Therefore, detailed knowledge of nucleosome structure and dynamics is crucial for understanding cell function and viability.

Over four decades of research, structural and biochemical approaches have provided profound insights into the structure and function of nucleosomes.\textsuperscript{13–17} More recently, such knowledge has been complemented by single-molecule studies which especially yielded substantial information concerning the dynamics of nucleosomes on the molecular scale.\textsuperscript{18} It is now known that nucleosomes are intrinsically dynamic by partially un- and rewrapping their DNA ends (breathing\textsuperscript{19–22}) and transiently opening the two turns of DNA along the axis of the superhelix (gaping\textsuperscript{23}). In addition, nucleosome composition, stability, and dynamics are altered by chemical modification of the histones (post-translational modifications\textsuperscript{24}) and by active remodeling enzymes (ATP-dependent remodelers\textsuperscript{25}). Furthermore, changes in nucleosome structure and dynamics are induced and regulated by the incorporation of histone variants with DNA-sequence or cell-cycle dependent expression, depositioning, and specific functions.\textsuperscript{26,27} Under extraneous causes in the form of force, torque or changes in buffer...
conditions, nucleosomes undergo structural rearrangements resulting in different conformations. In agreement with the stepwise assembly of nucleosomes, tetrasomes, consisting of 80 bp DNA wrapped around the (H3-H4)2 tetramer [Figs. 1(a) and 1(b)], have been observed as stable intermediates in various studies. Remarkably, tetrasomes have further been found to wrap DNA either in a left-handed or right-handed superhelix [Fig. 1(c)]. Recently, we have investigated this phenomenon by examining the dynamics of individual tetrasomes containing either the canonical *Drosophila* histone H3.1 or its main replacement variant H3.3. By directly measuring the DNA linking number, we observed spontaneous flipping of such tetrasomes between a predominant state of left-handed superhelix, like in the full nucleosome, and a less occupied right-handed conformation of DNA wrapping. The transition between the two states has been suggested to arise from the spontaneous reorientation of the (H3-H4)2 tetramer at the H3-H3 interface. However, experiments directed at pinpointing the mechanism underlying the handedness dynamics of tetrasomes via real-time measurements have been lacking.

In this work, we investigated the potential role of flexibility at the H3-H3 interface of the histone tetramers in the handedness flipping of tetrasomes at the single-molecule level. Using Freely Orbiting Magnetic Tweezers (FOMT), we studied the assembly and structural dynamics of individual NAP1-loaded, chemically modified (H3.1-H4)2 and (H3.3-H4)2 tetrasomes in real time. The (H3-H4)2 tetramers were treated with iodoacetamide (IA), which covalently binds to the sulphur atom of the single cysteine at position 110 of the H3 histones [Fig. 1(d)]. In a previous bulk study, this modification was found to form inherently left-handed tetrasomes and to block their transition to the right-handed conformation, potentially by generating a steric hindrance at the H3-H3 interface of the (H3-H4)2 tetramers. While IA-treated tetrasomes assembled with a very similar structure to untreated tetrasomes, we surprisingly found that the IA-treatment did not fully prevent the handedness flipping. However, the kinetics of IA-treated tetrasomes differed by 1.5-fold altered dwell times in the states of left-handed and right-handed DNA wrapping and by a 3-fold decrease of their ratio. These results indicate that the IA-treatment impacts the conformational flexibility and dynamics of tetrasomes. Our findings further suggest subnucleosomal dynamics as an intrinsic and tunable mechanism of chromatin to facilitate and regulate the impact of forces and torques on the genome. In the cell, such a mechanism could assist the corresponding activities by genome-processing enzymes such as the RNA polymerase and could be adjusted by histone core modifications that alter histone-DNA or histone-histone interactions.

**II. MATERIALS AND METHODS**

A. Materials

1. Preparation of DNA constructs

Linear double-stranded DNA fragments of 1.97 kilobase pairs (kbp) length were used as templates for tetrasome assembly in all experiments. This DNA fragment was generated by Polymerase Chain Reaction (PCR) from plasmid pBluescript (pBlue) 2.3 using primers 1 and 2 (Table S1 of the supplementary material). Subsequently, shorter fragments (handles) of 643 bp length containing nucleotides modified by either multiple biotin (Roche Diagnostics, Basel, Switzerland) or multiple digoxigenin (Roche Diagnostics) linkages were ligated to either end of the main DNA fragment at BsaI restriction sites. These handles were amplified by PCR from plasmid pBlueSKII" (Stratagene/Agilent Technologies, Santa Clara, CA, United States) using primers 3 and 5 or 4 and 5 (Table S1 of the supplementary material) in the presence of biotin-16-dUTP (Roche Diagnostics) or digoxigenin-11-dUTP (Roche Diagnostics) in a ratio of 1:5 with dTTP (Promega, Madison, WI, United States). The resulting DNA molecules contained no nucleosome-positioning sequences (Fig. S1 of the supplementary material).

2. Protein expression and purification

Expression and purification of recombinant *Drosophila* NAP1, histones H3.1-H4 and H3.3-H4 were performed as described in the respective previous studies. Example gel images of the histones after Sodium Dodecyl Sulfate
Polyacrylamide Gel Electrophoresis (SDS-PAGE) are shown in Figs. S2(a) and S2(b) of the supplementary material.

