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Revisit of reconstituted 30-nm nucleosome arrays reveals an ensemble of dynamic structures

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Abstract

It has long been suggested that chromatin may form a fiber with a diameter of ~30 nm that suppresses transcription. Despite nearly four decades of study, the structural nature of the 30-nm chromatin fiber and conclusive evidence of its existence in vivo remain elusive. The key support for the existence of specific 30 nm chromatin fiber structures is based on the determination of the structures of reconstituted nucleosome arrays using X-ray crystallography and single particle cryo-electron microscopy coupled with glutaraldehyde chemical cross-linking. Here we report the characterization of these nucleosome arrays in solution using analytical ultracentrifugation, nuclear magnetic resonance, and small angle X-ray scattering. We found that the physical properties of these nucleosome arrays in solution are not consistent with formation of just a few discrete structures of nucleosome arrays. In addition, we obtained a crystal of the nucleosome in complex with the globular domain of linker histone H5 that shows a new form of nucleosome packing and suggests a plausible alternative compact conformation for nucleosome arrays. Taken together, our results challenge the key evidence for the existence of a limited number of structures of reconstituted nucleosome arrays in solution by revealing that the reconstituted nucleosome arrays are actually best described as an ensemble of various conformations with a zigzagged arrangement of nucleosomes. Our finding has implications for understanding the structure and function of chromatin in vivo.

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Graphical abstract

Introduction

Genomic DNA in eukaryotic cells is packaged into chromatin that controls essentially all nuclear processes involving DNA, including transcription, DNA replication and DNA repair. The packaging of DNA into chromatin is primarily guided by core and linker histones. The first level of DNA packaging involves the association of DNA with the core histones and the formation of the nucleosome, the recurring structural unit of chromatin [1–3]. The core particle of the nucleosome contains an octamer of core histones (two copies each of H2A, H2B, H3 and H4), around which ~146 bp of DNA is wound in a left handed super helical manner [4, 5]. DNA is further packaged to form the next repeating structural unit of chromatin, the chromatosome, which consists of a linker histone bound to the nucleosome [6–10]. Linker histones also help chromatin fold to a more compact structural form with a diameter of ~30-nm, termed the 30-nm chromatin fiber [11]. However, the structural nature of the 30-nm chromatin fiber and whether it exists *in vivo* are still being debated [12–23].

The crystal structures of the nucleosome and chromatosome core particles have been solved at atomic and near-atomic resolution, respectively [8, 24]. The highest resolution structures that are available of the 30 nm chromatin fibers are a 9 Å resolution crystal structure of a tetra-nucleosome condensed with MgCl_2 [12] and an 11 Å resolution single particle cryo electron microscopy (cryo-EM) structure of the nucleosome array condensed by human linker histone H1.4 with glutaraldehyde cross-linking fixation [19]. These structures show that the nucleosome arrays have a specific zigzagged nucleosome organization, which also appears to be also supported by the spontaneous formation of a disulfide bond between two pre-engineered Cys residues in core histones (H4 V21C and H2A E64C) in the *i* and *i*+2 nucleosomes of the nucleosome array [25]. In contrast, earlier cryo-EM studies showed that the nucleosome arrays containing linker histone H5 are comprised of multiple different conformations with a common zigzagged and loosely packed nucleosome organization [26] or form an interdigitated nucleosome organization in the presence of additional 1.0 mM MgCl_2 [14].

However, uncertainties remain in each of the above structural studies. Specifically, crystal packing could conduce the formation of the specific tetra-nucleosome structure. Formation of disulfide bonds between *i* and *i*+2 nucleosomes might only occur in the transiently

formed conformational state of the nucleosome array rather than in the predominantly populated conformational state. Glutaraldehyde cross-linking might alter the nucleosome array conformation. Linker histone H5 used in the experiments, which is purified from chicken erythrocytes, might contain various post-translational modifications [27] that could affect the folding of chromatin. In addition, statistical analysis was not made for selection of the nucleosome array particles for structural determination in the cryo-EM studies.

We have previously used MgCl_2 to condense the nucleosome array and observed that its sedimentation coefficient changes nearly linearly as a function of MgCl_2 concentration in the range from 0.5 to 2.5 mM with a slope of $3.5 S_{20,w}/\text{mM MgCl}_2$, above which most of the nucleosome array forms insoluble aggregates [28]. Similar behavior has also been observed for the nucleosome array containing chicken linker histone H5/H1 in monovalent salt solution [23, 29, 30]. The linear behavior of the sedimentation coefficient as a function of MgCl_2 concentration suggests that the conformation of the nucleosome array can be changed by very small perturbations, and therefore the nucleosome array is unlikely to exist predominantly as a single unique conformation in solution. We reasoned that the specific structures of the condensed nucleosome arrays determined from the earlier experiments might not adequately represent the conformational variability of the nucleosome arrays in solution.

Here we used solution methods including sedimentation velocity analytical ultracentrifugation (SV-AUC), small angle X-ray scattering (SAXS) and nuclear magnetic resonance (NMR) to characterize reconstituted nucleosome arrays that lack post-translational modifications, chemical cross-linking fixation and crystal packing to avoid possible extrinsic perturbations to the conformation of the nucleosome arrays. Our experimental results reveal that cross-linking fixation and crystal packing can alter the population of the nucleosome array conformations. We also crystallized the nucleosome bound to the globular domain of H5, revealing nucleosome packing that suggests a possible alternative ladder-like nucleosome arrangement in chromatin. Our studies suggest that *in vitro* reconstituted nucleosome arrays comprise an ensemble of conformations with different nucleosome-nucleosome interactions whose population is sensitive to environmental perturbation. We discuss the implications of this ‘ensemble view’ of 30-nm chromatin fiber for understanding the *in vivo* structure and function of chromatin.

Results

Spontaneous Disulfide Cross-Linking Can Alter the Population of Nucleosome Array Conformations

A previous analysis of the nucleosome core particle crystal showed that the histone H4 N-terminal tail in one core particle interacts with the acidic patch residues of H2A in the neighboring core particle (Figure 1A) [5]. It was assumed that such interactions might also occur in the 30 nm nucleosome array and could be used as a tool to investigate the nucleosome array structure [25]. Indeed, two engineered cysteine residues, one in the H4 tail (V21C) and the other in the acidic patch of H2A (E64C) formed a disulfide bond in the condensed nucleosome array when oxidized. The nucleosome array stabilized by the disulfide bond displayed a zigzagged nucleosome organization when imaged using negative

stain electron microscopy. However, the DNA ends of the pair of stacked nucleosome core particles in the crystal faced opposite directions (Figure 1A, right), which made it difficult to rationalize the zigzagged nucleosome organization in the nucleosome array. Subsequent determination of the tetra-nucleosome crystal structure also showed that H4 V21 and H2A E64 are not sufficiently close to form the disulfide bond between the corresponding Cys mutations, and that the ends of the DNA in the closely packed nucleosomes face the same direction (Figure 1B) [12].

