Chapter 22

Sulfyhydryl-Reactive Site-Directed Cross-Linking as a Method for Probing the Tetrameric Structure of Histones H3 and H4

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Abstract

The structural characterisation of protein–protein interactions is often challenging. Where interactions are not amenable to high-resolution approaches, alternatives providing lower resolution information are often of value. One such approach is site-directed cross-linking. Here, through the introduction of cysteine residues at strategic locations in histone proteins, we use site-directed cross-linking to monitor the association of chromatin subunits. This approach is informative for the study of both recombinant and native chromatin complexes consisting either of histone subunits alone or in association with accessory proteins, in this case histone chaperones. The approaches described may be generally applicable for monitoring the interactions of a diverse range of multi-protein complexes.

Key words: Chromatin, Histone octamer, Tetramer, Nucleosome, Histone chaperone, Chromatin remodelling, Site-directed cross-linking

1. Introduction

Proteins and protein complexes are often not amenable to high-resolution structural analysis. Targeted cross-linking that utilizes the unique reactivity of cysteine side chains has provided valuable structural information regarding topology, conformation, and structural rearrangements of such proteins. The sulfhydryl group of cysteine has a valency of two and an ionization constant of ~8.2, conferring reactivity within a biologically applicable pH range. Cysteines are comparatively rare, accounting for only 0.6% of amino acids in *Saccharomyces cerevisiae* (1), which makes them ideal for site-directed cross-linking analysis. Protein domains of moderate size usually contain only a handful of cysteine residues

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that, generally, can be mutated to a homologous amino acid, such as alanine or serine, without affecting the overall structure or function of the protein. Using recombinant technologies, site-directed mutagenesis can be employed to substitute cysteine residues at strategic locations within the cysteine null protein of interest. The proximity of two cysteine residues can then be analysed using targeted cross-linking of their sulfhydryl groups, either through direct disulphide bond formation or through the use of a homo-bifunctional cross-linking reagent. Cross-linking generally affects the migration of the target protein when analysed by denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), even when cross-linking occurs between residues within the same polypeptide. Thus, SDS-PAGE is a convenient and cheap method for analysing the extent of cross-linking at any given location. Combining these approaches, proximity constraints within the tertiary structure can be placed upon the primary sequence, yielding valuable structural information.

The core histone octamer forms the scaffold for wrapping ~147 bp of DNA within the nucleosome core particle. The histone octamer is composed of two H2A–H2B dimers and an $(H3–H4)_2$ tetramer. Assembly of the nucleosome core particle follows an ordered series of events, H3 and H4 being deposited onto DNA first, proceeded by the deposition of two H2A–H2B dimers to form the nucleosome proper. In vivo assembly and disassembly involve accessory proteins known as histone chaperones. Histone chaperones have been shown to be important in moulding the thermodynamic landscape of histone–DNA interactions, thereby promoting correct nucleosome formation or dissolution (2, 3). In addition, these accessory proteins are often found as components of ATP-dependent, chromatin-remodelling enzymes and histone post-translational modifying complexes (4, 5).

One question that has arisen in the field is the conformation of soluble histones H3 and H4 prior to deposition while in complex with histone chaperones. Interestingly, although adopting a tetrameric conformation in the nucleosome, H3 and H4 exist as an obligate dimer while associated with the ubiquitous chaperone Asf1 (6–8). Recently, however, it has been shown that while in complex with another class of histone chaperone H3 and H4 can adopt their tetrameric conformation previously observed within the nucleosome (9). In this chapter, we describe a method which utilises a site-directed cross-linking strategy to probe the conformation of H3 and H4 (Fig. 1). This is also applicable to H3 and H4 while in complex with accessory proteins, such as histone chaperones, with the previously well-characterised interaction between Asf1 and H3–H4 used as an example of how the methodology can be implemented.