3. Histone treatment with iodoacetamide (IA)

Purified H3.1-H4 and H3.3-H4 histones were dialed overnight against a buffer containing 10 millimolar (mM) 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-KOH (Hepes-KOH, pH 7.6), 10 mM KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 10% (v/v) glycerol (buffer A) with two buffer changes to remove dithiothreitol (DTT). Subsequently, the samples were incubated with 1 mM IA in buffer A for 3 h in the dark at room temperature (RT). Afterwards, IA was removed by overnight dialysis against buffer A with 1 mM DTT at 4 °C with two changes of buffer. Aliquots of H3-H4 solutions at the different steps of treatment were analyzed by SDS-PAGE [Figs. S2(a) and S2(b) of the supplementary material]. IA was our reagent of choice because it allows for a robust and quantitative modification of histone H3 in a technically straightforward manner.34–45

4. Mass spectrometry analysis of IA-treated histones

The degree of derivatization of the core histones by incorporation of IA was quantified by mass spectrometry. The experimental procedure is detailed in the supplementary material together with the results shown in Figs. S2(c) and S2(d). We found that all H3.1 histones (100%) and virtually all H3.3 histones (99.3%) were derivatized upon IA-treatment. The underlying chromatograms from high-performance liquid chromatography (HPLC) and MS2-spectra are shown in Figs. S3 and S4 of the supplementary material, respectively.

B. Methods

1. Tetrasome reconstitution via salt-dialysis

The capability of the histones to successfully load onto the DNA constructs designed for use in the single-molecule experiments was confirmed by reconstituting tetrasomes with both untreated and IA-treated tetrasomes using salt-gradient dialysis.53,54 The details of the employed protocol can be found in the supplementary material together with the results shown in Fig. S5.

2. Sample preparation for tetrasome assembly in single-molecule experiments

In single-molecule experiments, tetrasome assembly was performed in flow cells consisting of a channel cut into a double-layer of paraffilm that was sandwiched between two cover glasses (24 × 60 mm/#1, Menzel-Glaser, Braunschweig, Germany). The details of the assembly and preparation of the flow cells are described in the supplementary material.

For the experiments with histones, the buffer was changed to the measurement buffer containing 50 mM KCl (Merck, Darmstadt, Germany), 25 mM Hepes-KOH (pH 7.5; Sigma-Aldrich, St. Louis, MO, United States), 0.1 mM EDTA, 0.1 mg/ml Bovine Serum Albumin (BSA; Sigma-Aldrich) for passivation, 0.25% Polyethylene Glycol (PEG; Sigma-Aldrich), and 0.25% Polyvinyl Alcohol (PVA; Sigma-Aldrich) as crowding agents. These buffer conditions with a 10-fold higher concentration of crowding agents compared to our previous studies46,49 were employed in most of the experiments (n = 13 out of N = 15) to increase tetrasome stability since tetrasomes had been observed to disassemble in the course of the first two experiments. For the NAPI-mediated assembly of tetrasomes, either 51 nanomolar (nM) of an equimolar solution of H3.1IA-H4 histones or 54 nM of an equimolar solution of H3.3IA-H4 histones were incubated with 192 nM NAP1 for 30 min on ice in a buffer containing 50 mM KCl, 25 mM Hepes-KOH, 0.1 mM EDTA, 1 mg/ml BSA, 0.25% PEG, and 0.25% PVA. The incubated protein solution was then diluted at least 1:100 and 100 μl of the diluted solution was flushed into the flow cell to achieve the controlled assembly of a few tetrasomes. Free proteins were not flushed out in most measurements due to the enhancing effect on tetrasome disassembly observed in the first two experiments. In our previous studies, the presence of NAPI was found to affect neither the stability of tetrasomes nor the flipping dynamics for (H3.1-H4)$_2$ tetrasomes, but to slightly increase the flipping probability of (H3.3-H4)$_2$ tetrasomes.46,49

3. Magnetic tweezers instrumentation

The NAPI-mediated assembly of tetrasomes was measured by directly monitoring the length and linking number of single DNA molecules using FOMT.50 The hardware of the magnetic tweezers setup used in this study is described in the supplementary material. The exerted force was calibrated for each experiment and amounted to values between 0.6 pN and 0.7 pN. All experiments were performed at RT (22 °C).

4. Data analysis

The acquired data were analyzed using custom-written scripts in Matlab (Mathworks, Natick, MA, United States) and its built-in functions. The traces were analyzed for stepwise changes (steps) in DNA length and linking number using a custom-written step-fitting algorithm that improves upon its previous version described in Ref. 55. In a subsequent analysis, steps coinciding in both time traces, and hence indicating assembly or disassembly of tetrasomes, were identified using these fits. The sizes of the coinciding steps in DNA length (N = 71) and linking number (N = 71) upon assembly and disassembly were then extracted as key quantities describing the structure of the tetrasomes.

The handedness dynamics was only analyzed in those parts of the time traces that had stable DNA length and linking number baseline, reflecting stably bound tetrasomes (N = 34). By fitting a corresponding number of Gaussian functions to the linking number data between two subsequent coinciding steps [Fig. 4(b)], the handedness flipping was characterized in terms of the associated alteration in tetrasome structure (n = 22). The differences between the mean values of these fits were used to determine the change in linking number upon flipping (N = 26). The relative peak area ratios of the individual Gaussian fits yielded the probabilities for the tetrasomes to occupy the corresponding states.

