Histone H3 disulfide dimers and nucleosome structure
(nuclease digestion/superciling of DNA/sedimentation velocity/nucleoprotein gel electrophoresis/chromatin)

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ABSTRACT The arginine-rich histone, H3, isolated from avian erythrocytes, can dimerize by forming a disulfide linkage between the single cysteine sulfhydryl residues at position 110 of the H3 polypeptide chain. The H3 dimer can be substituted for undimerized H3 in experiments in which the nucleosome is reconstituted from DNA and mixtures of the four “core” histones, H2A, H2B, H3, and H4. We report here that reconstituted nucleosomes containing H3 dimer are indistinguishable, by a number of criteria, either from native nucleosomes or from reconstituting H3 monomer. The criteria include the pattern of susceptibility of the complex to nuclease, the amount of DNA supercoiling induced by histone binding, and the hydrodynamic properties of reconstituted nucleosome “core” preparations.

The results suggest that the residues in the neighborhood of position 110 on each H3 molecule are in close contact in the nucleosome. If, as has been proposed, the nucleosome has a dyad axis, then the disulfide bridge between H3 molecules must lie on this axis.

An understanding of the biological activity of chromatin will require detailed knowledge of the internal architecture of its fundamental repeating subunit, the nucleosome.

Considerable evidence has accumulated that the DNA in the 140-base-pair nucleosome “core” is wrapped around (1, 2) a histone octamer consisting of two of each of the core histones H2A, H2B, H3, and H4 (3). Studies of histone-DNA interactions have shown that the arginine-rich histones, H3 and H4, play a central role in nucleosome organization (4–10). We are therefore interested in obtaining further information about the way in which these histones interact with each other within the nucleosome. An obvious approach is to use chemical cross-linking reagents to estimate the proximity of functional groups in the proteins; there have been many such studies of histone interactions in chromatin (3). However, most crosslinking reagents can react at more than one site, and so far it has not been possible to identify the specific amino acid residues within the histones that are involved in the crosslinking.

There is one crosslinking reaction that may avoid some of these problems: the formation of intermolecular disulfide bonds between cysteine residues. This is a particularly useful reaction for the study of histones because H3 is the only histone that has cysteine and in organisms other than higher mammals there is only one such residue per molecule (11–14). The cysteine at this one position, residue 110 of H3, has been preserved, with the single exception of yeast H3 (15), throughout evolution.

It is well known (11, 12, 16) that H3 molecules can dimerize in vitro through a disulfide bond between the cysteine residues at position 110. We report here that such a dimer is capable of entering into the formation of nucleosomes.

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MATERIALS AND METHODS
Colicin E1 plasmid (ColE1) DNA (both supercoiled and relaxed forms) and duck DNA were prepared and purified as described (4, 7). Monomer core-length DNA (140 base pairs) was purified from the DNA of staphylococcal nuclease digests of duck chromatin “stripped” of the lysine-rich histones (see below) by column chromatography on Bio-Gel A-5M (Bio-Rad) by a procedure to be described elsewhere (R. H. Simon, R. D. Camerini-Otero, and G. Felsenfeld, unpublished data). H2A, H2B, H3, and H4 were prepared by “stripping” the lysine-rich histones (H1 and H5) from duck or chicken erythrocyte chromatin (4) by dialysis against 0.7 M NaCl/5 mM Tris-HCl/0.5 mM EDTA, pH 8.0, at 4° followed by isolation of the stripped product by exclusion chromatography on Bio-Gel A-5M. Histones were either extracted with 2 M NaCl at 4° after the chromatin was bound to hydroxyapatite (R. H. Simon and G. Felsenfeld, unpublished data) or extracted directly with 0.2 M H2SO4 at 4°.

Histone–DNA complexes were prepared by mixing histones and the appropriate DNA in the presence of 50 mM NaCl/10 mM Tris-HCl, pH 8.0/1 mM EDTA; usually the DNA concentration was 50–250 μg/ml. For the reconstitution onto Col E1 DNA and duck DNA, a total of 1 g of a mixture of the four histones per g of DNA was added; in the reconstitutes onto monomer-length DNA, 1.2 g of the four histones per g of DNA was used (1.16 g/g of DNA corresponds to 2 mol of each of the four histones per mol of core-length DNA). Histone and DNA concentrations were determined as described (4). These solutions were then dialyzed for 12–16 hr at 4° against 5 M urea/2 M NaCl/5 mM Tris-HCl, pH 8.0/0.5 mM EDTA, with or without 10 mM 2-mercaptoethanol (Sigma Chemical Co.) as indicated. They were then subjected to gradient dialysis as described (4), except that all the dialysis steps included 5 mM Tris-HCl/0.5 mM EDTA, pH 8.0. At before, when 10 mM 2-mercaptoethanol was used, it was not included after the urea was removed. This reconstitution procedure has been shown to give reconstitutes with protein-to-DNA ratios within 10% of the input ratio. Furthermore, none of the histones has been selectively lost and all of the protein is bound to the DNA (4, 7).