Formation of the disulfide bond could occur in a minor population, for instance as a transient conformation of the nucleosome array, which could consequently cause the population of the nucleosome arrays in other conformations to shift towards it. Therefore, the observation of the zigzagged organization of the nucleosome array containing the disulfide bonds does not *per se* provide definitive evidence that the nucleosome array predominantly forms a specific structure with the zigzagged organization in solution. To test whether the nucleosome array with the disulfide bond represents the major conformation of the nucleosome array in solution, we performed velocity sedimentation analysis of a reconstituted nucleosome array consisting of 12 nucleosomes with a nucleosome repeat length of 207 bp under the same conditions used in the earlier study [12]. The nucleosomes were positioned using the '601' DNA sequence [31]. We found that the nucleosome array with the formation of the disulfide bond (oxidized form) had a much larger sedimentation coefficient than the array lacking the disulfide bond (reduced form) (Figure 1C). These results show that formation of the disulfide bond must have occurred in the more compact and transiently formed conformation, which is not representative of the predominant population of less condensed nucleosome array conformations.

Crystal Packing Conduces Tetra-Nucleosome Array Compaction

Formation of the disulfide bond between H4 V21C and H2A E64C, which is not consistent with the crystal structure of the tetra-nucleosome, suggests that in solution the tetra-nucleosome array could exist in multiple conformations in equilibrium and that crystal packing might conduce the formation of the specific more compact tetra-nucleosome structure. To test this possibility, we first performed sedimentation experiments with a tetra-nucleosome array in buffer containing various MgCl_2 concentrations used for crystallization [12]. We found that the sedimentation coefficient changed linearly as a function of MgCl_2 concentration within the range of 40 mM to 120 mM (Figures 2A, B). Below 40 mM, the nucleosome array predominantly formed aggregates. Next, we performed SAXS experiments and found that the scattering curves were different at various MgCl_2 concentrations (Figure 2C). The calculated radii of gyration from the scattering data, which reflect the overall sizes of the nucleosome arrays, also changed linearly as a function of MgCl_2 concentration and were larger than the value calculated based on the crystal structure of the tetra-nucleosome [12] (Figure 2D). We further calculated pairwise distributions from the scattering data and the tetra-nucleosome crystal structure (Figure 2E). We observed two major peaks in the pairwise distribution curve; the first one (centered at ~ 90 Å) is likely due to the distribution within the nucleosome core particle while the second peak (centered at ~ 140 Å) likely reflects the distribution between nucleosomes. The change in the shape of the curves largely comes from the second peak. These results indicate that the tetra-nucleosome

array does not exist predominantly as a well-packed specific structure under the solution conditions for crystallization.

To visualize the conformations of the tetra-nucleosome, we took electron microscopic images of the negatively stained tetra-nucleosome samples at 60 mM MgCl_2 and obtained 2D classification (Figures 2F and S2A, B). We identified 24 classes, with the majority showing open conformations (Figure 2F and Figure S2B). (Note that we were unable to obtain interpretable images of the tetra-nucleosome arrays using cryo-EM due to aggregation and dissociation of the nucleosome arrays on the grid.) To gain further insights into the tetra-nucleosome array conformation, we performed Monte Carlo (MC) simulations of the tetra-nucleosome array using a coarse-grained model [22]. To mimic the interaction of nucleosomes under different concentrations of MgCl_2 , simulations were performed with different stacking energies between nucleosomes. We calculated the sedimentation coefficient for each tetra-nucleosome conformation using the previously developed formalism [32]. Our MC-simulated sedimentation coefficients also showed a linear behavior in the range of the experimentally measured values from 23 to 27.5 S (Figures 2B and S2C). We note that a significant number of partially packed tetra-nucleosomes exist in the conformational ensemble obtained from the MC simulation at ~ 27.5 S (large standard deviation) (Figure 2B, H, I and S2C). A plateau only occurred at much higher stacking energy values, which corresponds to the condition in which the tetra-nucleosome array forms aggregates. Taken together, our results showed that the tetra-nucleosome array in solution does not form a predominant specific structure under the conditions used for crystallization, and that crystal packing is likely responsible for the compaction of the tetra-nucleosome array that is observed by crystallography.

Glutaraldehyde Cross-Linking Perturbs Nucleosome Array Conformation

The cryo-EM structure of the nucleosome array condensed by human linker histone H1.4 with glutaraldehyde cross-linking fixation showed a twisted double helix structure, with the tetra-nucleosome as the structural unit and the globular domain of the linker histone bound to the nucleosome off the dyad [19]. Chemical cross-linking occurs when the C=O groups of glutaraldehyde ($\text{O}=\text{CH}-\text{CH}_2-\text{CH}_2\text{CH}_2-\text{HC}=\text{O}$) react with the primary amine and guanidine groups of the side chains of Lys and Arg residues [33]. Since Lys and Arg residues are important for linker histones to interact with DNA, it is possible that glutaraldehyde crosslinking might alter the interaction and the structure of the nucleosome array.

To investigate the effects of glutaraldehyde on nucleosome array conformation, we determined the velocity sedimentation coefficients of the nucleosome arrays under the same buffer conditions used in the cryo-EM study [19]. We analyzed nucleosome arrays containing 12 nucleosomes with a nucleosome repeat length of 177 bp and the linker histone H1.0 or H1.4 in the presence or absence of glutaraldehyde. We found that the sedimentation coefficients of the nucleosome arrays displayed different values depending on the absence and presence of cross-linking (Figure 3A and B). For the nucleosome array containing H1.0, the sedimentation coefficient increased by ~ 2 S upon cross-linking, consistent with further compaction. Surprisingly, the sedimentation coefficient of the nucleosome array containing H1.4 decreased by ~ 1 S after cross-linking, but consistent with a similar result observed in

the earlier experiment [19]. These results suggest that the structure of the nucleosome arrays could be perturbed by glutaraldehyde cross-linking. To further test this possibility, we measured the sedimentation coefficients of the above nucleosome arrays in the presence of various concentrations of NaCl from 0 to 30 mM. We found that the sedimentation coefficients of the nucleosome arrays increased by more than ~10 S in a nearly linear manner (Figures 3C, D), indicating that the nucleosome arrays condensed by the linker histones alone do not form a highly compact structure.