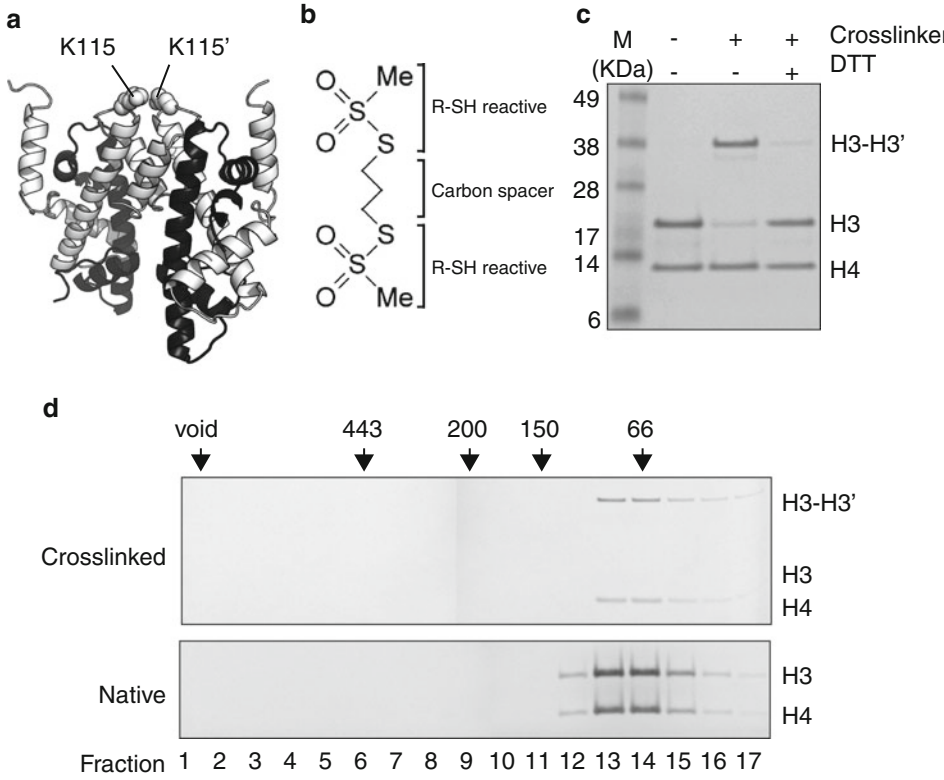

Fig. 1. Site-directed cross-linking as a probe of tetramer structure. (**a**) Histones H3 and H4 shown in their tetrameric structure observed within the nucleosome (coordinates taken from PDB: 1KX5). H3 is shown in *white*, whereas H4 is shown in *black*. The site-directed cross-linking positions, H3K115, are shown as *spheres*. (**b**) Chemical structure of the sulfhydryl-reactive cross-linker 1,3-propanediyl bismethanethiosulfonate (M3M). (**c**) Cross-linking of the $(H3–H4)_2$ tetramer at position H3 K115C. Cross-linking across the dyad interface of H3–H3′ results in a slower migrating species that can be resolved from non-cross-linked H3 by SDS-PAGE. Incubation with the reducing agent DTT reverses the cross-linking. (**d**) Gel filtration analysis shows that the cross-linked tetramer has the same size as non-cross-linked (native) tetramer, demonstrating the fidelity of cross-linking across the dyad interface of H3.

2. Materials

2.1. Preparation of Reduced Histone Tetramers

1. Recombinant histones H3 K115C and H4.
2. Unfolding buffer: 7 M guanidinium chloride, 20 mM HEPES, pH 7.5, 1 mM EDTA. Care should be taken when handling guanidinium chloride as it is toxic, and with EDTA as it is an irritant.
3. Refolding buffer: 1 M sodium chloride, 20 mM HEPES, pH 7.5, 1 mM EDTA.
4. β-mercaptoethanol (β-ME). Care should be taken when preparing β-ME as it is toxic.
5. Dialysis tubing (≤8,000 molecular weight cut-off, such as Spectra/Por® Dialysis Membrane MWCO: 6–8,000, Spectrum Laboratories Inc.).

6. Superdex™ S200 packed gel filtration column (such as GL 10/300, GE Healthcare) and chromatography system.
7. 4× SDS-PAGE loading buffer: 40% glycerol, 240 mM Tris–HCl, pH 6.8, 8% SDS, 0.04% bromophenol blue, 5% β-ME (or pre-made, e.g. by Invitrogen).
8. Dithiothrietol (DTT). Care should be taken when preparing DTT as it is harmful.
9. SDS-PAGE equipment for protein separation and analysis.
10. Centrifugal concentrators (≤10,000 molecular weight cut-off, such as Amicon® Ultra, Millipore™).
11. Liquid nitrogen. Personal protective equipment should be worn while handling liquid nitrogen.

2.2. Targeted Cross-Linking as a Probe of Tetramer Structure

1. Buffer A: 0.5 M sodium chloride, 20 mM HEPES–KOH, pH 7.5, and 1 mM EDTA.
2. Purified histone chaperone of interest in buffer conditions that allow binding to histones. The histone chaperone Asf1 is used as an example in this protocol. Buffer conditions that allow histone binding by Asf1 had previously been determined (buffer A).
3. 1,3-Propanediyl bis-methanethiosulfonate (M3M) (see Note 1).
4. A mono-reactive, small-molecule methanethiosulfonate (MTS) compound, such as propyl methanethiosulfonate (PMTS) (see Note 2).
5. SDS-PAGE equipment for protein separation and analysis.
6. Coomassie protein stain (for example, "Instant*Blue*", Expidion).
7. Dimethyl sulfoxide (DMSO). Care should be taken when handling DMSO as it is an irritant.