For a more detailed picture of the handedness dynamics, the times a single assembled and flipping (H3.1IA-H4)$_2$
tetrasome spent in the left-handed or right-handed state of DNA wrapping (dwell times) were analyzed using a custom-written algorithm based on Ref. 56. Smoothed linking number data from the corresponding time traces ($N=4$) were assigned to the two states with the help of a threshold zone set by the midpoint of the mean values and their standard deviation (STD) obtained from the Gaussian fits to the unfiltered data [Fig. 4(b)]. The times between subsequent transitions from one state to the other, i.e., intersections with the midpoint, were considered as the dwell times in the corresponding states. All data sets assigned to the left-handed state ($N=195$ for 3.4 s or $N=76$ for 18.4 s time averaging by filtering) and right-handed state ($N=199$ for 3.4 s or $N=81$ for 18.4 s time averaging) were combined and fitted by an exponential function to determine the mean dwell time in each conformation [Figs. 5(a) and 5(b)]. For comparison, dwell times in these traces were also determined using the recorded dwell times in the plateaus of the steps fitted by the step-fitting algorithm in a separate analysis ($N=65$ for the left-handed state, $N=64$ for the right-handed state). For a direct comparison to the behavior of untreated tetrasomes, we further re-analyzed the dwell times in the partial time traces ($N=6$) of one of our earlier experiments with untreated (H3.1-H4$_2$) tetrasomes published in the related article, using the same custom-written algorithm and settings ($N=158$ for 3.4 s or $N=69$ for 18.4 s time averaging for the left-handed state, $N=160$ for 3.4 s and $N=71$ for 18.4 s time averaging for the right-handed state).

Further details of the data analysis are described in the supplementary material together with the complementing results shown in Figs. S6 and S7. Overall, it should be noted that the values of the results obtained here for the dynamics and kinetics of the tetrasomes are an upper boundary due to the finite bead response time. The errors stated on the mean values determined in this study correspond to 1 STD based on the underlying distributions, unless indicated otherwise. The errors of computed quantities were calculated by error propagation.

III. RESULTS

A. NAP1-mediated assembly of iodoacetamide (IA)-treated tetrasomes results in proper complexes

Modified tetrasomes were assembled by flushing IA-treated histone (H3$_3$A-H4$_2$) tetramers pre-incubated with NAP1 chaperones into a flow cell containing individually tethered DNA molecules without specific nucleosome-positioning sequences. The formation of tetrasomes was monitored in real-time by measuring the length and the linking number of a single DNA molecule using FOMT. A magnetic bead-tethered DNA molecule is precisely aligned with the axis of the vertically oriented magnetic field generated by a cylindrical permanent magnet allowing controlled application of force without constraining the bead’s rotational motion [Fig. 2(a)]. In this study, constant stretching forces of 0.6-0.7 pN were applied, comparable to our previous studies with untreated tetrasomes. The assembly of tetrasomes upon flushing in histone/chaperone-complexes was reflected in stepwise decreases in both DNA length $z$ [in micrometers ($\mu$m)] [Fig. 2(b)] and linking number $\theta$ (in turns) [Fig. 2(c)] simultaneously. Histone tetramers or NAP1 alone did not interact with the DNA molecule under identical conditions (Fig. S8 of the supplementary material).

For improved statistics, different numbers of tetrasomes were assembled in several experiments ($N=15$) by changing the protein concentration. For the same purpose, the results
obtained for (H3.1\textsubscript{IA}-H4\textsubscript{2}) and (H3.3\textsubscript{IA}-H4\textsubscript{2}) tetrasomes were combined, as we previously found the properties of untreated (H3.1-H4\textsubscript{2}) and (H3.3-H4\textsubscript{2}) tetrasomes to be very similar.\textsuperscript{48,49} The total, simultaneous changes in DNA length $\Delta z_{\text{tot}}$ and linking number $\Delta \theta_{\text{tot}}$ upon assembly of different numbers of tetrasomes in several experiments follow a linear relation with a slope of $\Delta z_{\text{tot}}/\Delta \theta_{\text{tot}} = 33 \pm 6$ nm/turn (95% confidence interval for estimated values from a linear fit) [Fig. 3(a)]. Interestingly, some of the total changes in DNA linking number were smaller than the value expected from their corresponding change in DNA length, suggesting the assembly of right-handed tetrasomes. Therefore, such results ($n = 5$) were excluded from the fit. From the total changes, we determined that 11% ($n = 8$) of all modified tetrasomes ($N = 74$) assembled in the right-handed conformation. In contrast to untreated tetrasomes, 66% ($n = 49$) of the assembled modified tetrasomes were found to disassemble in the course of the measurements, regardless of the NAP1/histone ratio employed (Fig. S9 of the supplementary material), indicating their decreased stability. A destabilizing effect of the 10-fold higher concentration of crowding agents compared to our previous studies on tetrasomes\textsuperscript{48,49} seems unlikely given the observation that under the same conditions, untreated tetrasomes did not disassemble [Fig. S10(a) of the supplementary material].

While multiple IA-treated tetrasomes mostly assembled simultaneously as reflected in large steps, their disassembly mainly occurred in a one-by-one fashion, indicating proper formation of individual complexes rather than aggregates [Fig. 3(b)]. A possible reason for this behavior could be a cooperative binding mechanism that leads to a simultaneous or faster assembly of individual IA-treated tetrasomes than we can experimentally resolve. The several individual changes in DNA length $\Delta z_{\text{dis-lass}}$ ($N = 71$) and linking number $\Delta \theta_{\text{dis-lass}}$ upon tetrasome assembly or disassembly ($N = 71$) also follow a linear relation with a slope of $\Delta z_{\text{dis-lass}}/\Delta \theta_{\text{dis-lass}} = 26 \pm 4$ nm/turn (95% confidence interval for estimated values from a linear fit). Similar to the total changes, some of the changes in DNA linking number showed the opposite sign compared to those expected from the change in DNA length, indicating the assembly or disassembly of right-handed tetrasomes. Such data ($n = 14$) were likewise excluded from the fit.