To explore the possible cause for the decrease in the sedimentation coefficient due to cross-linking, we also performed SV-AUC experiments on the mono-nucleosome bound to the globular domain of H1.4 with and without glutaraldehyde cross-linking. We found that cross-linking decreased the sedimentation coefficient by ~0.7 S (Figures 3E and F), suggesting that glutaraldehyde cross-linking perturbed the interactions between the linker histone and the nucleosome. We also examined the effects of cross-linking on the histone tails in the nucleosome arrays and found that the NMR peaks of the histones became unobservable upon addition of glutaraldehyde, indicating that involvement of both core and linker histone tails in cross-linking (Figure S3). These results suggest that glutaraldehyde cross-linking could indeed alter the conformation of 30 nm nucleosome arrays.

Glutaraldehyde Cross-Linking Perturbs the Linker Histone Binding Mode

To further investigate the effects of glutaraldehyde cross-linking on the structure of the nucleosome bound to the globular domain of H1.4, we performed methyl-transverse relaxation-optimized spectroscopy (TROSY) NMR and spin label experiments in the absence or presence of glutaraldehyde. In these experiments, we linked H3 K37C to the spin label reagent (MTSL) through a disulfide bond and monitored its effects on the Ile, Leu, and Val methyl groups in the globular domain as we did in our previous studies [8, 10, 34]. The effect of the spin label on the NMR peak intensity depends on the distance between the spin-label and the methyl group. We found that the spin labels had small effects on the methyl groups of the globular domain of H1.4 in the absence of cross-linking (Figures 4A, B), indicating that the globular domain of H1.4 is distant from both spin labels and binds to the nucleosome on the dyad [8, 10].

In contrast, in the presence of glutaraldehyde, the methyl groups showed strong peak intensities and the methyl groups of the same residue type displayed essentially the same chemical shift (Figure 4C). These results indicate that crosslinking by glutaraldehyde disrupted the specific tertiary packing of the globular domain of H1.4, leading to unrestricted motions of the methyl groups. Importantly, the peak intensities of the methyl groups were substantially decreased due to the H3 K37C MTSL spin label (Figure 4D) and were only slightly higher than those predicted based on the location of the globular domain of H1.4 in the cryo-EM structure of the nucleosome array (Figure 4D) [19]. Thus, upon cross-linking the methyl groups must become closer to one of the two spin label sites and the globular domain moved away from the dyad of the nucleosome. To further confirm this surprising observation, we investigated the effects of cross-linking on the structure of the free globular domain of H1.4. We found that most of the NMR peaks in the ^1H - ^{15}N two-dimensional spectrum disappeared even though a significant amount of monomeric globular

domain remained in the solution, revealing a molten-globule-like feature of the crosslinked globular domain of H1.4 [35] (Figures S4A, B).

The observation that glutaraldehyde cross-linking can change the binding mode of the H1.4 linker histone in the mono-nucleosome suggests that cross-linking might also cause a similar binding mode change in the nucleosome array containing the linker histone H1.4 [19, 36]. To examine the binding mode of the globular domain of H1.4 in the nucleosome array, we performed folding experiments using the nucleosome array with 12 nucleosomes and a nucleosome repeat length of 177 bp (Figure 4E). In the presence of 0.3 mM Mg^{2+} , we found that the globular domain of H1.4 condensed the nucleosome array in the same way as the globular domain of H5 (Figure 4F), which binds to the nucleosome on the dyad. In contrast, the globular domain of *Drosophila* H1, which binds to the nucleosome off the dyad [10], does not significantly condense the nucleosome array [8]. Similarly, we found that full-length linker histones H1.4 and H1.0 condensed the nucleosome array to a similar extent (Figure 4G and 1C, D), whereas full-length *Drosophila* H1 had much less effect on the nucleosome array compaction [8]. These results show that it is the on-dyad binding mode of the linker histone, not the tails of the linker histones, that plays a dominant role in determining the folding of the nucleosome array, and that H1.4 behaves like H1.0.

Alternative Nucleosome Packing Modes

In a screen for crystals of the nucleosome bound to the globular domain of H5, we identified a crystal that diffracted at a resolution of 5.5 Å and solved its structure (Table 1). This structure is consistent with our earlier crystal structure at 3.5 Å resolution (Figure 5A). Interestingly, the nucleosomes are packed in two parallel columns in the crystal (Figure 5B). The ends of the DNA of a nucleosome in one column contact the ends of the DNA in the two neighboring nucleosomes in the other column. If such nucleosome packing occurs in a chromatin containing a continuous DNA, it would suggest a chromatin conformation in which nucleosomes pack in a zigzag manner to form a ladder-like structure. To investigate how a linker DNA may be accommodated between the nucleosomes in the two columns, we performed structural modeling in which we kept the same nucleosome packing while changing the distance between the two columns and rotating one column along its own central axis (Figure S5). We found that a nucleosome repeat length of 171 bp produced a nucleosome array structure with low elastic energy (less bending and twisting of DNA), suggesting that such a conformation is physically plausible (Figure 5C). In fact, the elastic energy of our model is much smaller than those of earlier 30-nm chromatin structural models. Furthermore, the calculated sedimentation coefficient of our model for a 12 nucleosome array (55 S) is close to the experimentally measured value of a 12×172 nucleosome array condensed by linker histone H5 (~56 S) [37]. Notably, heterogeneous structural states with different nucleosome packings for a tetra-nucleosome array have also been reported based on the low-resolution crystal structures [38]. More specifically, it was shown that in solution, there was a noticeable (albeit relatively weak) H2BQ44C-H2BQ44C cross-linking in 12×162 and 12×172 arrays, but no cross-linking in 12×157 , 12×167 and 12×177 arrays, which could not be explained based on the available X-ray and cryo-EM structures. Our structural model suggests a feasible explanation of their cross-linking results (Figures 5, 6).