2.3. Targeted Cross-Linking as a Method of Tetramer Stabilisation

1. Superdex™ S200 PC 3.2/30 column (e.g. GE Healthcare) and micro-chromatography system (see Note 3).
2. Microcentrifugal filter units (such as Ultrafree® 0.22-μm Centrifugal Filters, Millipore™) (optional).

3. Methods

3.1. Preparation of Reduced H3 K115C Tetramers

For purification of individually expressed recombinant core histones in bacteria and their refolding in vitro, we refer the reader to previous publications (10–12). *Xenopus laevis* histones were used in this study. The single, non-conserved cysteine (C110) of H3 was mutated to alanine and the K115C mutation inserted by site-directed mutagenesis. H3 and H4 are insoluble on their own, but highly

soluble after denaturation and refolding in equal stoichiometries. However, refolding is never 100% efficient, and requires a further chromatographic step to isolate correctly folded tetramers from misfolded aggregates and monomers. We have adapted this step to incorporate the removal of reducing agents from the H3 K115C mutant tetramer prior to cross-linking.

1. Dissolve purified, lyophilised histones H3 K115C and H4 at equal stoichiometries in unfolding buffer + 20 mM DTT at a final concentration of 20–100 μM (individual histone monomer).
2. After 1 h, transfer the unfolded histones to dialysis tubing with a molecular weight cut-off of ≤8,000, and dialyse overnight versus 2 L of refolding buffer + 5 mM β-ME at room temperature or 4°C.
3. Retrieve the refolded histones from the dialysis tubing and centrifuge at 10,000 × *g* to remove any precipitated material.
4. Transfer the soluble portion of the refolding reaction to a centrifugal concentrator (molecular weight cut-off of ≤10,000). The extent of concentration depends on the type of gel filtration column used during size-exclusion chromatography. Typically, resolution among soluble aggregates, histone monomers, and refolded tetramer is maintained up to a loading volume of 1/60th of the column's bed volume. We have found that using a Superdex™ 200 10/300 GL column with a 24-mL volume, a maximum load volume of 400 μL, works well.
5. After concentration, add 20 mM DTT and leave at room temperature for 1 h to ensure that cysteine residues are fully reduced.
6. Chromatography is carried out in refolding buffer without reducing agents. Thus, in addition to isolating the correctly folded tetramer, it serves as a desalting step to remove reducing agents from the sample prior to cross-linking. Equilibration of the gel filtration column can be carried out during the preparation of the refolded tetramer sample.
7. Collect fractions spanning the void to bed volumes of the column. We typically run a Superdex™ S200 10/300 GL column at 0.25 mL/min, collecting 0.5-mL fractions from 8 to 24 mL, using an ÄKTA™ purifier liquid chromatography system (GE Healthcare).
8. After gel filtration, it is important to work as quickly as possible to minimise the oxidising effect of dissolved atmospheric oxygen on the reduced cysteines. An indication of the fractions containing tetramer can be obtained by monitoring the absorbance at 276 nm. This can be confirmed by SDS-PAGE and Coomassie staining. Typically, the tetramer elutes with an

apparent molecular weight of 80–100 kDa, and with experience the appropriate fractions can be selected for step 9 before the SDS gel has been run.

9. Pool tetramer containing fractions and concentrate using a centrifugal concentrator (molecular weight cut-off of <10,000). Monitor the concentration of the tetramer during centrifugation by its absorbance at 280 nm (see Note 5). Once it has reached ~200 μM, distribute it into 10-μL aliquots, flash freeze in liquid nitrogen, and store at −80°C. We have found that the tetramer remains in its reduced state for at least 6 months under these conditions.

3.2. Targeted Cross-Linking as a Probe of Tetramer Structure

In this chapter, two complementary approaches for using site-directed cross-linking as a probe of H3–H4 conformation while in complex with histone chaperones are described. In the first approach detailed in this section, the histone tetramer containing the H3 K115C mutation is incubated with increasing concentrations of the chaperone of interest. After binding has equilibrated, cross-linking is carried out using a homo-bifunctional sulfhydryl reactive cross-linker. Free histone tetramers are covalently linked across the dyad interface at the introduced H3 K115C cross-linking site. As two covalently linked H3 molecules (H3–H3′) have twice the molecular weight of a single H3, these two species are easily separated by denaturing SDS-PAGE (Fig. 1c). A histone chaperone which disrupts the H3–H3′ interface, such as Asf1, hinders cross-linking. Thus, increasing the concentration of such a chaperone, prior to cross-linking, results in reduction of the H3–H3′ species and an increase in the H3 species. Alternatively, a chaperone which interacts with H3–H4 in their tetrameric configuration has minimal effect on the H3–H3′ species. Before starting, it is necessary to determine interaction conditions between H3–H4 and your chaperone of interest. It is also beneficial to know the stoichiometry of the interaction so that relevant titration points can be made.