Combining the absolute values from all measurements with (H3.1\textsubscript{IA}-H4\textsubscript{2}) and (H3.3\textsubscript{IA}-H4\textsubscript{2}) tetrasomes for improved statistics yielded a mean change in DNA length of $\Delta z_{\text{dis-lass}} = 28 \pm 8$ nm ($n = 49$) and a mean change in linking number of $\Delta \theta_{\text{dis-lass}} = 1.0 \pm 0.3$ turns ($n = 61$) upon the assembly or disassembly of IA-treated tetrasomes [Figs. 3(c) and 3(d)]. The individual distributions and results of the changes for the two types of tetrasomes are shown in Figs. S11 and S12 of the supplementary material. The mean values were determined from the data within the corresponding resolution limits, the contour length (50 nm) of nucleosomal DNA

![FIG. 3. Changes in DNA length and linking number upon dis-assembly/assembly of IA-treated tetrasomes. (a) Total changes in DNA length $\Delta z_{\text{tot}}$ (in $\mu$m) upon assembly of different numbers of (H3.1\textsubscript{IA}-H4\textsubscript{2}) (blue circles) or (H3.3\textsubscript{IA}-H4\textsubscript{2}) tetrasomes (dark green squares) in several experiments plotted against their corresponding total change in DNA linking number $\Delta \theta_{\text{tot}}$ (in $\mu$m) ($N = 15$). A linear fit (orange solid line) yielded a slope of $\Delta z_{\text{tot}}/\Delta \theta_{\text{tot}} = 33 \pm 6$ nm/turn (95% confidence interval for estimated values). Data involving right-handed assembly (cyan stars, $n = 5$) were excluded from the fit (see main text). (b) Changes in DNA length $\Delta z_{\text{dis-lass}}$ (in $\mu$m) upon disassembly/assembly of IA-treated tetrasomes plotted against their corresponding change in DNA linking number $\Delta \theta_{\text{dis-lass}}$ (in $\mu$m) ($N = 71$). A linear fit yielded a slope of $\Delta z_{\text{dis-lass}}/\Delta \theta_{\text{dis-lass}} = 26 \pm 4$ nm/turn (95% confidence interval for estimated values). Data involving right-handed disassembly/assembly (shaded areas, $n = 14$) were excluded from the fit (see main text). (c) Histogram of the changes in DNA length $\Delta z_{\text{dis-lass}}$ (blue bars) upon disassembly/assembly of IA-treated tetrasomes plotted together with the mean spatial resolution based on 1 STD (17 nm, green line). The mean change in length of $\Delta z_{\text{dis-lass}} = 28 \pm 8$ nm was determined from the data within the range bounded by the resolution limit (shaded area) and the DNA contour length wrapped in a full nucleosome (50 nm) ($n = 49$). (d) Histogram of the changes in DNA linking number $\Delta \theta_{\text{dis-lass}}$ plotted together with the mean spatial resolution based on 1 STD (0.5 turns, green line). The mean change in linking number of $\Delta \theta_{\text{dis-lass}} = 1.0 \pm 0.3$ turns was determined from the data within the range bounded by the resolution limit (shaded area) and the number of turns the DNA is wrapped around the histone core in a full nucleosome (1.7 turns) ($n = 61$).]
and the number of turns (1.7 turns) that it is wrapped around the histone octamer. Considering the above observed linear relation between the changes in DNA length and linking number, the mean values yield a ratio of $\Delta z_{\text{ass}}/\Delta \theta_{\text{ass}} = 28 \pm 12$ nm/turn, which is in good agreement with the results obtained from the linear fits to the two different data sets above.

Overall, these values agree well with previous studies in which tetrasmes were characterized as intermediates during un- and refolding of complete nucleosomes or by direct measurements. The linear dependency between the key quantities characterizing the structure of the modified tetrasmes further suggests that their conformation is independent of their number being assembled on a DNA molecule, as we previously observed for untreated tetrasmes as well. These results show that IA-treated tetrasmes assembled properly in our assay with a very similar structure to untreated tetrasmes.

B. IA-treated tetrasmes have reduced tendency towards handedness flipping

To observe tetrasm behavior after assembly over an extended period of time, the FOMT experiments were carri ed out for several hours. As mentioned above, most modified tetrasmes were observed to disassemble in the course of the experiments, unlike untreated tetrasmes. On average, the (H3.1IA−H4)2 tetrasmes were found to disassemble within 2499 ± 415 s [1 standard error of the mean (SEM), Fig. S7 of the supplementary material]. Therefore, the structural dynamics of tetrasmes was analyzed in partial traces with states of stable binding between two subsequent assembly and/or disassembly events. Quite unexpectedly, since IA-modification was previously reported to block the structural transition of tetrasmes, we found IA-treated tetrasmes to be dynamic in terms of their handedness. While the DNA length remained constant [Fig. 4(a)], the linking number of a DNA molecule loaded with a tetrasm continuously fluctuated between two states corresponding to a left-handed superhelix, like in the full nucleosome, and a right-handed conformation of DNA wrapping [Fig. 4(b)]. Such handedness flipping was observed in 86% ($n = 12$) of the analyzed partial traces ($N = 14$) with (H3.1IA−H4)2 tetrasmes. (H3.3IA−H4)2 tetrasmes were found to flip in 50% ($n = 10$) of the analyzed partial traces ($N = 20$). The associated change in tetrasm structure was quantified by the difference between the means of the corresponding number of Gaussian distributions fitted to the linking number data that show flipping ($N = 26$). On average, the change in DNA linking number associated with flipping $\Delta \theta_{\text{flipping}}$ equalled 1.6 ± 0.2 turns [Fig. 4(c)], which exactly corresponds to the values obtained for the two types of tetrasmes individually (Fig. S13 of the supplementary material). This value further agrees well with that determined previously for untreated tetrasmes and reaffirms our observation that IA-treated tetrasmes assembled into proper complexes.