To gain insights into the interactions that may stabilize nucleosome array structures, we examined nucleosome packing in the available crystal and cryo-EM structures. For the tetra-nucleosome structure, the H4 tails seem to mediate the interactions between the two stacked nucleosomes through interacting with neighboring DNA (Figure 6A). H4 residues K20 and R23 are close to the DNA of the neighboring nucleosomes, while residues K16, R17 and R19 interact with the DNA in its own nucleosome. We have previously shown that the H4 K16Q mutation can cause the unfolding of the basic patch of H4 in the nucleosome core particle [28], which is also known to cause a decrease in the sedimentation coefficient for the condensed nucleosome arrays. In the ladder-like nucleosome packing arrangement, a major interaction appears to occur between the DNA in one nucleosome and the histone H2B α helix in the neighboring nucleosome (Figure 6B). Three positively charged H2B residues in one nucleosome are close to the DNA in the neighboring nucleosome, whereas the H4 tails do not appear to contribute to direct nucleosome-nucleosome interactions. In the cryo-EM structural model, the packing interactions within the tetra-nucleosome unit are similar to those in the tetra-nucleosome crystal structure. However, between tetra-nucleosome units, no stabilizing interactions seem to exist since the neighboring nucleosomes are far apart (Figure S6).

Discussion

Our results reveal that the reconstituted 30-nm nucleosome arrays in solution can be easily perturbed by salt concentration, chemical cross-linking and crystal packing. This characteristic is indicative of heterogeneously folded non-specific structures. Using solution NMR, we found that glutaraldehyde cross-linking can even disrupt the tertiary packing of the globular domain of H1.4 and change its binding mode in the mono-nucleosome. Our finding that the globular domain of H1.4 binds on the dyad of the mono-nucleosome is consistent with a recent cryo-EM result that the globular domain of human linker histone H1.5, a paralogue of H1.4, binds to the mono-nucleosome on the dyad [36]. This agreement is expected since the globular domains of H1.4 and H1.5 share 95% sequence identity and all positively charged residues are conserved (Figure S6A). The on-dyad binding mode of H1.4 in the mono-nucleosome is, however, in direct contrast to its off-dyad binding mode in the cryo-EM nucleosome array structure [19]. It has been suggested that the different binding modes of H1.4/H1.5 in the cryo-EM structures of the mono-chromatosome and the nucleosome array could be caused either by glutaraldehyde cross-linking or by the folded structure of the nucleosome array in which re-orientation of the linker DNA forces the linker histone to move away from its location at the dyad [36].

Available experimental results appear to favor the cross-linking perturbation hypothesis. (i) Under the low salt condition used for determination of the nucleosome array structure by cryo-EM [19], the nucleosome array is only moderately condensed (Figure 3D). Atomic force spectroscopic experiments revealed that substantial extension occurs to reconstituted nucleosome arrays under low force (< 2.5 pN) in a highly compliant non-cooperative manner [39, 40]. In contrast, disruption of the chromatosome structure requires a much higher force (> 9 pN) [40]. It is unlikely that the weaker packing interactions between nucleosomes in the nucleosome array can disrupt the stronger intrinsic binding of the linker histone to the nucleosome. (ii) Human H1.0 or H1.4 condenses the nucleosome array to the

same extend and stronger than *Drosophila* linker histone H1, which binds off the dyad of the mono-nucleosome (Figures 4F, G), suggesting H1.0 and H1.4 bind on the dyad of the nucleosome in the nucleosome array. (iii) Glutaraldehyde cross-linking can change the on-dyad binding mode of the globular domain of H1.4 in the mono-nucleosome to an off-dyad binding mode (Figures 3B, E, F; Figure 4A-D). (iv) In the cryo-EM structure of the nucleosome array, the linker histone globular domain forms dimers between the tetra-nucleosome units. The large number of Lys and Arg residues on the surface of the globular domain of H1.4 made dimer formation energetically unfavorable for the dimer formation due to electrostatic repulsion, whereas they are prone to cross-linking by glutaraldehyde. In addition, H4 Arg23 in the nucleosome of one tetra-nucleosome unit does not make direct contacts with the acidic patch residues in the nucleosome of the neighboring tetra-nucleosome unit (Figures 6C and S6C). The observed density between H4 Arg23 and the region near the acidic patch in H2A/H2B might result from a cross-linking between H4 Arg23 and H2B Lys105 in the neighboring nucleosome [19] (Figure S6B), which in turn could contribute to the twisted feature of the nucleosome array structure. When H4 Arg23 is mutated to Ala and thereby could not be cross-linked, the nucleosome array displayed a less-distorted ladder-like structure [19]. Future cryo-EM studies of the nucleosome array in the absence of chemical crosslinking are needed to fully resolve this issue.

By re-examining the key evidence supporting the existence of specific structures of nucleosome arrays, our study shows that even the reconstituted 30-nm nucleosome arrays with well-positioned nucleosomes and the nucleosome repeat lengths favorable for nucleosome array compaction do not form uniquely folded structures. Structural heterogeneity of a tetra-nucleosome array has also been reported in a recent crystallographic study [38]. These results are in agreement with the observation that packing interactions between nucleosomes are, in general, weak [39–42]. The structures of the nucleosome arrays determined by X-ray crystallography and cryo-EM methods have relatively shorter nucleosome repeat lengths (187 bp or less) that favor compaction of nucleosome arrays. Nucleosome arrays with longer nucleosome repeat length is expected to have more flexible conformations as they show smaller sedimentation coefficients[37]. A common feature of these nucleosome array structures is the zigzagged nucleosome arrangement. This structural feature can be understood based on the intrinsic physical properties of DNA, the nucleosome and the chromatosome: (i) the DNA entry and exit sites are on the same side of the nucleosome core particle structure (Figure 1A); (ii) double stranded DNA has a persistence length (less bendable) of ~70 bp [43]; (iii) linker histones further restrict linker DNA orientation [8, 26, 36]. Therefore, reconstituted nucleosome arrays in solution are likely best described as an ensemble of different conformations with zigzagged arrangement of nucleosomes in dynamic equilibrium (Figure 7).