The quantities of chaperone required are dependent on the method of detection. In the approaches described below, histones were detected using Coomassie staining at a concentration of 20 pmoles (tetramer) per lane. Thus, assuming a 1:1 stoichiometry of binding and allowing for repetition, quantities in the range of ~0.5–1.0 nmoles of chaperone are required. This is typically well within the range of recombinant expression in bacteria and also within the range of TAP tag-purified yeast proteins of moderate abundance. However, if material is scarce, one can resort to immunoblotting for detection, thus lowering the required amount of chaperone by 10–100-fold.

As this methodology is based on exploiting the unique reactivity of the sulfhydryl containing side chain of cysteine, it is important that all buffers are free from reducing agents, such as DTT and

β-ME. Furthermore, the presence of cysteine residues within the chaperones can potentially complicate the interpretation of cross-linking. When using recombinant chaperones, such problems may be circumvented through mutation of native cysteines (to either alanine or serine) by site-directed mutagenesis. If this is not possible or if the chaperone is purified from an endogenous source, the chaperone can be incubated with PMTS to cap any reduced histones, followed by desalting or dialysis to remove the small molecule, before cross-linking. Cross-linking in this protocol details the use of MTS groups which form reducible disulphide bonds with cysteines separated by a carbon spacer. However, other methods of sulphydryl cross-linking are also possible (see Note 4).

In the following protocol, the binding of H3–H4 by Asf1 (*S. cerevisiae*) shall be used as a model histone–chaperone interaction that disrupts the H3–H3′ interface of the histone tetramer. The affinity of Asf1 for H3–H4 has previously been shown to be within the low nanomolar range (13), and insensitive to high ionic conditions (6, 8), of up to 1 M sodium chloride (9), with a binding stoichiometry of two Asf1 per $(H3–H4)_2$ tetramer (7).

1. Retrieve an aliquot of histone chaperone (in this case, Asf1 purified as described previously (9) and stored in aliquots dialysed into buffer A at −80°C). After thawing, make four 30 μL dilutions of 1, 2, 3, and 4 μM in buffer A and place on ice.
2. Retrieve an aliquot of refolded H3 K115C tetramer from −80°C storage, thaw, and place on ice. As the cross-linking reaction is sensitive to the stoichiometry of tetramer to cross-linker (Fig. 2b), it is worthwhile centrifuging the thawed aliquot, removing the supernatant and redetermining the concentration of tetramer by spectrophotometric analysis in case any precipitation has occurred during freeze thawing (see Note 5).
3. Dilute the tetramer to 2 μM (4 μM H3–H4 dimer) with buffer A. Note: Due to the large dilution factor, the salt content of the refolding buffer, in which the tetramer is dissolved, is negligible.
4. Aliquot 25 μL of the tetramer solution into six 1.5-mL microcentrifuge tubes labelled “0”, “0.5”, “1.0”, “1.5”, “2.0”, and “Control” with the suffix “T” (tetramer) and keep on ice. Add 25 μL of the 1, 2, 3, and 4 μM Asf1 dilutions to samples “0.5T”, “1.0T”, “1.5T”, and “2.0T”, respectively, to achieve the corresponding concentrations. To the remanding “0T” and “Control T” samples, add 25 μL of buffer A. Mix well by pipetting. Incubate at room temperature for 10 min to allow binding to equilibrate. Return to ice.
5. You should now have six sample tubes, all with a final tetramer concentration of 1 μM, four of which have Asf1 titrations of 0.5, 1.0, 1.5, and 2 μM. Remember that the binding stoichiometry