Nevertheless, the considerable remaining fractions of the analyzed partial traces did not show such handedness flipping. This indicates the existence of another, rather metastable population/state induced upon IA-modification. Along these lines, we also observed that the linking number data of multiple loaded IA-treated tetrasmes never showed the number of states that would be expected, if they all flipped simultaneously. At most three states were observed in 14% ($n = 2$) of the analyzed traces deduced from (H3.1IA−H4)2 tetrasmes and in 10% ($n = 2$) of the analyzed traces obtained with (H3.3IA−H4)2 tetrasmes. This implies that usually only one but not necessarily the same tetrasm exhibited handedness flipping.

Additionally, in 43% of the data from experiments with (H3.1IA−H4)2 tetrasmes, the lowest linking number state did not correspond to the value expected for all tetrasmes being in the left-handed conformation. While not observed for (H3.3IA−H4)2 tetrasmes, this phenomenon indicates that some (H3.1IA−H4)2 tetrasmes also stably dwelled in the

![FIG. 4. Handedness flipping of IA-treated tetrasmes. (a) Partial time trace of a DNA molecule’s length $z$ (in µm) after the assembly of a (H3.1IA−H4)2 tetrasm. The DNA length stays constant over time, as can be seen both from the fit to the time trace (red line) by the custom-written step-fitting algorithm and its histogram on the right panel. The data for the histogram were fitted by a gamma function (red line in histogram) after mirroring at the x-axis and offsetting to positive values (see supplementary material). (b) The corresponding part of the time trace of the same DNA molecule’s linking number $\theta$ (in turns). This shows spontaneous fluctuations between a predominantly occupied left-handed state and a less occupied right-handed state with a mean of $\theta_{\text{left}} = +0.77 \pm 0.01$ turns and $\theta_{\text{right}} = +0.86 \pm 0.06$ turns (95% confidence intervals for estimated values; orange dashed lines), respectively, as can be seen both from the fit to the time trace (red line) by the custom-written step-fitting algorithm and its histogram on the right panel. The data for the histogram were fit to two Gaussian functions (black lines in the histogram) underlying the full profile (red line in the histogram). For dwell time analysis, the time trace was smoothed (black) before categorizing the data into the two states based on a threshold (orange solid line) set at the average value of the means determined from the unfiltered data (orange dashed lines). The threshold was further extended to a zone (magenta striped area) bounded by 1 STD from the corresponding means (magenta dashed-dotted lines). Alternatively, the dwell times in the step plateaus from the step-fitting algorithm were analyzed after manual correction to obtain a better match to the data (cyan solid line). (c) Histogram of the changes in linking number upon handedness flipping of IA-treated tetrasmes. The data have a mean value of $\Delta \theta_{\text{flipping}} = 1.6 \pm 0.2$ turns ($N = 26$).]
right-handed state. From the DNA length and the corresponding linking number values, we determined that 18% \((n = 6)\) of all assembled (H3.1\(_{\text{IA}}\)-H4\(_{\text{2}}\)) tetrasomes \((N = 33)\) in the untreated tetrasomes assembled in the same conditions did not fully prevent tetrasomes from changing their handedness. Nonetheless, our findings clearly indicate that IA-treated tetrasomes have a reduced tendency towards flipping, which might arise from the incorporated IA molecules. This would support the idea that a potential rotation of the two H3-H4 tetramers against each other at the H3-H3 interface is the mechanism required enabling handedness flipping. The simultaneous flipping of multiple tetrasomes might be hindered by the increased stability of individual complexes in a cooperative setting. The differing behavior and statistics for (H3.1\(_{\text{IA}}\)-H4\(_{\text{2}}\)) and (H3.3\(_{\text{IA}}\)-H4\(_{\text{2}}\)) tetrasomes might result from subtle differences in their structure upon IA-incorporation. Similar to the case of tetrasome stability, the 10-fold higher concentration of crowding agents compared to our previous studies on tetrasomes\(^{48,49}\) is unlikely to affect tetrasome flipping, as untreated tetrasomes assembled in the same conditions did flip as previously observed [Fig. S10(b) of the supplementary material].

C. IA-treatment impacts the conformational plasticity of tetrasomes

The presence of non-flipping tetrasomes described above indicates that the H3-H3 interface of the histone tetramer plays an important role in tetrasome flexibility. However, since most IA-treated tetrasomes still exhibited handedness flipping, we looked more closely into its dynamics to obtain a more detailed picture of this process.

For this purpose, the linking number traces \((N = 4)\) of a single loaded, flipping (H3.1\(_{\text{IA}}\)-H4\(_{\text{2}}\)) tetrasome were first analyzed in terms of the times that it spent in each state (dwell times). The underlying data analysis is described in Sec. II, and the resulting mean dwell times of a (H3.1\(_{\text{IA}}\)-H4\(_{\text{2}}\)) tetrasome in the left-handed and right-handed conformations are shown in Figs. 5(a) and 5(b), respectively. These values, and those determined in a separate analysis based on the dwell times from the step-fitting algorithm, together with the results obtained for an untreated (H3.1-H4\(_{\text{2}}\)) tetrasome from the re-analysis of the partial traces of an earlier experiment\(^{48}\) are summarized for comparison in Table I. While the total dwell times for each type of tetrasome varied depending on the smoothing, which in the case of the step-fitting algorithm is caused by missed events, their ratio was essentially not affected. This result suggests
a reliable analysis that allows the direct comparison of the total dwell times obtained for an untreated tetrasome and an IA-treated tetrasome with the same settings as well as their ratios.