The ensemble view of nucleosome arrays has implications for understanding the structure and function of chromatin *in vivo*. Available *in vivo* experimental results are largely consistent with this view. For example, FRAP experiments revealed that the residence time of linker histones in chromatin is short, on the order of a minute [44], suggesting that chromatin containing linker histones has a dynamic structure. A number of studies have failed to reveal long regularly folded 30-nm fibers, including those using cryo-EM tomography [21], small angle X-ray [13], Micro-C [45], super-resolution light microscopy

[46], electron microscopy-assisted nucleosome interaction capture cross-linking [47], *in situ* spatially correlated DNA cleavage mapping [20] and ChromEMT [48]. The dynamic conformational states of chromatin *in vivo* could also be readily regulated by other protein factors and/or posttranslational modifications to perform biological functions. For example, H4 K16 acetylation (K16Ac) is a prevalent posttranslational modification and plays important roles in the maintenance of euchromatin and transcriptional activation [49–51]. In particular, H4 K16Ac has implicated in de-condensing the chromatin structure [52, 53]. In the ensemble view of chromatin structure, the chromatin conformation with nucleosome packing, like those in the tetra-nucleosome crystal structure containing unacetylated H4 K16 histones, might represent a substantial population (Figure 6A). Upon acetylation, the ladder-like nucleosome packing population could increase, since H4 K16Ac would disrupt the tetra-nucleosome-like packing but would have little effects on the ladder-like nucleosome packing (Figures 6A, B). In comparison with the helical structural models of 30-nm chromatin fibers, the ladder-like conformation is more open and accessible to transcription factors. In addition, our modeling shows that the ladder-like nucleosome packing is favored by a linker length of ~25 bp, which belongs to the topological family of chromatin fibers with linker length of $10n+5$ bp and may be more transcriptionally active. In contrast, the tetra-nucleosome and cryo-EM structural models belong to the topological group with linker length of $10n$ bp, which appears to be more transcriptionally repressed [54].

EXPERIMENTAL PROCEDURES

Cloning and Purification of Linker Histones

E. coli-codon optimized DNA fragment encoding residues of full length human H1.4 was synthesized (BioBasic, Ontario). The cDNA of human H1.0 was obtained from Origene (Rockville, MD). These were cloned into the pET42b vector in frame with a C-terminal His6 tag. The globular domain of H1.4 (GH1.4) and H5 (GH5) was sub-cloned into the pET15b vector in frame with a N-terminal His6-tag followed by a thrombin cleavage site. Mutations were generated using the Quikchange kit (Stratagene, CA) and the DNA sequences were verified by DNA sequencing (Genewiz, NJ). Linker histones were expressed in *Escherichia coli* BL21(DE3) RIPL cells and purified by metal-affinity chromatography with NiNTA resin (Qiagen, CA) under denaturing conditions. The his6-tag was removed by Thrombin digestion at room temperature. The protein was further purified by reverse phased HPLC with a Protein-RP column (YMC, Japan) and lyophilized. Purification of wild type *Drosophila* core histones and their mutants (H2AE64C, H3C110A, H3C110A/K37C, H4V21C) followed the procedure described previously [55]. $^{15}\text{N}/^2\text{D}$ labeled core histones, linker histones, and $^{15}\text{N}/\text{ILV}$ methyl labeled GH1.4 were expressed in M9 medium with the desired isotopes [10].

DNA, Nucleosomes and Nucleosome Array Reconstitution

A redesigned 167 bp DNA (see sequence below) based on the 147 bp “601” DNA (kindly provided by Tan Song lab) was released from pUC19 plasmid by EcoRV digestion (New England Biolabs, MA). Tandem repeat 4×167 bp and 12×177 bp “601” DNA were released from the plasmid (Kindly provided by Timothy Richmond lab) by EcoRV digestion (New England Biolabs, MA)[12, 25]. Tandem repeat 12×207 bp “601” DNA (Kindly

provided by Karolin Luger lab) was released from the plasmid by HindIII, XbaI, and DraI digestion (New England Biolabs, MA). Further purification of DNA using a Prep Cell (BioRad, CA) followed the procedure described previously [56]. 167 bp mono-nucleosome reconstitution and purification followed the procedures described in previous study [55]. 12×177 bp, 12×207 bp nucleosome arrays, and 4×167 tetranucleosome were assembled according to Dorigo et al [25]. Briefly, Drosophila histone octamer, “601” sequence in array DNA and 147-bp competitor DNA (from pUC18 plasmid backbone, with lower histone octamer affinity) were mixed at 1.25 : 1 : 0.3 molar ratio in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 (TE 10/1) buffer with 2 M NaCl and 10 mM DTT. The nucleosome array was assembled by dialyzing against TE 10/1 buffer with 2M NaCl, and the salt concentration of dialysis buffer was continuously diluted to 0.6 M by adding in TE 10/1 buffer using a P1 pump over 16h, followed by a final 6-h dialysis step in 10 mM Hepes, 0.1 mM EDTA, pH 8.0 (HE 10/0.1) buffer. The nucleosome array was separated from mono-nucleosome and competitor DNA using a Superose 6 size exclusion chromatography in HE 10/0.1 buffer. The saturation of the reconstituted nucleosome array by histone octamers was confirmed by ScaI (12×177 nucleosome array and 4×167 tetra-nucleosome) or EcoRI (12×207 nucleosome array) digestion, which produced only mono-nucleosomes. For linker histones incorporation, an equal molar amount of linker histone (relative to mononucleosomes in the array) was mixed with the array in TE 10/1 buffer with 0.6 M NaCl, then the salt was removed by a final overnight dialysis step in HE 10/0.1 buffer.

Redesigned 167 bp DNA sequence (“601” sequence underlined):

ATCGGCCGCCATCGAGAATCCCGGTGCCGAGGCCGCTCAATTGGTCGTAGACAGC
TCTAGCACCGCTTAAACGCACGTACGCGCTGTCCCCGCGTTTTAACGCCAAGG
GGATTACTCCCTAGTCTCCAGGCACGTGTCAGATATATACATCCGATGCATGTAGAT

Chemical Cross-linking

Disulfide bond crosslinking of linker histone-bound nucleosome array was followed the procedures published previously [25]. Briefly, 12×207 arrays containing histone mutants (H2AE64C/H3C110A/H4V21C) were reduced in TE 10/1, pH 8.0, 100 mM DTT for 2h. After removing DTT by G-20 buffer exchange spin column, the nucleosome array solution was mixed 4:1 with a solution containing 125 mM Tris-Cl, pH 9.0, 2.5 mM oxidized glutathione, 2.5 mM reduced glutathione, and 330 mM NaCl at 37° C. After overnight incubation, the remaining free thiols were blocked by adding 10 mM iodoacetamide (FLUKA) to the solution at 22° C for 1 h in the dark. The resulting array was extensively dialyzed against TE 10/1 buffer and subjected to SDS-PAGE gel and AUC analysis.