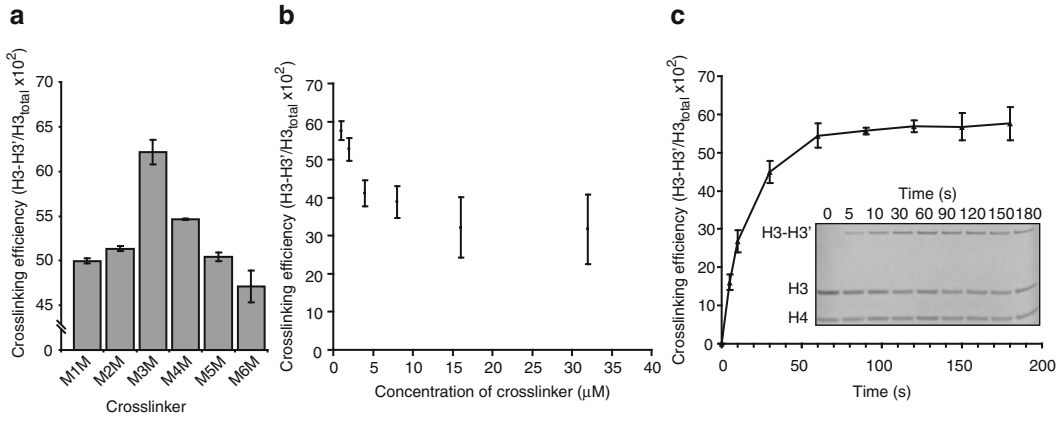

Fig. 2. Parameters affecting cross-linking across the dyad interface of histone H3. (**a**) Cross-linking efficiency with respect to cross-linker length. Methanethiosulfonate cross-linkers containing 1–6 carbon spacers (M1–6M, respectively) were tested for their cross-linking efficiency against 1 μM tetramer at equal stoichiometry by quantification of Coomassie stained bands. A 3-carbon spacer (M3M) was determined to be the most efficient. (**b**) Cross-linker efficiency with respect to the stoichiometry of cross-linker to cross-linking sites. Histone tetramer at a concentration of 1 μM was incubated with increasing amounts of M3M. Cross-linking was found to be the most efficient at a stoichiometry of one cross-linker per tetramer or one cross-linker per two cysteine residues. (**c**) The cross-linking reaction monitored over time. 1 μM tetramer was incubated with 1 μM M3M and cross-linking allowed to proceed for the displayed times before quenching. Cross-linking occurs rapidly and is all but complete after 60 s. The insert is a gel from one of the time courses. In all three experiments, cross-linking reactions were carried out in triplicate, with error bars representing the standard deviation.

of Asf1 to the histone tetramer has been previously determined as 2:1; therefore, the final titration represents an equal stoichiometry in binding.

6. In the meantime, retrieve a 10-nmole aliquot of dried cross-linking reagent M3M (see Note 6) and dissolve in 250 μL DMSO to achieve a 40 μM solution. Retrieve a 2-μmole aliquot of quenching reagent PMTS and dissolve in 10 μL of DMSO to achieve a 200 mM solution.
7. Aliquot out 1 μL of the cross-linking reagent to five micro-centrifuge tubes labelled "0", "0.5", "1.0", "1.5", and "2.0" (the final concentration of Asf1) with the suffix "XL" (cross-linker), 1 μL of the quenching reagent to a sixth labelled "control Q". Additionally, aliquot out 1 μL of quenching reagent to five micro-centrifuge tubes, again, labelled "0", "0.5", "1.0", "1.5", and "2.0", but with the suffix "Q" (quencher), and 1 μL of cross-linking reagent to a sixth (labelled "control XL"). Do not put these tubes on ice as the solvent, DMSO, freezes and prevents rapid mixing. It is helpful to organise these tubes into two corresponding rows in a micro-centrifuge tube rack: "0 XL", "0.5 XL", "1.0 XL", "1.5 XL", "2.0 XL", and "Control Q" (top row) and "0 Q", "0.5 Q", "1.0 Q", "1.5 Q", "2.0 Q", and "Control XL" (bottom row).
8. 40 μL of each of the Asf1 tetramer samples ("0.5T", "1.0T", "1.5T", and "2.0T") and the tetramer-alone control ("0T") is,

in turn, rapidly mixed with the cross-linking reagent ("0 XL", "0.5 XL", "1.0 XL", "1.5 XL", and "2.0 XL"), left for 60 s at room temperature (see Note 7) and then rapidly mixed with the quenching reagent. The second tetramer control ("Control T") is mixed first with the quenching reagent ("Control Q"), left for 60 s and then mixed with the cross-linking reagent ("Control XL"). This serves as a control to monitor the effectiveness of the quenching reagent. As MTS groups are highly reactive (see Note 8), it is important that mixing of the protein and cross-linker is as rapid as possible. We find that the following procedure works well. (a) Remove 40 μL of the protein sample and keep it in the pipette tip. (b) Start a timer at 70 s. (c) Open the corresponding micro-centrifuge tube which contains the cross-linking reagent and ready the tip at the bottom of the tube just above the aliquot of cross-linker. (d) As the timer counts down to 60 s, pipette rapidly up and down ten times. As the protein concentration is low and no detergents are used in the buffer, bubbles are not usually a problem. (e) Recover the sample in the tip of the pipette and discard the tube. (f) Open the corresponding micro-centrifuge tube containing the quenching reagent and ready the tip just above the aliquot, which should be sitting at the bottom of the tube. (g) As the timer hits 0 s, pipette rapidly up and down ten times, as before. We have found that this procedure works more effectively, and is more reproducible than other methods of mixing, such as using a vortex. With regards to the quenching control, the same procedure is carried out, just reversed: first, the tetramer is mixed with quenching reagent, and then cross-linking reagent.