The nicking-closing extract (NCE) was isolated from duck reticulocyte nuclei. Methods of isolation, the conditions for the nicking and closing reactions, and the analysis of the products by agarose gel electrophoresis have been described (7). For this study, the NCE was dialyzed against the reaction buffer used in the nicking-closing reaction.

Abbreviations: ColE1, colicin E1 plasmid; NCE, nicking-closing extract; NaDodSO4, sodium dodecyl sulfate.
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Staphylococcal nuclease digestions were carried out as described (4). Bovine pancreatic DNase (3750 units/mg, RNase-free; Worthington Biochemical Corp.) digestions were carried out at a DNA concentration of 100–150 µg/ml in 5 mM Tris-HCl, pH 8.0/0.1 mM MgCl₂/0.1 mM CaCl₂ at 37° with 0.3–1.5 Worthington units of enzyme per ml for 15 min.

Electrophoresis of DNA fragments on 8 and 12% polyacrylamide slab gels was carried out as described (4). Electrophoresis of nucleoprotein complexes was carried out in 6% polyacrylamide gels prepared in the same manner as the DNA gels and in the same buffer (90 mM boric acid/90 mM Tris base/3 mM EDTA, pH 8.3 (17)).

The histones were routinely monitored for integrity and for the extent of H3 dimerization by gel electrophoresis at each stage of the experimental procedures; sodium dodecyl sulfate (NaDodSO₄) 18% polyacrylamide stacking gels were prepared according to the modification of the method of Laemmli (18) by Weintraub et al. (19), with the exception that the reducing agent (2-mercaptoethanol) was added only when indicated.

Boundary sedimentation velocity experiments were carried out in a Beckman model E ultracentrifuge equipped with ultraviolet optics. The sedimentation velocity data were corrected for radial dilution. In all cases the solvent was 50 mM Tris-HCl, pH 8.0/0.1 mM EDTA, and the DNA concentration of the samples was approximately 50 µg/ml.

RESULTS

To examine the effects of H3 disulfide dimers on nucleosome structure, our approach was to reconstitute nucleosomes from DNA and the four core histones, with H3 present either as the monomer (with a free thiol) or as the disulfide-linked dimer. We found that the reconstitutes with dimer had the same structure as reconstitutes involving H3 monomers, and that both of these reconstituted nucleosomes were indistinguishable from those isolated from cells as judged by various assays: (i) response to biochemical probes such as staphylococcal nuclease and pancreatic DNase; (ii) the effect of bound histones on the supereiling of DNA; and (iii) the effect of the binding of histones on the electrophoretic and hydrodynamic properties of nucleosome core-length DNA.

H3 Disulfide Dimers. When preparations of the four core histones are handled extensively during the purification, H3 dimerizes spontaneously (11, 12, 16) even when the preparations are kept at 4°. A preparation of such “core” histones is shown in Fig. 1 left. On the same gel were also run total chromatin histones and a preparation of core histones in which H3 was not dimerized. In Fig. 1 middle are shown the same three samples after a brief exposure to 0.1% NaDodSO₄ and 1 M 2-mercaptoethanol at pH 8.0.

Fig. 1 right shows the result of a mixing experiment. Preparations of core histones containing both H3 dimers and monomers were electrophoresed; when these two preparations were mixed and electrophoresed on the same NaDodSO₄ gel, the pattern obtained was the simple sum of the electrophoretic patterns of the two samples. This experiment rules out the possibility that dimer is an electrophoretic artifact formed only in the absence of such a putative reducing substance. Similar mixing experiments have indicated that, when dimerized histones are treated with 2-mercaptoethanol during reconstitution, the histones are reduced by this treatment, rather than by their exposure to NaDodSO₄ and remaining mercaptan just prior to electrophoresis. Under the conditions used for electrophoresis, there is not enough 2-mercaptoethanol at the end of a reconstitution to reduce an existing H3 dimer. For all the experiments shown, the extent of dimerization of H3 dimers was greater than 70% and usually greater than 90% (as in Fig. 1 left).

Digestion of Reconstitutes by Staphylococcal Nuclease and Pancreatic DNase. The accessibility of the DNA in nuclei and chromatin is presumably a reflection of rather precise interactions between the histones and the DNA (4, 5, 10, 20–24).