Overall, a (H3.1IA-H4)2 tetrasome dwelled 1.5 ± 0.3 times shorter in the left-handed state compared to an untreated (H3.1-H4)2 tetrasome, while the opposite was the case for the right-handed state with a likewise longer dwell time. This indicates that left-handed and right-handed (H3.1IA-H4)2 tetrasomes are energetically less and more stable, respectively, than their untreated counterparts, while the transition barrier between the two states remains essentially unaffected. The overall impact of IA-treatment is clearly illustrated by the 3 ± 1-fold decrease of the left-handed versus right-handed dwell time ratio, which suggests a change in the free energy difference between the two states of (H3.1IA-H4)2 and untreated (H3.1-H4)2 tetrasomes.

The free energy difference between the two states can be determined from the ratio of the respective dwell times by computing $\Delta E = -k_B T \ln(\tau_{D,right}/\tau_{D,left})$. This calculation yields a value of $\Delta E_{IA} = 1.5 \pm 0.1$ kB T, which is considerably different from the values resulting from dwell time analyses for an untreated (H3.1-H4)2 tetrasome, i.e., the previously reported value $\Delta E_0 = 2.6 \pm 0.8$ kB T (Ref. 48) or the corresponding value $\Delta E_0 = 2.4 \pm 0.1$ kB T determined via our updated analysis algorithm. Thus, the cumulative change in the free energy difference between the left-handed and right-handed states of IA-treated and unmodified tetrasomes by $\Delta E_0 - \Delta E_{IA} = 1.1 \pm 0.3$ kB T is consistent with the 3 ± 1-fold difference in the dwell time ratio.

For validation purposes, the same linking number data were also analyzed in terms of the probability for a (H3.1IA-H4)2 tetrasome to occupy either the left-handed or right-handed state. This was achieved by considering the peak areas of the fitted Gaussian distributions [Fig. 4(b)] whose relative ratios give the probabilities $p$ and $1-p$ to occupy the corresponding states. By this means, a single flipping (H3.3IA-H4)2 tetrasome was found to obtain the left-handed conformation with an average probability of $p_{av} = 0.85 \pm 0.11$ ($N = 4$), corresponding to an average probability of $1-p_{av} = 0.15 \pm 0.11$ for occupying the right-handed conformation. Likewise, the free energy difference between the two states of $\Delta E_{IA} = 1.7 \pm 0.7$ kB T which is deduced from the ratio of the probabilities according to $\Delta E = -k_B T \ln((1-p)/p)$ is similar to the value obtained from the dwell time ratio above.

Alternatively, the free energy difference of the two states was also calculated from the probabilities of all data sets including multiple assembled (H3.1IA-H4)2 tetrasomes ($N = 12$) by fitting to a binomial distribution [Fig. 5(c)]. In this approach, the probabilities based on the relative peak area ratios of the Gaussian distributions for each data set with varying number of assembled tetrasomes were assigned to their corresponding states in terms of the number of tetrasomes being in the left-handed state. Non-observed states were assigned a probability of zero. These data were fit to a binomial distribution with the number of assembled tetrasomes, i.e., the expected number of states being fixed, and the probability of a tetrasome to have the left-handed conformation treated as the free parameter.

Averaging over all obtained values yields a mean probability of $p_{av} = 0.76 \pm 0.15$ of a (H3.1IA-H4)2 tetrasome to occupy the left-handed state. This value corresponds to a free energy difference between the two states of $\Delta E = 1.2 \pm 0.6$ kB T which agrees well with the values determined from the two other approaches above. An untreated (H3.1-H4)2 tetrasome, however, was previously found to occupy the left-handed conformation with a probability of $p_{av} = 0.90 \pm 0.08$ (Ref. 48) corresponding to a free energy difference between the two states of $\Delta E_0 = 2.3 \pm 0.8$ kB T (Ref. 48). Taken together, the results from different analysis approaches consistently indicate a decrease in the free energy difference between the states of left-handed and right-handed DNA wrapping in (H3.1IA-H4)2 versus (H3.1-H4)2 tetrasomes by 1 kB T.

For a (H3.3IA-H4)2 tetrasome, only the latter approach by fitting all probability data ($N = 10$) to a binomial fit was used (Fig. S14 of the supplementary material) because the structural dynamics of (H3.3-H4)2 tetrasomes was previously observed to be very similar to (H3.1-H4)2 tetrasomes and no dwell time data are available for direct comparison. On average, a mean probability of $p_{av} = 0.88 \pm 0.08$ was found for a (H3.3IA-H4)2 tetrasome to be in the left-handed state, which corresponds to a difference in free energy between the two states of $\Delta E_{IA} = 2.0 \pm 0.7$ kB T. In contrast to the observation with (H3.1IA-H4)2 tetrasomes above, these values agree well with those obtained previously for untreated (H3.3-H4)2 tetrasomes [$p_{av} = 0.91 \pm 0.03$ and $\Delta E_0 = 2.3 \pm 0.4$ kB T (Ref. 49)]. The differing results for (H3.3IA-H4)2 tetrasomes and (H3.1IA-H4)2 tetrasomes, as also observed for their flipping behavior, might arise from subtle structural differences upon IA-incorporation. However, it was also observed that the handedness dynamics of (H3.3-H4)2 tetrasomes was slightly stimulated by the presence of NAP1, in contrast to (H3.1-H4)2 tetrasomes.48,49

In our experiments, NAP1 was present in solution throughout the measurements due to the observed trend of enhanced