Glutaraldehyde crosslinking of GH1.4, GH1.4-bound nucleosome or linker histone-bound nucleosome array was performed according to Song et al. [19]. 0.2 mg/ml (by DNA concentration) purified mono-nucleosome or nucleosome array, and 0.5 mg/ml GH1.4 in HE10/0.1 were mixed with 10% glutaraldehyde (EM Science) 50:1. After incubating on ice for 30 min, the reaction was quenched by mixing 1:1 with 100 mM Tris-HCl, pH8.0. Crosslinked samples were then dialyzed against TE 10/1 buffer overnight before analyzing by SDS-PAGE gel, AUC and NMR.

Analytical Ultracentrifugation Analysis

Sedimentation velocity data were collected at 20 °C by centrifugation at 15,000 rpm in a Beckman XL-A analytical ultracentrifuge following standard protocols [57]. The absorbance was monitored at 260 nm or 280 nm. Data were analyzed in SEDFIT [58] in terms of a continuous $c(s)$ distribution spanning a sedimentation coefficient range of 0 to 100 (or 150) S with a resolution of 200 (or 300) points and a confidence interval of 68%. The sedimentation coefficient reported was the value at the peak maximum. A partial specific volume of 0.65 cm³/g was used [59]. Solution densities and viscosities were calculated in SEDNTERP [60], and sedimentation coefficients were corrected to standard conditions $s_{20,w}$.

Nuclear Magnetic Resonance

Isotope labeled core and linker histones were incorporated into the nucleosome array as described above. After reconstitution and glutaraldehyde crosslinking, the array samples were buffer exchanged into NMR buffer (10 mM sodium phosphate buffer, pH 6.3, 0.5 mM EDTA with 10% D₂O) using a 30 Kda cut-off centrifugal filter unit (EMD Millipore, MA). ¹H-¹³C HSQC NMR spectra were collected on Bruker 700 and 900 MHz NMR instruments equipped with cryoprobes.

To determine the binding mode of GH1.4 on the nucleosome, H3 Lys37 was mutated to Cys (the H3 Cys110 was mutated to Ala simultaneously) and linked to MTSL (S-(2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl) methyl methanethiosulfonate) as described previously [10]. The completion of the labeling was confirmed by mass spectrometry. Nucleosomes bearing the MTSL spin labels were reconstituted in the buffer without reducing agent. Leu, Val, and Ile methyl-labeled globular domains were mixed with spin labeled nucleosomes at a ratio of 0.75 in the NMR buffer (20 mM Tris-D11-DCI, pD 7.4, 0.1 mM EDTA, 99.8% D₂O). ¹H-¹³C HMQC spectra of the globular domain-nucleosome complex were recorded before and after the reduction of the MTSL with 5 μl of 1 M DTT. The same NMR experimental procedure was followed for samples with glutaraldehyde crosslinking. The data was analyzed using NMRPipe (Delaglio et al., 1995). The peak intensities of the well-separated peaks were measured using NMRViewJ (One Moon Scientific Inc. MD) or UCSF Sparky software (<http://www.cgl.ucsf.edu/home/sparky/>).

Small-Angle X-ray Scattering Analysis

SAXS data were collected at beamline BioCAT (18ID-D) at the Advanced Photon Source at the Argonne National Laboratory. Purified 4×167 tetranucleosome in the presence of various concentrations of Mg²⁺ were run in-line on a Superose 6 10/300 column at a flow rate of 0.5 mL/min directly connected to the sample chamber, and images were collected during 1-s exposures. Two-dimensional diffraction images were reduced to one-dimension scattering data, and buffer scattering was subtracted. Data analysis and radius of gyration (R_g) and pairwise distance distribution function $P(r)$ calculations were performed using PRIMUS [61].

Negative Staining Electronic Microscopy (EM) analysis

4×167 tetranucleosome in 10mM Tris-HCl, pH 8.0, 50mM KCl and 60 mM Mg²⁺ were diluted to 60 ng/ml using the same buffer. Negative-staining samples were prepared using 1.0% uranyl acetate. Carbon coated copper grids were glow discharged for 30s, and then 5 µl of sample were loaded. The excess sample on the grid was first blotted using filter paper, then washed with water and uranyl acetate, sequentially. Images were recorded on a 200 kV Tecnai F20 microscope (FEI) equipped with a 2000 × 2000 Eagle CCD camera at a magnification of ×62,000, corresponding to pixel size of 3.54 Å on the images. A total of 34,000 particles from 1000 images were picked using XMIPP [62]. Two-dimensional classification was performed with RELION 1.4 [63].

Crystallization and Structure Determination

The H5₂₂₋₉₈- nucleosome complex was reconstituted at 1.1 : 1 ratio in 10 mM Tris-HCl, 1 mM EDTA and 1 mM DTT buffer at pH 8.0. Small amounts of aggregation were removed by high-speed centrifugation at 4 °C. The complex was concentrated to 10–15 mg/ml and used for crystal screening without further purification. Crystals were screened using the crystallization robot, Mosquito (TTPLabTech.com, UK). Crystals were optimized using hanging drop method at 18 °C. The reservoir (0.5 ml) contained a solution of 0.1 M NH₄NO₃ and 10% MPD (v/v) at pH 4.0. The complex (0.5 µl at ~10 mg/ml) was mixed with 0.5 µl of the reservoir solution. A dehydration process was applied to crystals by addition of 10–12% of MPD (v/v) to the crystal drop and then soaking for 10–20 minutes [64]. We then added 20% glycerol (v/v) to the drop for cryo-protection immediately before picking the crystal for flash freezing using liquid nitrogen. The crystal yielded the 5.5 Å resolution diffraction data. The X-ray diffraction data was collected at GM/CA-CAT (beamline 23ID-B) at the Advanced Photon Source (APS) with a wavelength of 1.033 Å. The diffraction data were processed with XDS [65]. Data analysis by XTRIAGE in the PHENIX suite indicated a possible 2fold merohedral twin operator (*h*, *-h-k*, *-l*) [66]. The estimated twinning fraction was 0.34.

The structure solution was found using the molecular replacement method (Phaser) [67] with the model of the GH5 and nucleosome complex (pdb ID: 4QLC) [8] by removing GH5 (Phaser scores: TFZ=27.9, LLG=815). Two noncrystallographic copies are found in asymmetry unit. These two nucleosomes were connected by the DNA linker and form a "zigzag" twist. The crystallographic packing revealed a ladder-like structure with two parallel columns. After the rigid body refinement, the electron densities on the difference maps clearly showed that GH5 was located in the middle of two DNA linkers near the dyad-axis. We then manually located the globular domain (PDB code: 1HST chain B) to fit the difference map, and further refined using the DEN-based strategy for low resolution [68]. The model of 4QLC was used as the reference model in PHENIX. The data collection and refined model quality is shown in Table 1.