Noll (20) has shown that a set of single-stranded DNA fragments that are exact multiples of 10 nucleotides can be obtained if the products of digestion of nuclei by pancreatic DNase are denatured before gel electrophoresis. We have obtained the same pattern from reconstitutes of core histones onto duck DNA of high molecular weight (5); the same discrete fragments and characteristic fragment intensity distribution were obtained whether H3 dimers or monomers were used in the reconstitution (Fig. 2).

We have shown previously that digestion of chromatin by staphylococcal nuclease gives rise to a large array of double-stranded DNA fragments of discrete size (4, 21). Similar results were obtained when the four core histones were reconstituted with duck DNA of high molecular weight (4). We examined the nuclease digestion products by gel electrophoresis, and found that the results are the same whether H3 dimers or monomers are used in the reconstitution. The same series of fragments was obtained from both of these digests as was obtained from digests of chromatin that had been stripped of its lysine-rich histones (data not shown).

In all above experiments the histones of the reconstitutes were examined by NaDodSO₄/gel electrophoresis after reconstitution and nuclease digestion; the dimer or monomer status of the H3 was not altered by the digestion.

Supereiling of the DNA. It is now well-known (25) that, when an equimolar mixture of the four core histones is reconstituted onto relaxed covalently closed circular DNA and the complex is treated with a NCE, the subsequently isolated DNA is supereiled. In reconstitution experiments using CoE1 DNA, we have previously shown (7) that full supereiling occurs when the histone-DNA weight ratio is greater than 0.8. Fig. 3 shows that the DNA was fully supereiled whether H3 monomers or dimers were used. The nucleosomes formed from these two species of H3 have identical effects on DNA topology.

Control experiments showed that the NCE had no effect on
the oxidation state of H3. Exposure of the reconstitutes to the NCE did not reduce H3 dimers, form H3 dimers from monomers, or change the electrophoretic mobility of the histones on NaDodSO₄ gels even when a 10-fold excess of extract was used for periods up to 30 times as long as required for the completion of the supercoiling reaction.

**Nucleoprotein Gel Electrophoresis and Sedimentation Velocity Analysis.** The DNA in a nucleosome core is 140 base pairs in length (26-28). Fig. 4 shows the electrophoretic pattern of core-length DNA, reconstituted nucleoprotein complexes made with this DNA, and nucleoprotein complexes isolated from a staphylococcal nuclease digest of stripped chromatin. Clearly the structure formed, as monitored by this assay, was the same whether H3 dimers or monomers were used in the reconstitution. Furthermore, both of these reconstitutes had the same electrophoretic mobility as native nucleosome cores.

Nucleosomes and nucleosome cores are known to have a sedimentation coefficient (s₂₀,W) of approximately 11 S, whereas the corresponding value for naked DNA is about 5.3 S. Fig. 5 shows that, whether the reconstitution was carried out with H3 dimers or monomers, the four core histones compacted about 90% of the DNA into an 11S particle. The heterogeneity of sedimentation coefficient values is not greater than is found for nucleosomes isolated from nuclei. The small percentage of fast-sedimenting material in the complex formed with H3 monomers is a reproducible finding which has also been reported by Tatchell and Van Holde (29).

**DISCUSSION**

It has been well known for some time that the thiols of H3 can react to form intermolecular -S-S- bridges (11, 12, 16). On the
other hand, the evidence that such disulfides exist in vitro has been less than convincing. Several workers have claimed to have isolated H3 dimers linked by disulfide bridges and have attributed to them important biological roles in the regulation of mitotic chromosome condensation (30–32) and transcription (33). The difficulties encountered in attempting to prove the existence of such dimers in vitro have been discussed in a careful study by Garrard and coworkers (34). They were unable to determine conclusively whether such dimers exist or not, and they showed that dimers can be both created and destroyed artifactualy. As they pointed out, the uncertainty probably arises because of the high levels of glutathione in mammalian cells (35) and because of the highly reactive nature of the thiols in H3, for which the $pK_{a(app)}$ is 4.5 units lower than that for a freely ionizing SH group (36).

In view of these difficulties we asked a simpler question: Can H3 disulfide dimers be accommodated in the structure of the nucleosome? For this purpose we used the techniques of chromatim and nucleosome reconstitution. Even in this simple system we must be wary of artifacts. It is well known that the thiols of denatured proteins are highly reactive and that disulfides can be formed easily when these SH groups are exposed to aerated solutions at a pH such that the thiolate ion is the predominant species. The converse is also true. Exposure of denatured H3 to traces of reducing agents can readily reduce disulfides (R. D. Camerini-Otero, unpublished data). For these reasons we have taken care to show that the dimers and monomers of H3 that we have examined by NaDodSO4 gel electrophoresis were not generated accidentally during sample preparation for electrophoresis or during electrophoresis itself.