9. Allow quenching to continue for a minimum of 5 min. Remove 20 μL of each sample and mix with SDS-PAGE loading buffer. Heat the sample at 65°C to ensure complete denaturation before separating the polypeptides by SDS-PAGE (see Note 9). After separation, stain the gel with Coomassie to visualise the extent of the cross-linking. Non-cross-linked H3 migrates slightly slower than its theoretical molecular weight, ~18 kDa, whereas H3–H3′ migrates at ~38 kDa (Figs. 1c and 3b). Asf1 is a chaperone that binds to the dyad interface of H3–H4, disrupting the H3–H3′ interaction. Thus, as would be expected, as the concentration of Asf1 is increased, the H3–H3′ at ~38 kDa gradually disappears (Fig. 3b). If quantitation of cross-linking is required, at 20 pmoles, tetramer histones H3 and H4 are well within the linear range for densitometric analysis (see Note 10).

3.3. Targeted Cross-Linking as a Method of Tetramer Stabilisation

The second approach capitalises on the observation that covalent linkage across the dyad interface of H3 and H3′ effectively stabilises the conformation of the histone tetramer (9). Using pre-cross-linked tetramer as a substrate, histone chaperones that

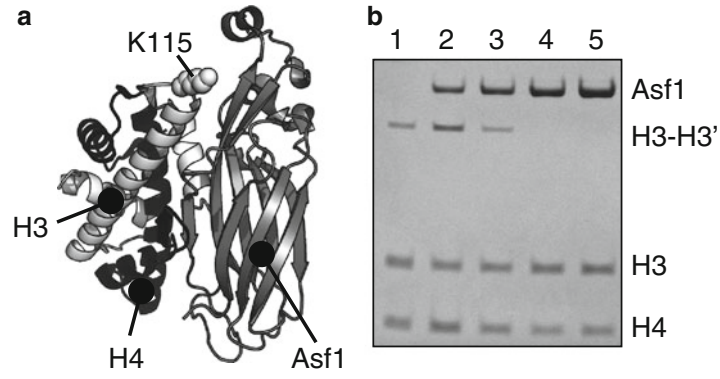

Fig. 3. Probing the structure of histones H3 and H4 while in complex with the histone chaperone Asf1. (**a**) Co-crystal structure of the histone-binding domain of *S. cerevisiae* Asf1 in complex with *X. laevis* histones (PDB code: 2HUE). (**b**) Pre-incubation with increasing amounts of Asf1 results in the inhibition of H3–H3′ formation. *Lane 1*, no Asf1; *lane 2*, 0.5 μM Asf1; *lane 3*, 1.0 μM Asf1; *lane 4*, 1.5 μM Asf1; *lane 5*, 2.0 μM Asf1; all lanes containing 1.0 μM $(H3–H4)_2$ tetramer.

specifically interact with a dimer of H3–H4 (such as Asf1) display reduced binding compared to that of non-cross-linked histones. Following the same line of reasoning, histone chaperones whose binding is not affected by the presence of the covalent linkage do not require access to the H3–H3′ interaction interface for binding (9). Binding or, more correctly, the loss of binding could be monitored thermodynamically using quantitative methods, such as isothermal titration calorimetry, surface plasmon resonance, or Forster resonance energy transfer. However, we detail here a simpler approach utilising gel filtration chromatography to monitor the association of H3–H4 with the histone-binding domain of Asf1 (Asf1g).

Before binding and separation of cross-linked tetramer and chaperone can be carried out, a number of controls must first be implemented: one must determine the elution volumes of the chaperone alone, H3–H4 alone, and the H3–H4–chaperone complex (without cross-linking). This protocol is set out with the assumption that the researcher is competent in using gel filtration chromatography to separate protein complexes of different sizes. In this protocol, a Superdex™ S200 PC 3.2/30 column attached to a SMART system (Amersham Pharmacia) micro-chromatography unit is used (see Note 3).