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time average in filtering (s)</th>
<th>$\tau_{D,left}$ (s)</th>
<th>$\tau_{D,right}$ (s)</th>
<th>$\tau_{D,left}/\tau_{D,right}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(H3.1-H4)2 tetrasomes</td>
<td>3.4 (N = 340)</td>
<td>177 ±15–12a</td>
<td>16 ± 1</td>
<td>11 ± 1a</td>
</tr>
<tr>
<td>18.4 (N = 1840)</td>
<td>366 ±51–39a</td>
<td>29 ±4–3</td>
<td>13 ± 2a</td>
<td></td>
</tr>
<tr>
<td>(H3.1IA-H4)2 tetrasomes</td>
<td>3.4 (N = 340)</td>
<td>105 ±48–7</td>
<td>24 ± 2</td>
<td>4 ± 0a</td>
</tr>
<tr>
<td>18.4 (N = 1840)</td>
<td>244 ±32–25</td>
<td>52 ±6–5</td>
<td>5 ± 1a</td>
<td></td>
</tr>
</tbody>
</table>

*Errors calculated by error propagation.*
disassembly of IA-treated tetrasomes upon flushing out free proteins. Therefore, the mean probability and free energy difference for \((H3.3_{IA}-H4)_2\) tetrasomes might also be smaller than the obtained value and similar to those determined for \((H3.1_{IA}-H4)_2\) tetrasomes.

Overall, these results indicate that IA-modification influences the stability of and the kinetics between the two tetrasome conformations. The shorter dwell time in the left-handed state suggests its decreased stability, while the right-handed conformation with a dwell time increased to the same extent is more stable compared to untreated tetrasomes. These effects are also reflected by corresponding differences in the probabilities of finding an IA-treated tetrasome in a certain state. Thus, the IA-treatment results in a decrease of the free energy difference between the two states.

### IV. DISCUSSION AND CONCLUSION

Since four decades, chromatin research continues to reveal various aspects of the structure, function, and dynamics of the nucleosome as the fundamental DNA-protein complex in increasing detail. The chemically, force-, or torque-induced partial or full removal of H2A/H2B dimers by changes in buffer conditions, mechanical manipulation, or genome-processing enzymes, such as the RNA polymerase, \(^{57-59}\) makes subnucleosomal structures a topic of great interest. As stable intermediates, tetrasomes were investigated early on by biochemical approaches. \(^{42,43}\) These pioneering experiments demonstrated the high affinity of the \((H3-H4)_2\) tetramers for either negatively or positively supercoiled DNA, resulting in mutually convertible tetrasome conformations of a left-handed superhelix, like in the full nucleosome, or a right-handed DNA wrapping, respectively. Additional studies have suggested that this transition in tetrasome handedness results from the spontaneous reorientation of the \((H3-H4)_2\) tetramer at the H3-H3 interface. \(^{44-47}\) Our previous studies of the assembly and structural dynamics of tetrasomes at the single-molecule level have confirmed the existence of two handedness states and revealed their dynamic nature. \(^{48,49}\) The tetrasomes were observed to continuously flip between a predominant left-handed and a less occupied right-handed conformation. However, these studies did not directly investigate the underlying mechanism of this phenomenon.

In this work, we sought to address this issue by interfering with the potential flexibility of the histone tetramer at the H3-H3 interface and monitoring the effects at the single-molecule level. We have investigated the NAP1-mediated assembly and structural dynamics of individual \((H3.1-H4)_2\) and \((H3.3-H4)_2\) tetrasomes modified with iodoacetamide (IA) at the single cysteine at position 110 of the H3 histones in real time using FOMT. \(^{50}\) In biochemical analyses of bulk tetrasome assemblies, this modification was previously found to block the handedness flipping of tetrasomes by potentially generating a steric hindrance at the H3-H3 interface of the histone tetramers. \(^{44}\) The IA-treated histone \([(H3_{IA}-H4)_2]\) tetramers had been reported to only form inherently left-handed tetrasomes lacking the structural transition to the right-handed conformation. In agreement with this biochemical study, IA-treated tetrasomes assembled with a similar structure to untreated tetrasomes in our assay, which indicates the formation of proper complexes. However, in contrast to untreated tetrasomes, we observed IA-treated tetrasomes to disassemble in the course of the experiments, which suggests their decreased stability, possibly due to changes in their properties upon IA-incorporation. In the previous biochemical analyses, IA-treated tetramers have also been found to exhibit a low affinity to relaxed circular DNA templates and modified tetrasomes migrated faster on a gel than untreated tetrasomes. However, unlike the observation of only left-handed IA-treated tetrasomes in that study, we found \((H3_{IA}-H4)_2\) tetramers to also form right-handed tetrasomes. The varying results likely arise from the use of different approaches: single-molecule experiments, performed in highly diluted conditions, are known to be more sensitive than bulk assays, especially in terms of revealing transient intermediates and the dynamics of biomolecules. Another reason for this discrepancy could be the difference in the topology of the employed DNA constructs, which might be critical. The different topological restriction of tethered linear DNA fragments being subject to (low) force in our assay and of supercoiled circular DNA molecules employed in the biochemical analyses might bias tetrasome assembly. Likewise, apart from the right-handed assembly, we also observed IA-treated tetrasomes to exhibit spontaneous changes in their handedness. However, while their structural rearrangement upon handedness flipping was very similar to that of untreated tetrasomes, the kinetics were found to be different.