Modeling of a Ladder-Like Nucleosome Array Structure

The side view of the X-ray structure showed two stacks of nucleosomes organized in such a way that the end-to-end distances between the adjacent nucleosomes from the opposite stacks alternate between 67 and 77 Å (Figure S5A). When the nucleosomes were connected

by DNA linkers, the energy of DNA bending and twisting (the so-called elastic energy) proved to be rather high (~20 kT) for nucleosome repeat length (NRL) between 168 and 171 bp. To relieve this tension in DNA linkers, we rotated the right stack by 8° counterclockwise, thereby making the two-start fiber symmetric. In addition, we displaced the right stack from the original position by a variable distance from 5 to 10 Å (Figure S5B). Simultaneously, the NRL was varied from 168 to 173 bp, and the elastic energy of DNA was optimized for each NRL value and for each displacement as described earlier [22]. As a result of this optimization, the energetically favorable array conformation had an NRL of 171 bp and was selected as our model (Figure 5C). Note that the elastic energy of the linker DNA in our model is ~5 kT per nucleosome, which is comparable to the energy in optimal two-start chromatin fibers calculated without spatial restraints [22].

Computing Sedimentation Coefficients

The sedimentation coefficient $S_{20,w}$ for an oligo-nucleosomal array is calculated using the following equation [32]:

$$\frac{S_N}{S_1} = 1 + \frac{R}{N} \sum_i \sum_j \frac{1}{R_{ij}}$$

Here, S_N represents $S_{20,w}$ for a rigid structure consisting of N nucleosomes of radius R , R_{ij} is the distance between the centers of two nucleosomes, and S_1 is $S_{20,w}$ for a mono-nucleosome. This approach assumes that nucleosomes are spherical particles with radius $R = 5.5$ nm and $S_1 = 11.1$ S.

Monte Carlo Simulations

We used the crystal structure (pdb ID: 3MVD) as the nucleosome core particle [69], which was fixed during simulation and could only go through rigid body motions. The dynamic part of the tetra-nucleosomes in MC simulations are the DNA linkers. The DNA was modeled at the level of dimeric steps, and its trajectory was described by the six base-pair step parameters [70]. The geometry of the linker DNA fluctuates around the regular B-DNA parameters (Twist, Tilt, Roll, Shift, Slide, Rise) = (34.5°, 0, 0, 0, 0, 3.35 Å) on average. We used the following four energy terms: elastic (DNA bending and twisting), electrostatic, stacking interactions between nucleosomes and the steric hindrance terms as specified in detail elsewhere [22]. 200,000 successful MC cycles were performed. The system was equilibrated after 30,000 MC cycles. Statistical averaging of various parameters of the system (*e.g.*, sedimentation coefficient) was done for the last 150,000 cycles taken well after the system was equilibrated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- Disulfide cross-linking can alter nucleosome array conformation population
- Crystal packing conduces tetra-nucleosome array compaction
- Glutaraldehyde cross-linking can perturb nucleosome array conformation
- A novel ladder-like nucleosome packing mode
- 30-nm nucleosome arrays exist as an ensemble of dynamic structures

Figure 1. Alteration of Nucleosome Array Conformation by Disulfide Bond Cross-Linking
 (A) Structural illustration of the nucleosome core particle packing in the crystal (pdb ID: 1AOI). Left: H4 V21 in the top nucleosome core particle is close to the H2A E64 in the bottom nucleosome core particle. Right: a different view of the structure; the red arrows indicate the ends of the DNA. DNAs are colored purple for the top nucleosome core particle and cyan for the bottom nucleosome core particle. Core histones are shown in grey except for H4 (green) in the top nucleosome and H2A (orange) in the bottom nucleosome that are close to each other.

(B) Structural illustration of the nucleosome core particle packing in the crystal structure of the tetra-nucleosome (pdb ID: 1ZBB). Labels and colors are the same as in (A).

(C) Normalized sedimentation velocity $c(s)$ profiles of the nucleosome arrays with core histone WT and Cys mutations (H4 V21C and H2A E64C) under reduced (red) and oxidized (blue) conditions.

Figure 2. Flexible Conformations of the Tetra-Nucleosome in Solution

(A) Normalized Sedimentation velocity $c(s)$ profiles for the tetra-nucleosome in the presence of various concentrations of MgCl_2 .

(B) Sedimentation coefficients of the tetra-nucleosome at various concentrations of MgCl_2 (solid circles) and those calculated from Monte Carlo simulation with various stacking energies for nucleosome packing (open circles). The error bars are the standard deviations for the sedimentation coefficients obtained from the Monte Carlo simulation. The solid line is the linear fitting result for the measured sedimentation coefficients. The curve with a dashed line illustrates a hypothetical cooperative folding of the tetra-nucleosome.

(C) X-ray scattering intensity profiles of the tetra-nucleosome in the presence of various concentrations of MgCl_2 .

(D) Radius gyrations derived from the SAXS results (solid circles). The solid line is the fitting result to a linear function. The calculated radius gyration of the tetra-nucleosome crystal structure (pdb ID: 1ZBB) is indicated by open circles and is plotted on the extrapolated line (dashed) from the fitting curve.

(E) Pairwise distributions derived from SAXS results. The dashed line is the result calculated from the crystal structure of the tetra-nucleosome (pdb ID: 1ZBB). The two vertical parallel lines are at the two peak values of the distribution.

(F) Representative 2D classifications of the negatively stained tetra-nucleosome images in the presence of 60 mM MgCl_2 . The scale bar is 10 nm.

(G) PyMol cartoon representation of the tetra-nucleosome crystal structure (pdb ID: 1ZBB). The atoms are shown as spheres with linker DNA removed.

- (H) Cartoons of representative conformations of tetra-nucleosomes obtained from the Monte Carlo simulation. The yellow and white balls represent the histone core. DNA is shown as cyan and orange lines. The small black balls were used to indicate the ends of DNA.
- (I) Cartoon representation of the tetra-nucleosome crystal structure (pdb ID: 1ZBB).

Figure 3. Glutaraldehyde Cross-Linking Perturbs the Conformation of the Nucleosome Arrays and the Mono-nucleosome bound to GH1.4.

(A) Sedimentation velocity $c(s)$ profiles for the nucleosome arrays containing linker histone H1.0 with (blue) and without (red) glutaraldehyde cross-linking, respectively.