1. Equilibrate the gel filtration column with buffer A. Retrieve an aliquot of histone chaperone (Asf1g) from −80°C storage. To avoid precipitates that may have formed during freeze thawing being loaded on the micro-gel filtration column, either centrifuge the thawed sample in a micro-centrifuge and remove the supernatant to a fresh tube or apply the sample to a centrifugal filter unit. Asf1g was previously purified and stored at 400 μM

in buffer A. Make a 20 μM dilution of Asf1g in 40 μL in buffer A. Load 20 μL of this into the pre-washed injection loop of the micro-chromatography system using a 100-μL Hamilton syringe. Handling such small volumes can be tricky. To precisely inject 20 μL without any air bubbles, the full 40 μL is taken up into the syringe. 10 μL is then expelled to remove any air bubbles from the needle and insure drop-to-drop contact with the injection loop. The needle is inserted into the loop and the remaining sample volume noted (~30 μL). 20 μL can then be precisely injected leaving ~10 μL of residual sample in the syringe so that no air bubbles follow the sample into the loop.

2. Chromatography is then carried out at a flow rate of 20 μL/min. 50-μL fractions are collected spanning the void to bed volume and collected in 0.5-mL tubes: using a Superdex™ S200 PC 3.2/30 column, this amounts to 30 fractions spanning an elution volume of 0.8–2.4 mL. Once all fractions have been collected, add 17 μL of 4× SDS-PAGE loading buffer to each, mix, and heat at 65°C for 10 min before storing at –20°C.
3. Carry out the same procedure for H3–H4 alone (carrying the H3 K115C mutation, but not cross-linked). The 10-μL aliquots stored at –80°C in refolding buffer can be used for gel filtration analysis. Again, centrifuge or filter the sample before chromatography to remove any precipitation that may have occurred during freeze thawing. Dilute the tetramer to 10 μM in 40 μL of buffer A and apply to the injection loop. Carry out chromatography as detailed above. Again, to each of the 30 fractions, add 17 μL of SDS-PAGE loading buffer, heat at 65°C for 10 min, and store at –20°C.
4. Next, the elution volume for the H3–H4–chaperone complex is determined. Retrieve an aliquot of H3 K115C tetramer and an aliquot of chaperone from –80°C storage. Centrifuge or filter the samples before chromatography to remove any precipitation that may have occurred during freeze thawing. Dilute the tetramer to 10 μM in buffer A and transfer 40 μL to a fresh tube. To this, add 2 μL of Asf1g and leave to equilibrate for 10 min at room temperature (Asf1g binding to H3–H4 is rapid). Carry out gel filtration analysis as before, collecting 50-μL fractions and storing in 1× SDS-PAGE loading buffer at –20°C.
5. Now, the effect of the covalent cross link on histone chaperone binding can be analysed. Retrieve an aliquot of H3 K115C tetramer and an aliquot of chaperone from –80°C storage. Centrifuge or filter the samples before chromatography to remove any precipitation that may have occurred during freeze thawing. Redetermine the concentration of histone tetramer by spectrometric absorption, dilute to 10 μM in buffer A, and transfer 50 μL to a fresh tube.

6. Now, to cross-link the histone tetramer across the dyad interface: Dissolve a 10-nmole aliquot of cross-linking reagent (M3M) in 25 μL DMSO to make a 500 μM stock solution, and dissolve a 2-μmole aliquot of quenching reagent (PMTS) in 10 μL to make a 200 mM stock solution. Add 1 μL of the cross-linker and 1 μL of quenching reagent to two labelled micro-centrifuge tubes. To the tube containing 1 μL of cross-linking reagent, add 40 μL of the diluted histone tetramer and mix rapidly by pipetting up and down. After 60 s, transfer the reaction to the tube containing 1 μL of quenching reagent and mix rapidly by pipetting. In the previous experiment, cross-linking was carried out at 1 μM tetramer, which resulted in ~60% cross-linking (Fig. 2). At 10 μM tetramer and keeping the tetramer–cross-linker ratio stoichiometric, the cross-linking efficiency is increased to ~80%, which aids in the analysis of the dimer–tetramer preference of the chaperone.
7. After quenching has proceeded for 5 min, add 2 μL of Asf1g to the cross-linked tetramer and leave for 10 min at room temperature to allow binding to equilibrate. Addition of cross-linker and quenching reagent slightly dilutes the sample. However, this has negligible effect on the elution profile and, as the stoichiometry is retained, minimal effect on the cross-linking efficiency. Carry out chromatography as before. Once all fractions are collected, again, mix with 17 μL 4× SDS-PAGE loading buffer and heat for 10 min at 65°C.
8. Comparison of the cross-linked and control chromatograms: The four gel filtration runs can be compared in two ways. Firstly, if the chromatography system is equipped with a UV flow cell, peak profiles from each experiment can be compared directly. Secondly, fractions can be analysed for their content by separation using SDS-PAGE. As the peak profile does not directly report on the component of each peak and as multiple complexes can be formed, we suggest that SDS-PAGE analysis of each chromatographic profile is carried out. The UV trace can aid in determining which fractions to be analysed by SDS-PAGE.
9. Interpretation of the results: The three control runs show the elution profiles of (a) the histone chaperone alone, (b) H3–H4 alone, and (c) H3–H4–chaperone complex. If stabilisation of the tetramer structure (through the covalent linkage across the dyad interface introduced by cross-linking) inhibits the binding of the histone chaperone, an increase in the free histone chaperone and free histone tetramer species should be observed. Whereas, if cross-linking does not affect the interaction between histone tetramer and histone chaperone, the elution profile should be identical to that seen for the non-cross-linked tetramer in complex with the histone chaperone (9).