We used various assays to show that, whether H3 is dimerized or not, reconstituted complexes of the four core histones with DNA give rise to structures very similar to those isolated from cells. The ability to form an appropriately compacted structure is probably the most direct and stringent test for the reconstitution of the nucleosome. DNA 140 base pairs long behaves in solution approximately like a stiff rod 450 Å in length (37). The same length of DNA in the nucleosome core is compacted by the core histones into a nucleoprotein disk 110 Å in diameter and 57 Å in height (24). The electrophoretic properties (reflecting both hydrodynamic and charge properties) of reconstitutes onto nucleosome core-length DNA (Fig. 4) suggest that H3 monomer and dimer are equally effective in causing compaction. Nucleosome compaction can be studied more directly by measuring the sedimentation coefficient of the complex after reconstitution (ref. 29; Fig. 5). If an octamer’s worth of core histones binds to each DNA molecule without any accompanying compaction, it can be calculated that the sedimentation coefficient of the complex will not exceed about 8.5 S. Nucleosomes, however, have a sedimentation coefficient of about 11 S, which can be taken as direct evidence for compaction of the DNA. Analysis of the shape of the boundary makes it easy to determine quantitatively the fraction of DNA that has in fact formed a nucleosome core particle. This is an advantage not shared by such methods as electron microscopy, for example. The results shown in Fig. 5 make it clear that, whether H3 dimers or monomers are used in the reconstitution, 90% of the DNA is appropriately compacted.

The results of Lewis (38) indicate that the formation of intramolecular disulfides, involving the thiols on cysteine 96 and 110 in calf H3, abolishes the ability to form an H3-H4 tetramer. His study complements those carried out by Böhm et al. (39) which show that residues 42–120 of H3 are essential in forming such a tetramer, which is in turn crucial to the formation of the nucleosome (4–10). These studies strongly suggest that interactions involving these segments of H3 are important to nucleosome structure. It therefore seems unlikely that intermolecular dimerization of avian H3 in the nucleosome (via its only thiol on cysteine 110 (40)) would involve a major conformational alteration in this region. This suggests that the thiols in the two H3 molecules are normally very close. Evidence from fluorescent energy transfer experiments (41, 42) indicates that the thiols are at most 15–20 Å apart. We can now add that they may be as close as the length of a disulfide bridge.

Where are the SH groups at position 110 of H3 located in the intact nucleosome structure? Recent studies show that they are ordinarily inaccessible to small molecules that react with thiols, and they become available only when the nucleosome structure is disrupted (43, 44). For example, the SH groups of H3 become reactive at salt concentrations that dissociate all of the histones from the DNA (44). There is some evidence suggesting that under such conditions the histones exist as heterotypic tetramers, containing one molecule of each of the four core histones (19). Because these tetramers retain much of the histone secondary structure (19, 45), Olins et al. (44) have suggested that the cysteine residues are buried between the contact surfaces of two heterotypic tetramers in the intact nucleosome and that dissociation into separate tetramers exposes the SH groups and that whatever the structure of the nucleosome, our results indicate that the residues in the neighborhood of position 110 on each H3 molecule are in close contact. Furthermore, if we accept the hypothesis, for which some evidence exists (24, 46, 47), that the nucleosome core possesses a dyad axis, then a much stronger statement can be made: The disulfide bridge between H3 molecules must lie on this dyad axis.

It should be kept in mind that all of our conclusions depend upon the validity of our criteria for the intactness of nucleosome structure. Although we have not been able to detect structural differences between nucleosomes containing H3 dimers and those with H3 monomers, it is possible that more sensitive assays

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The calculation is made by using the appropriate mass and partial specific volume and assuming that the complex has the same frictional coefficient as the DNA.
would reveal such differences. Even if H3 dimerization perturbs the nucleosome structure very little, it may very well affect the ability of the nucleosome to unfold. Experiments to measure the stability of these structures are needed.

What biological role could such disulfide linkages play? Considerable evidence has accumulated over the last few years that the DNA in chromatin undergoing transcription and replication is covered by histones. It has been suggested that, among the several ways in which polymerases might deal with nucleosomes in their path, there might be advantages to a mechanism involving nucleosome unfolding along a dyad axis (47). We have calculated that such an unfolding is energetically feasible and might involve a delicate balance between the opposing forces of DNA bending and the interaction of one or two histone pairs (7). It is tempting to speculate that H3 intermolecular disulfides might play a role in further stabilizing nucleosomes that need not be unfolded (perhaps in transcriptionally inactive regions). Whether in fact H3 dimers can affect the transcriptional activity of a template chromatin is a question that can now be answered in vitro by using the appropriate systems.

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