(B) Sedimentation velocity $c(s)$ profiles for the nucleosome arrays containing linker histone H1.4 with (blue) and without (red) glutaraldehyde cross-linking, respectively.

(C) Sedimentation coefficients of the nucleosome arrays containing linker histone H1.0 in the presence of various concentrations of NaCl. Solid and open circles represent results from two independently reconstituted nucleosome arrays, respectively.

(D) Sedimentation coefficients of the nucleosome arrays containing linker histone H1.4 in the presence of various concentration of NaCl. Solid and open circles represent results from two independently reconstituted nucleosome arrays, respectively.

(E) Native gel electrophoresis of the nucleosome containing the globular domain of H1.4 in the presence and absence of glutaraldehyde cross-linking.

(F) Sedimentation velocity $c(s)$ profiles for the nucleosome containing the globular domain of H1.4 in the presence and absence of glutaraldehyde cross-linking.

Figure 4. Glutaraldehyde Cross-Linking Alters the Binding Mode of Linker Histone H1.4.

(A) Methyl-TROSY spectra of the globular domain of H1.4 bound to the nucleosome without glutaraldehyde cross-linking in the presence (left) or absence (right) of spin label. (B) Bar graphs showing the effects of the spin labels on the methyl groups. The error bars represent the deviations from the averaged values of two experiments. The diagram (right) illustrates the on-dyad binding mode and the red oval represents the globular domain of H1.4 in the absence of crosslinking. The black lines represent DNA in the nucleosome. (C) Methyl-TROSY spectra of the globular domain of H1.4 bound to the nucleosome in the presence (left) or absence (right) of spin label. The lack of peak dispersion suggests that the tertiary structure of the globular domain was disrupted. (D) The measured effects of the spin labels on the methyl group types in the presence of glutaraldehyde cross-linking and those predicted based on the location of the globular domain of H1.4 in the cryo-EM structure of the nucleosome array [19]. The diagram (right) illustrates the off-dyad binding mode and the blue oval represents the globular domain of H1.4. (E) Diagram illustrating nucleosome array folding by the globular domain in the presence of 0.3 mM MgCl_2 (to mimic the effects of the linker histone tail) or the full-length linker histone. (F) Sedimentation velocity $c(s)$ profiles of nucleosome arrays condensed by the globular domains of linker histone *Drosophila* H1 (GH1), Chicken H5 (GH5), and human H1.4 (GH1.4) in the presence of 0.3 mM MgCl_2 . The data for *Drosophila* GH1 and chicken GH5 are from previously published results [8].

(G) Sedimentation velocity $c(s)$ profiles of nucleosome arrays condensed by the full-length linker histones: *Drosophila* H1, human H1.0 and H1.4. The data for *Drosophila* H1 is from previously published results [8]. Error bars represent the range of the values measured in two independent experiments.

Figure 5. A Ladder-Like Nucleosome Packing in the Crystal and Modeling of a Nucleosome Array Structure.

(A) Omit map of the globular domain of H5 and linker DNA. Green color represents the $F_o - F_c$ map at $\sigma=3$ level and the gray color represents the $2F_c - F_o$ at $\sigma=1$.

(B) Ladder-like packing of the nucleosomes in the crystal. The ends of the DNA in one nucleosome are packed against the ends of the DNA in the nucleosome in the neighboring column.

(C) The ladder-like structural model of a nucleosome array based on the observed nucleosome packing in the crystal.

Figure 6. Comparison of Nucleosome Packings in Available Structural Models of Nucleosome Arrays.

(A) Nucleosome packing in the tetra-nucleosome structure.

(B) Nucleosome packing in the ladder-like structure.

(C) Nucleosome packing between tetra-nucleosome units in the cryo-EM structure. H4 R23 and the acidic patch in H2A/H2B are not in direct contacts.

Figure 7. Diagram Illustration of the Ensemble of Dynamic Structures of 30nm Nucleosome Arrays.

Nucleosome arrays in the presence of linker histones show large conformational fluctuations and can be condensed to more compact forms by addition of salt. The process of condensation is not cooperative and the condensed structures have local conformational fluctuations, which are prone to environmental perturbation. The nucleosome arrays are likely to have a zigzagged nucleosome arrangement, which is determined by the structural features of the nucleosome or chromatosomes.

Table 1

Data collection and refinement statistics

	5WCU (pdb ID)
Data collection	
Space group	P1
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	65.93, 108.54, 180.77
α , β , γ (°)	100.79, 90.08, 89.94
Resolution (Å)	50–5.53 (5.73–5.53) *
Rmerge	0.089(1.71) *
I/ σ (I)	7.3 (0.7) *
Completeness	97.5 (95.7) *
(%)	2.2 (2.2) *
Redundancy	0.079 (1.53) *
Rpim	0.998 (0.227) *
CC1/2	
Refinement	
Resolution (Å)	48–5.53(5.73–5.53) *
No. reflections (unique)	15265
<i>R</i> _{work} / <i>R</i> _{free} (%)	18.8(34.5) */24.0(36.7) *
No. atoms	26640
Core histones	11922
DNA	13568
Globular domain	1150
B-factors	
Core histones	208
DNA	248.2
Globular domain	224.2
R.m.s deviations	
Bond lengths (Å)	0.012
Bond angles (°)	1.51
Ramachandran favor/outlier	99.0/0.06

* Asterisk numbers correspond to the last resolution shell.

$R_{\text{merge}} = \frac{\sum_h \sum_i |I_i(h) - \langle I(h) \rangle|}{\sum_h \sum_i I_i(h)}$, where $I_i(h)$ and $\langle I(h) \rangle$ are the *i*th and mean measurement of the intensity of reflection *h*.

$R_{\text{pim}} = \frac{\sum_h [(1/n-1)/2] \sum_i |I_i(h) - \langle I(h) \rangle|}{\sum_h \sum_i I_i(h)}$, where $I_i(h)$ and $\langle I(h) \rangle$ are the *i*th and mean measurement of the intensity of reflection *h*, and *n* is the redundancy of reflection *h*.

$R_{\text{work}} = \frac{\sum_h |F_{\text{obs}}(h) - F_{\text{calc}}(h)|}{\sum_h F_{\text{obs}}(h)}$, where $F_{\text{obs}}(h)$ and $F_{\text{calc}}(h)$ are the observed and calculated structure factors, respectively.

R_{free} is the R value obtained for a test set of reflections consisting of a randomly selected 5% subset of the dataset excluded from refinement.

** Values from Molprobit server (<http://molprobit.biochem.duke.edu/>).