The different kinetics and energetics between IA-treated and untreated tetrasomes are shown by the schematic energy diagram presented in Fig. 5(d), based on the here obtained results. Setting the barrier energy \(E_{\text{barrier}}\) to the same value for a common reference, the free energy of left-handed IA-treated tetrasomes \(\Delta G_{l_{IA}}\) is by a small amount higher than that for untreated tetrasomes \(\Delta G_{0_{l}}\), as reflected in the 3-fold decrease in the corresponding dwell time \(\tau_{D_{l}}\). Conversely, the 1.5-fold increase in the dwell time \(\tau_{D_{r}}\) indicates a likewise lower free energy of right-handed IA-treated tetrasomes. These differences indicate that IA-treated tetrasomes are slightly less stable in the left-handed state and slightly more stable in the right-handed conformation than untreated tetrasomes due to the incorporated IA molecules. This effect might also play the central role in the 3-fold decreased ratio of the dwell times for IA-treated tetrasomes, which corresponds to a change in the free energy difference between the two states of 1 k_BT compared to untreated tetrasomes, as determined from the three different approaches. Knowing the transition rates \(k_{l\rightarrow r}\) and \(k_{r\rightarrow l}\), related to the corresponding dwell times \(\tau_{D_{l}}\) and \(\tau_{D_{r}}\), the height of the respective transition barriers can be calculated according to \(\Delta G = -k_BT \ln(k_0)\) with the rate \(k_0\) for spontaneous transitions in tetrasome structure at zero force. Considering a rate of \(k_0 \sim 10^7 s^{-1}\) based on the value of \(\sim 3 \times 10^6 s^{-1}\) previously estimated for spontaneous structural transitions of full nucleosomes, \(^{46}\) the energy barrier for the transition from the left-handed state to the right-handed state can be estimated to \(\Delta G_{0_{l\rightarrow r}} \sim 21.3 k_BT\) for untreated tetrasomes and to \(\Delta G_{l_{IA\rightarrow r}} \sim 20.8 k_BT\) for \((H3.1_{IA}-H4)_2\) tetrasomes. The same calculation gives an estimate of \(\Delta G_{r_{l\rightarrow r}} \sim 18.9 k_BT\) and \(\Delta G_{r_{IA\rightarrow r}} \sim 19.3 k_BT\) for the transition from the
right-handed to the left-handed state for untreated and (H3.1IA-H4)2 tetrasomes, respectively. The results consistently indicate that the energies of the two states are altered, while the energy barrier for the structural transition of tetrasomes is essentially unaffected.

On the whole, our findings show that the IA-treatment did not affect the overall structural properties of tetrasomes but had some impact on their stability, flexibility, and dynamics. The unexpected occurrence of continuous handedness flipping and assembly of IA-treated tetrasomes into the right-handed conformation indicate that the incorporation of the IA molecules does not fully prevent these dynamics. Our results are directly comparable to our previous studies of untreated tetrasomes, as the same technique and essentially the same conditions have been used (Table II). Based on the observations in other single-molecule assays,61 we do not expect such low volume concentrations (<1%) of crowding agents to have a significant impact on the kinetics and energetics of the biological system under study. For this reason, we conclude that the observed changes in the energetics and flipping kinetics of tetrasomes are due to the IA-treatment. The results suggest that IA-modification enhances the conformational plasticity of tetrasomes, while their structural dynamics is affected to a lesser extent. Considering the very low forces of 0.6-0.7 pN employed in our experiments, possible contributions from other phenomena, such as DNA flexibility, cannot be excluded.

In a broader context, our previous and current findings suggest the handedness dynamics of tetrasomes as an intrinsic and tunable mechanism of chromatin to regulate the impact of other phenomena, such as DNA flexibility, cannot be excluded. This suggests that tetrasomes are due to the IA-treatment. The results suggest that IA-modification enhances the conformational plasticity of tetrasomes, while their structural dynamics is affected to a lesser extent. Considering the very low forces of 0.6-0.7 pN employed in our experiments, possible contributions from other phenomena, such as DNA flexibility, cannot be excluded.

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**SUPPLEMENTARY MATERIAL**

See supplementary material for further details.

**ACKNOWLEDGMENTS**

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**TABLE II.** Summary of the properties of IA-treated and untreated tetrasomes for comparison.

<table>
<thead>
<tr>
<th>Quantity</th>
<th>(H3.1IA-H4)2 tetrasomes</th>
<th>(H3.1-H4)2 tetrasomes</th>
<th>(H3.3-H4)2 tetrasomes</th>
<th>(H3.3IA-H4)2 tetrasomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\Delta E_{\text{diss,att}}) (nm)</td>
<td>24 ± 3(^a)</td>
<td>29 ± 8</td>
<td>25 ± 6(^b)</td>
<td>26 ± 8</td>
</tr>
<tr>
<td>(\Delta \theta_{\text{diss,att}}) (turns)</td>
<td>0.73 ± 0.05(^a)</td>
<td>1.1 ± 0.3</td>
<td>0.8 ± 0.2(^b)</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>(\Delta \theta_{\text{flipping}}) (turns)</td>
<td>1.7 ± 0.1(^a)</td>
<td>1.6 ± 0.2</td>
<td>1.7 ± 0.1(^b)</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>(\tau_{\text{D,lef}}) (s)</td>
<td>177 ±15±12</td>
<td>105 ±8±7</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>(\tau_{\text{D,right}}) (s)</td>
<td>16 ± 1</td>
<td>24 ± 2</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>(\Delta E_{\text{dwell-time}}) (kJ T)</td>
<td>2.4 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>(\Delta E_{\text{hysteresis-flip}}) (kJ T)</td>
<td>2.3 ± 0.8(^a)</td>
<td>1.2 ± 0.6</td>
<td>2.3 ± 0.4(^b)</td>
<td>2.0 ± 0.7</td>
</tr>
<tr>
<td>(\Delta E_{\text{peak-area ratio}}) (kJ T)</td>
<td>...</td>
<td>1.7 ± 0.7</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

\(^a\)Values taken from our previous study of (H3.1-H4)2 tetrasomes.\(^{48}\)

\(^b\)Values taken from our previous study of (H3.3-H4)2 tetrasomes.\(^{49}\)