4. Notes

1. MTS-based cross-linking reagents lose their reactivity when in an aqueous environment for extended periods. Typically, minute quantities of this compound are needed per reaction. Therefore, weighing out milligrams of dry material each time, the experiment is to be carried out is not cost-effective. In the interests of economy and accuracy, stock material received from the supplier can be dissolved in trichloroethane or dimethylformamide, distributed into 10-nmole aliquots in 1.5-mL micro-centrifuge tubes, and vacuum dried. The dried aliquots are then stored at –20°C.
2. As for the cross-linking reagent, this compound is moisture sensitive; thus, we tend to store dried-down aliquots of 2 μmole at –20°C (see Note 1, above).
3. The micro-chromatography system we use is a discontinued SMART system produced by Amersham Pharmacia, which has subsequently been taken over by GE Healthcare. GE Healthcare has superseded the SMART system with the ÄKTAmicro™. In addition, a number of other manufacturers have produced chromatography system amenable to micro-gel filtration. We have also used the same Superdex™ 200 PC 3.2/30 column (GE Healthcare) attached to a Dionex manufactured Ultimate® 3000 Quaternary Micro LC System, with similar results. If material is not limiting and a micro-chromatography system is not available, everything can be scaled up tenfold for use with a 10/300 GL column (GE Healthcare), or similar, attached to a standard FPLC system.
4. For some applications, reducing conditions may be required after cross-linking is carried out. For this, we recommend using a maleimide-based cross-linker whose thioether linkage is non-cleavable with reducing agents. We have found bis-maleimidoethane (BMOE) to work just as efficiently and with similar characteristics to M3M (9). Additionally, a disulphide bond can be catalysed between the two K115C residues using copper chelated with 1,10-phenanthroline. We refer the reader to the following sources for more information on using this approach (9, 14). Oxidised and reduced glutathione have also been used in disulphide bond formation between cysteine residues introduced to histones (15).
5. Absorption coefficients for calculating protein concentration were determined from the primary sequences of *X. laevis* H3 and H4 using the tool ProtParam (http://expasy.org/tools/protparam.html) as 4,470 and 5,960 M^{-1} cm^{-1} for H3 and H4, respectively.

6. We have investigated the effect of the length of the cross-linker with respect to cross-linking efficiency using the H3 K115C tetramer as a substrate. We tested cross-linkers with 1–6 carbon atoms in the spacer region and found that cross-linking efficiencies peaked at the 3 carbon spacer mark (Fig. 2a). Although there were significant improvements in cross-linking between cross-linkers, the overall increase in cross-linking efficiencies were not drastic, representing ~15% increase from most efficient to least efficient (Fig. 2a). This is most likely due to the conformational flexibility of both the cross-linker (16) and the L2 loop region in which K115C resides (Fig. 1a).
7. We have found that cross-linking at 1 μM tetramer and 1 μM M3M is complete by 60 s (Fig. 2c). Shorter time periods for the reaction may be less accurate as a few seconds' difference in quenching times may significantly alter the cross-linking efficiency.
8. We have found that cross-linking efficiency is significantly affected by the ratio of cross-linker to cross-linking sites. There is a tendency to err towards a higher concentration of cross-linker in order to achieve a more efficient cross-linking ratio. However, upon titrating cross-linker against the H3 K115C tetramer, we found that cross-linking efficiency was maximal at a ratio of one cross-linker per cross-linking site (or one cross-linker per two cysteine residues) (Fig. 2b). This can be explained by saturation of the cysteine residues with mono-reacted cross-linker or the so-called hanging cross-linkers, highlighting the reactivity of MTS groups.
9. For SDS-PAGE analysis, we typically use the NuPAGE® Novex® precast bis–tris 1.0 mm mini gels (Invitrogen) with an MES buffer system (Invitrogen). However, the more traditional Laemmli tris–glycine systems also work fine. As the molecular weight difference between H3 and H3–H3′ is so large, they can be efficiently separated over a wide range of polyacrylamide concentrations; however, we routinely use 12%.
10. For densitometric analysis, we use the software AIDA (Raytest). Separation on a single-percentage acrylamide gel, as opposed to a gradient, helps in defining the background baseline.

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